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Studies using the anti-idiotypic monoclonal antibody 105AD7 in patients with Advanced and Primary Colorectal Cancer.

by Charles Alan MAXWELL-ARMSTRONG
MB ChB, FRCS (Eng).

Thesis submitted to the University of Nottingham, for the Degree of Doctor of Medicine, January 1998.

This work is dedicated to my Mother.

To T. Pruen Esqr

Let the usual Puncture be made in the arm with a Lancet- then introduce the coated extremity of the ivory point, & suffer it to remain near a minute, supporting it in its place by the gentle pressure of the finger, when the oozing fluids will dissolve the concreted vaccine Virus and Patient be probably infected. I say probably, because the dried vaccine matter tho' quite fresh, like that I consign to you, sometimes fails to infect while that which is taken in its fluid state in some early stage of the Pustule & inserted immediately from arm to arm, does not disappoint me once in five thousand times.

EJ

Letter to Thomas Pruen from Edward Jenner (date unknown).

Contents.

ABSTRACT.

Introduction. The anti-idiotypic monoclonal antibody 105AD7 mimics the tumour associated antigen 791T/gp72, present on approximately 80% of colorectal cancer cells. A Phase I study using 105AD7 in 13 patients with advanced colorectal cancer has shown that it is non-toxic, and conferred a survival advantage on patients who received it [Denton GWL 1994].

Aim. There were two aims of this work. The first was to assess whether the survival advantage seen in the Phase I study was reproducible in a Phase II study. The second was to immunise patients with primary colorectal cancer, in a non-randomised adjuvant study, and explore further the immune responses generated.

Materials and Methods. Patients with advanced colorectal cancer were recruited to a randomised, double-blind, placebo controlled survival study. The first patient was recruited to this Phase II study in April 1994, and the last in October 1996. Four trial centres were used- Nottingham, Hull, Leeds, and Newcastle. Eligible patients had a life expectancy of 3 months, and none had received radiotherapy or chemotherapy in the preceding 1 and 3 months, respectively. Patients attended on 3 occasions, 6 weeks apart, receiving 10μg of 105AD7/alum i.d. followed by 100μg i.m. Venous blood was assayed for blood count and differential, liver function, urea and electrolytes, and CEA. Chest X-rays and CT scans were performed at trial entry and week 12 where possible. Dates of death were recorded following consultation with General Practitioner or referring clinician.

In addition, patients with primary colorectal cancer were recruited to a non-randomised adjuvant study, whereby they received 105AD7 before surgery. Venous blood samples were taken between immunisation and operation, and assayed for lymphocyte subsets. Samples taken from resection specimens were analysed immunohistochemically. Fresh

tumours were in addition disaggregated, and separated TIL labelled with a panel of monoclonal antibodies, and analysed by flow cytometry. Control tumours were similarly labelled. All analysis was performed blind.

Results. 162 patients were randomised to the Phase II study, between April 1994 and October 1996. 85 received 105AD7 and 77 placebo. The mean ages and sex-ratios of the two groups were comparable, as was the time from diagnosis of advanced disease to trial entry (172v179 days). Median survival from date on study was 124 and 184 days, in 105AD7 and placebo arms, respectively ($p=0.38$). Survival from date of diagnosis of advanced disease was 456 and 486 days ($p=0.82$). Chemotherapy and radiotherapy all prolonged survival in a multivariate analysis. Only one serious adverse event was seen in the 105AD7 arm, and this was felt unlikely to be attributable to the vaccine.

Twenty-four patients were recruited to the adjuvant study. Immunohistochemical analysis of tumour sections from 16 patients showed increased infiltration of CD4 and CD8 expressing lymphocytes, relative to a well matched control group ($p<0.05$). Infiltration of CD4, CD8 and CD56 expressing lymphocytes combined was significantly higher, as was that of the mitochondrial antigen 7A6, expressed on cells undergoing apoptosis ($p<0.005$). The activation marker CD25 was also significantly increased ($p<0.05$). Flow cytometric analysis of disaggregated tumours from 16 trial and 22 control patients, confirmed the increased expression of CD25 on TIL in the 105AD7 group ($p<0.01$). Peripheral blood phenotyping failed to show any significant increase in any lymphocyte subset, following immunisation.

A separate analysis was performed comparing 2 year survival and recurrence in 23 patients immunised by the previous CRC Fellow, with 97 matched controls from the Trent Audit. No significant difference was seen between the two groups.

Discussion. No survival difference was seen between patients receiving 105AD7 and placebo, in the Phase II study. This suggests that any immune responses generated by 105AD7 are insufficient to have a significant effect on tumour growth, in patients with advanced disease. Work has therefore focused on immunising patients with primary colorectal cancer. Patients receiving 105AD7 prior to resection of their primary tumours, showed an increased number of activated lymphocytes, and apoptosis, at the tumour site, relative to a well-matched control group. The numbers in the survival analysis based on patients recruited by the previous CRC fellow, are insufficient to show whether any of these immunological changes confer a survival advantage. This question can only be answered in a large, prospective, placebo-controlled study in patients with primary colorectal cancer.

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I am indebted to Professor JD Hardcastle, Dr Lindy Durrant and Mr. John Scholefield for their unstinting support, and giving me the opportunity to complete this work. I would like to thank Dr Adrian Robins for the advice he has given me over the last two years.

This work would also not have been possible without the help of Professor Jim Carmichael, and Professor Herb Sewell from the University Departments of Clinical Oncology and Immunology respectively.

I would also like to acknowledge the role played by the previous CRC Fellow, Mr Declan Buckley. He set up the Phase II study, and recruited the first 60 patients. He also immunised 23 patients with primary colorectal cancer, on whom the survival analysis in Part 3 is based.

The Phase II study would also not have been completed without the support of the other trial centres involved. These included Professor JRT Monson and Mr. Nick Abdullah in Hull, Professor P Guillou, Mr. Shaw Somers and Mr. Al Windsor in Leeds, and Professor H. Calvert and Kevin Fishwick in Newcastle. Christos Pagonis in the Data Centre at CRC, and the monitoring of Sarah Armstrong and Lucinda Light, also contributed to the satisfactory conclusion of the study. Statistical analysis was performed by Dr Katherine Fielding, at the Trent Institute of Health Services Research, Nottingham University, to whom I am indebted for the advice she has given me on all aspects of this work. I would also like to thank the Cancer Research Campaign and Imclone systems Ltd for funding this study. I should also acknowledge the unforgettable contribution made by Dr Neil Rotherham at the randomisation company 'S-cubed'.

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The immunohistochemistry staining using APO2.7 was performed by Rula Quaitafan, and subsequently submitted to Nottingham University as part of her MSc thesis. This work was however supervised by myself and Adrian Robins, and funded by money I raised from the Special Trustees of the Queens Medical Centre.

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On a more personal note I would like acknowledge the support of Dr. Anne Thomas, and to take this opportunity to formally thank my guardians, June and Alan Parsons. I owe them a lot.

HYPOTHESIS

The anti-idiotypic monoclonal antibody 105AD7 mimics the tumour-associated antigen 791T/gp72, present on 80% of colorectal cancer cells. Alternative presentation of this epitope should stimulate a naive immune system, inducing T-cell responses. A Phase I study in patients with advanced disease has shown that 105AD7 is non-toxic. In addition 9/13 patients showed evidence of IL-2 production, or evidence of a T-cell blastogenesis against 791T/gp72 expressing cell lines. Immunisation also conferred a survival advantage on patients who received the vaccine, relative to a contemporary group of patients [Denton GWL 1994].

A Phase II, double-blind, placebo-controlled survival study has been performed, to test these results. In addition further studies on patients with primary colorectal cancer, have assessed whether immune responses can be seen in the peripheral blood, and at the tumour site of patients who receive the vaccine prior to surgery.

ABBREVIATIONS

Ab	Antibody.
AUC	Area under the curve.
CEA	Carcinoembryonic Antigen.
CR	Complete Response.
CRC	Cancer Research Campaign.
CTL	Cytotoxic T Lymphocyte.
DTH	Delayed Type Hypersensitivity.
ELISA	Enzyme Linked Immunosorbent Assay.
FA	Folinic Acid.
FCS	Fetal Calf Serum.
FITC	Fluoroscein isothiocyanate.
5-FU	5-Fluorouracil.
HLA	Human Leucocyte Antigen.
ICAM-1	Intercellular Adhesion Molecule.
IFN	Interferon.
IL	Interleukin.
i.d.	Intradermal.
i.m.	Intramuscular.
i.v.	Intravenous.
LAK	Lymphokine Activated Killer.
LFA-1	Leucocyte Function Antigen - 1.
MAb	Monoclonal Antibody.
MHC	Major Histocompatibility Complex.
NC	No Change.
NK	Natural Killer.
PBL	Peripheral Blood Lymphocyte.
PBS	Phosphate buffered saline.

PD	Progressive Disease.
PE	Phycoerythrin.
PR	Partial Response.
RPMI	Rosswell Park Media Institute.
SIP	Standardised Isotonic Percoll.
TAA	Tumour Associated Antigen.
TBS	Tris Buffered Saline.
TGF β	Transforming Growth Factor β.
TIL	Tumour Infiltrating Lymphocyte.
TNF α	Tumour Necrosis Factor.
TSA	Tumour Specific Antigen.
VCAM-1	Vascular cell adhesion molecule-1.

Chapter 1.

INTRODUCTION.

Immunotherapy of colorectal cancer.

CONTENTS.

The history of cancer vaccines.

The concept of attempting to vaccinate against tumours was originally proposed several hundred years ago. One of the first people to attempt it was Louis XIV of France. He allegedly injected himself with fragments of tumour, in order to induce an immune response [A. Dalgliesh 1996]. Work in the late nineteenth century by Hericourt and Richet showed that sera could be raised against tumours in dogs and donkeys. Further work showing that 'magic bullets', or antibodies, could be used in the treatment of malignancy was proposed by Paul Ehrlich in 1900. It wasn't however until the work of Coley, a New York surgeon, that the foundations of modern immunotherapy were established. He noted that patients who recovered from severe post-operative septicaemia, sometimes had regression of their residual tumours. He coined the phrase 'Coley's toxins', and went on to induce prolonged survival in a small number of patients with osteogenic sarcoma, by means of extracts prepared from haemolytic streptococci, and Bacillus prodigiosus [Coley 1911]. He also established some important principles, namely that a minimum dose of vaccine is required, and that repeated doses should be given.

It had been observed that mice recovering spontaneously from successfully implanted tumours usually resist reinnoculation with the same neoplasm. It was due to this observation that a method of active immunization against tumours was devised [Besredka 1935]. This consisted of innoculating susceptible animals intradermally with a small amount of tumour cell suspension. In a number of cases, the resulting tumours will regress, and the animals that recover remain immune to a further tumour challenge. In order to eliminate the possibility that the immunity obtained was caused by genetic differences between tumour and recipient, work was done using in-bred mice [Gross 1943]. One hundred

and fifteen mice of the C3H strain were innoculated i.d. with a cell suspension of a sarcoma that had been induced by methylcholanthrene in an animal of the same line. Spontaneous regression of the intradermal tumours was seen in 21 animals. Repeated intradermal reinnoculation with the same tumour was generally unsuccessful, showing that immunity resulting from the intra-dermal dose was specifically against the tumour, and does not depend on genetic differences. This premise was further substantiated in work by Prehn, Klein and Old.

Despite all this work there still remained a further question to be answered. Scientists had observed that while oncogenic mutations are common, the development of cancer was relatively rare. This led to the concept of 'immunosurveillance', or the abilty of the body to 'mop up' malignant cells, before tumours developed [Burnett 1970]. In addition spontaneous tumour regression was seen in malignant melanomas and Grawitz tumours, further substantiating a link between the immune system and tumour development [Fairlamb 1981][McGovern 1975].

Tumour associated and tumour specific antigens.

There are two categories of antigen, or epitope, present on the surface of cancer cells - tumour associated (TAA), and tumour specific (TSA). The former may occur due to over-expression of a normally expressed antigen, re-expression of antigens normally repressed in differentiated tissue, or the occurrence of antigen on tissue where it is not normally present. They are not confined to a single type of malignancy - CEA for example is a TAA that occurs on colorectal, gastric, breast and pancreatic. TSA's such as k-*ras* form either through cellular mutations or by the expression of viral glycoprotein envelopes on cell membranes [Lennox ES 1982].

There are a number of different immunotherapeutic strategies currently under evaluation, all of which will be discussed. They include active non-specific immunotherapy, adoptive immunotherapy, monoclonal antibody therapy and active specific immunotherapy (ASI).

Active Non Specific Immunotherapy.

Active non specific immunotherapy aims to augment the body's immune response, without directing it against any individual TSA or TAA. This followed on from the work of Coley, and to date a number of approaches have been assessed.

Intraperitoneal administration of Bacille Calmette-Guerin (BCG) to 19 patients with advanced colorectal cancer showed encouraging results, with minimal toxicity [Falk RE 1976]. A median survival of 13.2 months was observed, though patients did in addition receive 5-FU. A significant prolongation of disease-free interval and survival, was seen in 83 patients with Dukes C tumours, randomised to receive BCG+/- 5-FU [Mavligit GM 1976]. The comparison however was made with a historical control group, and results therefore need interpreting with caution. BCG has been used prospectively in conjunction with autologous colorectal tumour cells in patients with Dukes B and C cancers [Hoover HC 1993]. There was however no significant difference in terms of survival or disease-free survival between the 41 patients who received the vaccine, and the 39 unimmunised patients in the control group. Other work using BCG in patients with Stage II and III disease, has confirmed the lack of benefit, in terms of overall, and disease-free survival [Richards F 1979].

IL-2 is a 15.5 kDa glycoprotein that plays a central role in immune regulation [Smith KA 1988]. Rosenberg's group administered the cytokine alone to 155 patients with advanced malignancy [Rosenberg SA 1989]. While objective response rates of 22% and 24% were seen for renal cell adenocarcinoma and melanoma, there was no regression of any colorectal cancer metastases. Four patients died of therapy related complications, and many experienced nausea, vomiting, diarrhoea, and malaise. Other side effects noted included a decrease in peripheral vascular resistance,

hypotension, oliguria, and increased capillary permeability. IL-2 has been given pre-operatively to 50 patients with metastatic colorectal cancer [Brivio F 1996]. The cytokine effectively neutralised any post-operative lymphocytopaenia, and a prolonged survival time was seen. It should however be noted that all patients received 5-FU and folinic acid. Murine studies have suggested that IL-2 may act synergistically with IL-1 [Proietti E 1993]. Administration of both cytokines to 14 patients with advanced colorectal cancer, showed objective responses in 7, with toxicities similar to those described above [Triozzi P 1995]. A combination of IL-2 with IL-4, and use of the killer cell growth factor IL-12 have also been proposed as potential forms of active non-specific immunotherapy [O'Hara RJ 1997][Yamaue H 1996].

Levamisole is minimally toxic, and has been shown to augment the immune response by potentiating T cell, macrophage and neutrophil function. Results in the clinical setting have however been disappointing, with 2 randomised trials showing no survival benefit, when compared with placebo [Arnand JP 1959][Chlebowski RT 1988].

Recent work presented at the 1997 American Association of Cancer Research, has suggested that diphtheria toxoid may act as an immunostimulatory agent in patients with high risk cancer [Buzzi S 1997]. Significant increases in serum levels of IL-2, IL-6, IL-8 and TNF-α were seen following administration, with only 2 of the 22 patients showing evidence of recurrence at 5 years.

Adoptive Immunotherapy.

Adoptive immunotherapy is a treatment approach in which cells with anti-tumour reactivity are administered to a tumour-bearing host, in which they mediate, either directly, or indirectly, the regression of the established tumour [Rosenberg SA 1988]. This approach showed encouraging results in animal tumour models, and as such was extended to the treatment of humans [Fefer A 1976][Kedar E 1983]. There are broadly speaking two strategies. The first involves leukapherising mononuclear cells from the peripheral blood, stimulating them with IL-2, and reinfusing them into the patient. The second requires lymphocytes to be separated from fresh tumour specimens, stimulated in IL-2, and then infused back into the patient.

Incubation of human peripheral blood lymphocytes with IL-2, generates lymphoid cells capable of lysing fresh NK resistant tumour cells. These have been termed LAK cells. Adoptive transfer of LAK cells in combination with IL-2 caused regression of pulmonary and hepatic metastases from MC-38 murine colon adenocarcinoma [Lafreniere R 1985]. Provisional work showed partial responses in 3 of 26 colorectal cancer patients receiving LAK + IL-2 [Rosenberg SA 1987], with toxicity confined to hypotension, weight gain, and oliguria. Further work has confirmed these findings, with one complete, and four partial responses seen in a total of 30 patients [Rosenberg SA 1989].

A combination of freshly isolated expanded TIL, infused with cyclophosphamide and IL-2, caused regression of all hepatic metastases in mice bearing the MC-38 colon adenocarcinoma [Rosenberg SA 1986]. In view of this sixty-six patients were treated with this regime, of whom 2 had colorectal cancer. Objective responses were seen in up to 50% of

patients, though these were generally in melanomas, and renal cell carcinoma [Rosenberg SA 1989].

Adoptive immunotherapy suffered a setback when work was published suggesting that expanded TIL become trapped in liver, lungs and spleen, rather than at the tumour site [Griffith KD 1989]. Despite some work to the contrary, suggesting that TIL could be seen in 68% of melanomas, Rosenberg's work has now concentrated on adhesion of TIL to endothelium [Pockaj BA 1994][Adams DH 1997].

Monoclonal Antibody Therapy.

The work of Ehrlich was further continued in 1953 by Pressman D and Korngald L. They showed that radiolabelled polyclonal sera could be used to image rat osteosarcoma, thus showing that specific anti-tumour antibodies could be raised *in vitro*. Further work along these lines was made possible by the development of hybridomas for the production of monoclonal antibodies [Kohler 1975]. Examples include 17-1A, a murine IgG2a antibody against a 26kDa polypeptide tumour associated antigen, known as GA 733-2 (or CO 17-1A), present on the surface of colorectal cancers, and CA 19-9, now more commonly used in pancreatic carcinoma [Herlyn M 1979][Koprowski H 1981][Ross AH 1986]. 791T/36 is a murine monoclonal antibody against a 72 kDa glycoprotein expressed on a human osteosarcoma cell line [Embleton MJ 1981]. Further work has shown that this TAA is present on ovarian and bladder cell lines, and more importantly is present on 70% of primary colorectal tumours, and 85% of secondaries [Armitage NC 1984][Farrands PA 1982].

Hybridoma techniques have enabled antibodies against tumour-antigens to be manufactured in unlimited quantities. Therapeutic applications of these antibodies may centre on their use as immunotherapy agents, in radioimaging, or as carriers of cytotoxic agents.

Antibody coated tumour cells may be destroyed by a variety of mechanisms, including apoptosis, complement-dependent cytolysis, and antibody-dependent cell-mediated cytotoxicity (ADCC). A review of 8 trials using 17-1A in over 200 patients with colorectal cancer showed a response rate of around 5%. The effect was short-lived, though associated toxicity was low [Wadler S 1991]. A further 5 of 24 patients with metastatic colorectal cancer showed evidence of tumour regression [Fagerberg J 1995a]. As ADCC is one of the effector mechanisms for tumour cell death,

then the action of the antibody should be potentiated by granulocyte macrophage-colony stimulating factor (GM-CSF). This was thus tested on 20 patients with metastatic cancer. Two patients achieved complete remission, with one showing a minor response. A further two patients had stable disease [Ragnhammar P 1993]. 17-1A antibody has been used as post-operative adjuvant therapy in 189 patients with Dukes C tumours. Patients receiving 17-1A had a 30% and 27% reduction in death and recurrence rate, respectively. Toxic side effects were infrequent, consisting of only mild constitutional and gastrointestinal symptoms [Riethmueller G 1994]. An update of this work was presented at the American Society of Clinical Oncology in 1996, confirming reductions in mortality rate and tumour recurrence by 32% and 23% respectively, after a median follow up of 7 years [Riethmueller G 1996]. This data is currently being tested in a randomised, multicentre Phase III study, recruiting patients with Dukes C tumours to one of three arms 5-FU and FA, m17-1A and 5-FU and FA + m17-1A [Pullyblank AM 1997].

Monoclonal antibodies may also be bound to radionuclides, and used in conjunction with other imaging modalities for the detection of colorectal malignancy. Initial work was performed on resected specimens, in order to confirm that antibody had localised within the tumour [Farrands PA 1982]. Imaging using the same radionuclide bound to 791T/36 demonstrated a specificity of 56% in primary tumours, and 87% in disseminated disease [Armitage NC 1984]. Monoclonal antibodies may also be used to deliver therapeutic doses of radiation direct to tumours - radioimmunotherapy. The concept has been investigated primarily in haematological malignancies, but has been extended to advanced colorectal cancer [Press OW 1993]. A Phase I study using chimeric T84.66 (an anti-CEA IgG1) labelled with ^{90}Y has been performed in 3 patients [Wong JYC 1995]. They found no evidence of antibody related toxicity, and

concluded that this was potentially a valid therapy. A recent review has called for a multicentre study using this approach, and for further work to optimise antigen targetting, radionucleotide, and fractionation [Bischof Delaloye A 1995]

In a similar vein to the work outlined above, it has been possible to use monoclonal antibodies to deliver cytotoxic agents to the site of the tumour. *In vitro* experiments have been performed using the immunotoxin XMMCO-791-RTA, a conjugate of 791T/36, and the plant derived toxin ricin A. The immunotoxin has shown specific effects against gp72 expressing cell lines, and human tumour xenograft [Byers VS 1987][Embleton MJ 1986]. A phase I study on 17 patients with advanced colorectal cancer showed evidence of response, but up to a 25 % incidence of mental disturbance [Byers VS 1989]. An immunoconjugate of 30.6,I-1, JGT and n-acetylmelphelan has been used in a phase I study on patients with identical disease to above. Three patients showed minor responses, with acceptable, minor levels of toxicity, following hepatic artery infusion [Tjandra JJ 1989].

Antibody-directed enzyme prodrug therapy (ADEPT) is a new treatment for cancer, in which an antitumour antibody conjugated to an enzyme is given intravenously. The antibody causes the enzyme to concentrate in the tumour after it has been cleared from normal tissues. A prodrug is then given, which is converted by the enzyme to an active cytotoxic drug within the tumour. One such enzyme is the bacterial carboxypeptidase G2, which may be used in conjunction with the prodrug CMDA, a monomesyl benzoic acid mustard alkylating agent, inactivated by linkage to a glutamate. The CRC is due to launch a trial of ADEPT in patients with advanced colorectal cancer in 1997 [Leonard PC 1997].

The majority of antibodies used in the aforementioned work are murine in origin. These have the propensity to be recognised as foreign by

the patients immune system, leading to HAMA (Human anti-mouse antibodies) in 30-50% of patients [Courtney-Luk LS 1986][Larson SM 1983] [Schroff RW 1985]. The formation of HAMA can hasten blood clearance, and thus compromise the imaging or therapeutic efficacy of the antibody [Klein JL 1988][Pimm MV 1985]. There are several ways of removing this problem. The first is to link the animal variable region to the human constant domain. These 'chimeric antibodies' are not as immunogenic, and are thus less likely to form HAMA [Khazaeli MB 1990][LoBuglio AF 1992]. The desired specificity is however retained by the variable region protein [Winter 1991]. Another way of reducing immunogenicity is to use single-chain Fv antibodies. These consist of variable heavy and light regions bound by a short synthetic peptide, and have the advantage that protein that is not required for antigen binding is not included. There is also the added advantage that these antibodies may penetrate further into tumour, due to their lower molecular weight. MFE-23 is a high affinity scFv against the tumour antigen CEA. Nine patients with colorectal cancer were recently given MFE-23 labelled with iodine-123 [Begent RHJ 1996]. All known tumour deposits were located, and the authors conclude that this method might be extended to encompass antibody-directed therapy, in addition to imaging.

Active Specific Immunotherapy (ASI).

Individual T-cell receptors are genetically determined, and are capable of binding specifically to any one of a large number of antigens, which have been proteolytically cleaved and bound to cell surface glycoproteins. These glycoproteins are encoded in a cluster of genes termed the Major Histocompatibility Complex (MHC), and may be either Class I or Class II. CD8+ lymphocytes recognize peptide bound to Class I MHC, causing them to proliferate and differentiate into effector Cytotoxic T-lymphocytes . CD4+ lymphocytes interact with antigen in association with MHC Class II, forming either Inflammatory T_H1 cells or Helper T_H2 cells (Figure 1). The former interact with CTL, macrophages, and NK cells, and secrete a wide variety of cytokines including IL-2, TNFα and IFN-γ. The latter produce cytokines such as IL-4 and IL-10, that stimulate B cell proliferation. It is T-cell responses, rather than antibody responses, that are the most effective against tumours [Robins 1986].

Tumour antigens are incapable of eliciting an immune response per se, for a number of reasons. To be recognised by T-cells, antigen must be presented by specific Antigen Presenting Cells (APC), with a co-stimulatory signal delivered through the T cell surface molecule CD28, by its natural ligand B7.1 [Hodge JW 1994]. B lymphocytes, macrophages and dendritic cells are all capable of acting as APCs, presenting such epitopes to T lymphocytes. Without this co-stimulatory signal, anergy occurs, and no immune response is generated. Colorectal tumour cells are very poor at presenting antigen to T-lymphocytes. This may be due to their inability to process epitope, an absence of adhesion or co-stimulatory molecules, the presence of inhibitory cytokines, or the fact that these tumours have low

expression of the MHC molecules necessary for interaction with the T cell receptor [Gimmi C 1996].

As the name suggests, ASI attempts to stimulate the immune system to target a specific antigen, on the surface of tumour cells. A number of different approaches have been adopted. Anti-idiotypic antibodies mimic antigen, and elicit T cell responses. Polynucleotide vaccines (DNA and RNA) encode the tumour antigen, while vaccines based on viral vectors provide an alternative way of altering the host genome. Oncogene products may act as TAAs, against which vaccines may be developed, and autologous tumour may be processed to form mucin or heat shock protein based vaccines. Adjuvants aiming to enhance ASI are also discussed.

Heat Shock Proteins (HSP).

Heat shock proteins are a group of proteins present in all living cells. HSP preparations contain a broad array of peptides tightly bound to HSP molecules [Li Z 1993][Udono H 1993]. They offer a number of advantages, as cancer vaccines. If a lasting therapeutic effect is to be conferred by a vaccine, then a cytotoxic T-cell response needs to be generated [Leclerc JC 1973][Rouse BT 1972][Topalian SL 1990]. HSP's can not only generate this, but can in addition can show evidence of a memory T-cell response [Janetzki S 1994]. Vaccination with HSP-peptide complexes circumvents the necessity for identification of the antigenic epitopes of cancer cells, as HSP's are naturally complexed with the entire repertoire generated in the cell. Another advantage of such an approach is that an immune response will be generated against all antigens present in the tumour. In addition they require no adjuvants in order to elicit a CTL response, and the complexes can be purified rapidly. As the vaccine is

autologous, no material is innoculated into the patient, that they haven't already been exposed to, thus reducing the chance of toxicity.

A number of studies have shown that injection of apparently homogeneous HSP preparations from a given tumour to syngeneic rats or mice, renders the animal resistant to that particular tumour [Srivastava PK 1984][Srivastava PK 1986][Ullrich SJ 1986][Palladino MA 1987]. For this treatment modality to be successful, each vaccine would have to be "custom built" for individual patients, using autologous tumour. Though toxicity would be minimal with this approach, if it proves technically difficult, excessively time consuming, or expensive, then it will not be viable as a vaccine for colorectal cancer. Despite these limitations, Phase I studies are currently ongoing [Srivastava PK 1996].

Mucins.

Human epithelial mucins are a family of high molecular weight glycoproteins that lubricate and protect the underlying gastro-intestinal mucosa. They are characterized by a large number of O-glycosylated tandem repeat domains which vary in length, number, and extent of O-glycosylation [Fontenot JD 1993][Strouss GJ 1992]. Novel mucin epitopes are expressed by tumour cells, due to aberrant glycosylation of pre-existing mucins [Itkowitz S 1991][Jerome KR 1992]. This results in shorter sugar side chains, with concomitant exposure of peptide antigens (Figure 3).

Evidence has accumulated showing that T cells specific for native epitopes on the mucin polypeptide core tandem repeat can be expanded *in vitro* [Barnd DL 1989][Jerome KR 1991]. Further work has also shown that a humoral response may be generated, with B cells recognizing the mucin tandem repeats [Kotera Y 1994]. Thus antibodies have been detected in the

blood of patients with colonic carcinomas, breast and pancreatic tumours [Gourevitch MM 1995].

A vaccine has been formed by transfecting the gene for the tumour associated antigen (MUC-1) into Epstein Barr virus immortalized B cells [Pecher 1996]. The latter act as Antigen Presenting Cells priming cytotoxic T-cell precursors and Delayed Type Hypersensitivity (DTH) responses in the two chimpanzees immunized. A Phase I study using a 105αα MUC-1 peptide admixed with BCG has recently been used in 30 patients with advanced colorectal cancer [Goydos JS 1996]. A number experienced ulceration at the injection site, and systemic symptoms such as fever, rigors and malaise. Immunologically, DTH responses were seen against mucin-specific peptides, and 7 out of 22 patients tested showed a 2-4 fold increase in CTL [McKolanis JR 1996]. Clinically, however, only two patients had stable disease. Eleven patients with advanced colorectal cancer have been immunized with Theratope® sialyl-Tn-KLH cancer vaccine in Detox™ adjuvant, following low dose cyclophosphamide therapy [Reddish MA 1996]. This Phase II study showed that patients with higher anti-Sialyl-Tn IgG antibody titres following vaccination survived longer than patients with lower titres, thus suggesting an immune response.

Recently it has been reported that the gene MUC-1 can be expressed in baculoviruses, leading to the expression of underglycosylated mucin molecules [Ciborowski P 1996]. This may remove the need to process autologous tumour, making this approach more attractive.

Peptides.

Peptide vaccines can bind to MHC molecules and elicit immune responses, as described. Generation of CTL would be further enhanced if

the peptide was presented by an APC, such as a Dendritic cell (DC) [Steinman RM 1993]. *In vitro* work using a murine model has shown that antigen-specific Cytotoxic T-Lymphocytes may be generated following subcutaneous administration of irradiated bone-marrow derived DC, pulsed with OVA peptide *in vitro* [Young 1996]. These results have been confirmed in a separate study, which in addition showed that RMA-S cells and normal syngeneic adherent splenocytes were effective in eliciting CTL in the B16/F10.9 melanoma tumour model [Nair SK 1997]. Immunisation of mice with mutant p53 peptide-pulsed DC generated from stem cells of other tumour bearing mice can induce effective anti-tumour CTL responses, and lead to significant antitumour effects [Gabrilovich DI 1996]. If the T-cell epitope is as yet undefined, as is the case for a number of cancers, then CTL can still be generated using unfractionated acid-eluted tumour peptides in conjunction with the method outlined above [Zitvogel L 1996].

Mutations in codon 12 of K-*ras* are frequently found in pancreatic adenocarcinomas [Gjertsen MK 1995]. Mutant p21 ras is therefore a tumour specific antigen, that can be recognised by human T-cells [Jung 1991]. Synthetic *ras* peptides have been used in conjunction with antigen-presenting cells as a vaccine for pancreatic cancer, with encouraging results. This approach could also be applied to colorectal carcinomas, which also show mutations in codon 12 of K-*ras* . As with heat shock proteins, this approach necessitates formation of vaccine from autologous tumour, with its attendant difficulties. It clearly is advantageous when the tumour antigen has not been identified, or is difficult to purify. One potential drawback of immunising with unfractionated tumour material, as compared with defined antigens is the theroretical risk of developing autoimmune disease [Nair SK 1997].

Polynucleotide-mediated immunisation

Intramuscular delivery of DNA or RNA vaccines has been shown to lead to gene expression in myocytes and myofibroblasts. This will lead to a continuous intracellular production of protein antigens that may be presented in association with Class I MHC molecules, thus eliciting CTL responses [Wolff JA 1990][Ulmer JB 1993].

The advantages of DNA vaccines are numerous. They can, for example, be easily purified, coated on gold particles, and given directly into tissues by gene gun (bolistics). DNA may also be combined with genes for cytokines such as IL-2, IL-6 or IL-7, or GM-CSF, in order to enhance the immune response generated [Irvine KR 1996][Syrengelas AD 1996].

Work has shown that mice may be immunised with a plasmid encoding the full length of complementary DNA for CEA [Conry RM 1994]. Evidence of humoral and cellular responses against the glycoprotein were seen in all of the 5 mice immunized, and 3 generated CEA-specific memory T cells. In addition a further 2 had IL-2/IL-4 release in response to CEA. Use of a minigene coding for a single antigen derived from mutant p53 has been shown in a mouse model to elicit CTL [Ciernik IF 1996]. Clearly evidence exists supporting this approach as a potential vaccine strategy. There are however no Phase I studies relating to its use in colorectal cancer, as yet.

Viral Vectors

Viruses may be used as vectors, to transfect cells with genes encoding tumour associated antigens. The aim of this gene therapy approach is to co-present a weak immunogen, such as CEA, with a highly immunogenic viral protein, in order to enhance the immune response. The DNA

encoding CEA is inserted into viruses, such as retroviruses, adenoviruses, baculoviruses, herpes, pox and vaccinia viruses. Cells infected express a protein product, recognised by anti-CEA antibodies. Animal work has shown that effective humoral and cell mediated responses can be generated, that correlate with delayed tumour growth [Kaufman H 1991] [Kantor J 1992][Kantor J 1993]. Phase I studies have used vaccinia encoding CEA, in patients with colorectal cancer [Hamilton JM 1994]. Toxicity was confined to inflammation at the injection site, and further work has shown that cytolytic T cell responses can be generated using this approach [Conry RM 1995][Tsang KY 1995].

The antigen 17-1A has recently been cloned, and expressed in baculovirus [Herlyn D 1997]. Alum precipitated recombinant antigen induced in mice, in conjunction with peritoneal macrophages as effector cells, has shown DTH responses. This vaccine has been administered to 7 patients with pancreatic and colorectal cancer. Four developed antibody responses.

Rosenbergs' group has recently shown that the most effective route of administration of a viral vector is intravenous. They postulate that once systemic, the virus is capable of infecting a larger number of cells, especially those in the reticuloendothelial system [Irvine KR 1997]. In spite of the interesting results *in vivo*, there may however be potential problems associated with the use of live attenuated, or recombinant vaccines, and it has been shown that immune responses generated after the first immunisation may inhibit replication of recombinant vaccinia virus innoculated at subsequent injections [Hodge JW 1994].

Safety and regulation are key issues in this area of gene therapy, as highlighted in a recent editorial in the Lancet (12th July 1997). Concerns have been raised about lymphomas developing in monkeys exposed to a retrovirus, and spongiform encephalomyelopathy occurring in mice

innoculated intraperitoneally with amphotropic murine leukaemia virus [Munk 1997]. A safer alternative would be to use non-replicating viruses such as avipox, especially if the vector is to be given IV. There is however good evidence that viruses may be used as vectors for gene delivery, to generate CTL responses against tumour cells. However unless toxicity and regulatory issues are addressed, it seems likely that non-viral approaches, such as liposomes, molecular conjugates, and naked DNA injection may be more promising.

Adjuvants.

The aim of an adjuvant is to augment the intended immune response. There have however been concerns relating to their potential toxicity [Gupta RK 1993]. Reports of sterile abscesses, autoimmunity, and in some cases cancer have all been documented [Hardegree. 1966][Hilleman 1966]. An increased understanding of the properties of oil-based formulations, and the emergence of novel vehicles such as liposomes, and other particulate carriers, has however resurrected interest [Alving CR 1995].

In view of the fact that the adjuvant field has become progressively more involved, a new classification has been proposed [Edelman 1990]. In this scheme, immunostimulating formulations can be divided into three primary categories: adjuvants, carriers and vehicles. The adjuvants include direct immunostimulating substances such as aluminium salts, lipopolysaccharides, lipid A, and muramyl dipeptide derivatives. The carriers are molecules that provide T-cell help for attached antigens. Vehicles, such as liposomes and oil-based emulsions, provide a "substrate"or platform for adjuvant, or carrier effects. They also provide a delivery mechanism, or depot site for entry into antigen presenting cells.

The classification may also include the term "adjuvant formulation", referring to mixtures of the aforementioned categories. An example would be liposomes adsorbed onto aluminium hydroxide. As can be imagined there is a considerable degree of overlap between the sub-divisions described.

As advances are made in adjuvant technology, their ability to enhance an immune response will increase. If this can be combined with any of the vaccine strategies outlined below, then a more efficacious form of therapy will be developed.

Anti-idiotypic antibody immunisation.

Immunisation with anti-idiotypic monoclonal antibodies may offer an alternative immunological approach to tumour therapy. The theoretical basis of this treatment modality is outlined in the network hypothesis [Lindenmann J 1974]. The premise is that antibodies (Ab1) against tumour associated antigens have specific idiotypes in their variable regions. Ab2 is an antibody against this idiotype. The anti-idiotypic monoclonal antibody may therefore 'mimic' the antigen on the surface of the tumour cell (Figure 2). The concept of the anti-idiotypic antibody acting as an 'internal image' of the antigen implies that this novel presentation of tumour epitope should elicit an immune response [Nisonoff A 1981][Roitt IM 1981][Chattopadhyay P 1992]. The Jerne hypothesis also predicts the development of anti-anti-idiotypic monoclonal antibodies (Ab3).

This approach has a number of advantages over other forms of immunotherapy. Anti-idiotypic monoclonal antibodies may be presented by antigen-presenting cells in the context of Class I and II MHC, thus eliciting both cytotoxic and helper responses. Presentation of the epitope

in a different molecular environment may also act to break any tolerance that may have developed to the weakly immunogenic TAA's [Raychaudhuri 1989]. Anti-idiotypic monoclonal antibodies have a longer half life in the peripheral blood, and are more resistant to proteolytic digestion. They can also be used when the TAA is either unknown, or difficult to purify in the required quantities. In addition there is evidence that the anti-idiotypic antibody may be more effective in eliciting an immune response than the actual antigen. Neonatal mice, incapable of responding to a bacterial capsular polysaccharide, were able to mount an immune response to the antigen, when vaccinated with anti-idiotypic MAb [Stein KE 1984]. Furthermore *in vitro* human antigen-specific B-cell responses were more effectively induced by anti-idiotypic antibody vaccine than immunisation with group A streptococcal carbohydrate antigen [Bloem AC 1988]. The framework of the anti-idiotypic antibody is unlikely to express competing T cell epitopes, and the Fc region itself may be preferentially internalised and processed by Fc receptors expressed on antigen presenting cells. Anti-idiotypic antibodies are also cheaper, and less likely to give rise to autoimmune reactions [Bhattacharya Chatterjee M 1994]. Furthermore they are free from the potential dangers of retroviruses and genetic manipulations. Clearly the advantages to this form of therapy are numerous.

The premise that anti-idiotypic monoclonal antibodies could stimulate immunity has been tested. In addition to showing induction of helper and suppressor T cells for humoral immunity [Eichmann K 1978], various workers found that delayed-type hypersensitivity (DTH) responses could be elicited in animal models. Such responses to *p* -azobenzenearsonate (ABA) were observed in A/J mice following iv injection of anti-cross reactive idiotypic (CRI) antibodies, providing that the animals had been pre-treated with cyclophosphamide [Sy M-S 1980]. T

cells from lymph nodes taken from vaccinated mice could also transfer immunity to naive recipients. Similar results have been obtained in A/J mice, using 14A, an anti-idiotypic monoclonal antibody against a determinant on anti-ABA antibody [Thomas WR 1981]. Hyperimmunisation of BALB/c mice with MCA-1490 sarcoma produced an antibody (4.72) that could induce DTH responses upon transfer to naive mice [Forstrum JW 1983]. This response was both antigen-specific, and allotype restricted, confirming that it was anti-idiotypic,even though the Ab1, or antibody against MCA-1490 hadn't been established. The work did however add further support to the concept of anti-idiotypic immunisation, and show that immunity could be transferred.

The abilty of anti-idiotypic monoclonal antibodies to elicit an immune response that could be protective against a tumour challenge has also been established [Dunn PL 1987]. HIM/1/230 is an antibody (Ab2) against an idiotype on 11/160 (Ab1). The latter is an antibody against an epitope on the Hooded rat sarcoma HSN. Vaccination with 3 challenges of anti-idiotype stimulated production of Ab3, indistinguishable in antigen specificity from 11/160. Immunised animals also showed reduced tumour take following an i.v. challenge with HSN cells.

Polyclonal anti-idiotypic antibodies to CO17-1A and GA733 have been developed [Herlyn D 1985][Herlyn D 1986]. When rabbits were immunised with CO17-1A, antibodies were produced which bound to human tumour cells expressing the TAA recognised by CO17-1A. In view of these provisional results, 30 patients with advanced colorectal cancer were immunised with between 0.5mg and 4mg of alum-precipated polyclonal goat anti-id antibody [Herlyn D 1987]. Humoral responses were seen, and all showed evidence of Ab3 production. This antibody showed identical binding of tumour cells as that observed with Ab1. Six patients showed partial clinical remission and a further seven showed arrest of

metastases following treatment. Of these 13 patients, 9 also received chemotherapy, making conclusions about the efficacy of Ab2 more difficult. A follow up trial by the same group used a different goat polyclonal antibody in 12 patients who had undergone resection of their primary tumours [Herlyn D 1991]. Six of these patients developed Ab3, and 2 had antigen specific T cells, which proliferated in culture on stimulation with the GA733 antigen. In addition 7 of the original 12, showed tumour remissions which lasted between 1.1 and 4.1 years following immunisation. In support of Herlyn's work, evidence of cellular immunity has been seen in a further patient with advanced colorectal cancer [Samonigg H 1992]. This group used SCV106, a goat anti-idiotypic monoclonal antibody that mimics the TAA 17-1A. The patient concerned had two lung metastases from previously resected colonic carcinoma. These were removed after completion of the antibody course, and subjected to analysis. Antibodies eluted from resected tissue were confirmed to be anti tumour antigen in conventional ELISA, and immunohistochemical analysis of tissue confirmed "massive" infiltrate of T-helper, and cytotoxic T cells.

An anti-idiotypic monoclonal antibody mimicking the TAA GA733-2 has been given to patients with primary colorectal cancer [Fagerberg J 1995 b]. DTH responses, IL-2 and IFNγ were seen in all patients, indicating T cell immunity. Five patients had evidence of Ab3 production, suggesting a humoral response against the anti-idiotypic antibody.

Recent work has shown how passive immunotherapy with unconjugated monoclonal antibodies may give rise to an idiotypic network response, that correlates with clinical response [Fagerberg J 1995 a]. Twenty-four patients with metastatic colorectal cancer were treated with MAb 17-1A. After completion of therapy, five of the patients had peripheral blood T cells specifically recognizing human anti-MAb 17-1A

idiotypic antibodies. These same five patients were the only ones in the study who had any objective tumour regression, following MAb therapy. The association between the presence of anti-idiotypic reactive T cells and clinical response was statistically significant (p=0.00002). Clearly this adds further support to the concept of anti-idiotypic antibody immunisation.

The evidence outlined above suggests that anti-idiotypic antibodies are capable of eliciting cytotoxic and helper T cell responses. It is likely that following immunisation intradermally or intramuscularly, antibodies are taken up by Langerhans cells, a subset of immature tissue Dendritic cells (DC). Following antigenic stimulation these cells resume their migratory behaviour, travel to draining lymph nodes, where they arrive as mature DC. Such DC are particularly adept at antigen presentation because they express high levels of MHC, co-stimulatory molecules, and adhesion molecules. It was initially thought that peptides generated in the cytosol could only be presented in association with MHC Class I molecules, and those in intracellular vesicles with MHC Class II. More recent work has shown that antigen from the extracellular fluid, and on liposomes/adjuvants may also stimulate CTL [Kovacsovics-Barkowski S 1993]

The anti-idiotypic monoclonal antibody 105AD7.

The anti-idiotypic monoclonal antibody 105AD7 was originally developed at the University of Nottingham [Austin EB 1989]. A patient with advanced colorectal cancer received the murine monoclonal antibody C46 (Amersham), against CEA, and subsequently suffered a Type1 hypersensitivity reaction. It transpired that he had previously been given radiolabelled 791T/36 in order to image liver metastases. In the ensuing search for HAMA, an anti-idiotypic antibody that inhibited

binding of 791T/36 to gp72 positive tumour cells was discovered. This was termed 105AD7.

Pre-clinical studies showed that 105AD7 could induce DTH responses to human tumour cells in experimental animals [Austin EB 1991]. In view of these preliminary results, a Phase I study was performed. Thirteen patients were recruited over a twelve month period beginning in February 1990. All had liver metastases from previously resected colorectal cancer, and two of these had evidence of extra-hepatic disease [Denton GWL 1994].

A control group consisted of forty-five contemporary patients with advanced colorectal cancer. These were entered into 'treatment', and 'no treatment' arms of a multicentre Phase III study using an oral chemotherapeutic agent. Thirty-seven of these patients had liver metastases.

The monoclonal anti-idiotypic antibody 105AD7 was produced *in vitro* according to CRC/NIBRC guidelines [Robins RA 1991]. It was purified by affinity chromatography, and sterilised by filtration and heat treatment. Skin test doses of 10μg in 0.1 ml in sterile saline, and intramuscular doses of 100μg on aluminium hydroxide gel (Alhydrogel 85, SuperPhos Biosector a/s Vedbaek, Denmark) were formulated. Samples of the seed lots passed testing for viral contamination, and sterility.

Patients were immunised with an intradermal skin test dose of 10μg of 105AD7. They returned 24 hours later, and 7 received 100μg, and 6 patients 200μg of 105AD7 in aluminium hydroxide. Patients were admitted to hospital for the first week post immunisation.

After discharge they were seen on a weekly basis for the following five weeks. Patients were examined at each visit, and any new symptom investigated. In addition to this peak expiratory flow rates, urinalysis, a

full blood count, serum urea & electrolytes, liver function tests, complement levels , and immunological analysis were all performed. A chest X-ray and electrocardiogram were done pre-immunisation, at 1 week, and on completion of the study. In order to demonstrate disease progression/regression, computerized tomography was used to image chosen indicator lesions at trial entry, and after each treatment period. Patients whose clinical condition was satisfactory at the end of the study were re-entered into further six week treatment cycles. Levels of IL-2 were measured at each treatment cycle, and blastogenesis experiments were carried out using cryopreserved lymphocytes. Patients serum was also screened for the development of antibodies to 105AD7.

A total of 35 immunisations were given to 13 patients recruited during the study period. Each had a skin test dose of 10μg of 105AD7 with a further 100μg, or 200μg given intramuscularly. A second skin test dose was subsequently given several days later. The most any individual patient received was 7 treatment cycles.

The main aim of the Phase I study was to show that there was no toxicity associated with immunisation with 105AD7. As such a number of clinical observations were made following 105AD7 administration. All patients remained stable following treatment with the study drug. The only laboratory features of note were an elevation of lymphocyte count 2 days post-immunisation with the 100μg dose, and a concomitant decrease in serum urea. Interestingly, none of these changes were noticed in those patients receiving 200μg. The only adverse event however, was one patient who presented 5 weeks post-immunisation with melaena, secondary to a bleeding duodenal ulcer.

Despite anti-idiotypic therapy, there was no evidence of regression of malignant disease. The survival analysis was not performed until the study had been completed, and was done from the date of diagnosis of

advanced malignant disease. There was no significant difference between this date, and the time of the original operation, in the immunised and unimmunised groups (medians of 5.5 & 6 months respectively). Survival was significantly better in the former group for both patients with hepatic metastases (p=0.007), and those with advanced disease (p=0.022). Analysis of the time to disease progression similarly showed a significant differance in favour of the 105AD7 group in the two groups with advanced disease (p=0.023, and p=0.013 respectively).

In terms of the immunological analysis, nine out of the thirteen study patients had a significant blastogenetic response to gp72 expressing tumour cells, or raised levels of IL-2 in their plasma. Six of these patients had a response in both assays, though three failed to show a rise in either. The highest level of IL-2 was seen following the first immunisation in five patients, the third in one, and the fourth course in a further two. IL-2 levels were seen to be raised between 1 and 3 weeks post-immunisation in all but one patient. Peak blastogenetic responses mirrored the findings for IL-2 in that a maximum was reached after the first dose of 105AD7 in five patients, and after the second in two. The five longest surviving patients showed a response in the immunological assays , thus showing agreement between the two sets of results.

Further analysis of plasma samples failed to show any development of anti-anti-idiotypic antibody (Ab3), or antibody development to tumour. There was no evidence of hypersensitivity to 105AD7 on intra-dermal skin-testing.

The most important point raised in the Phase I study was the lack of toxicity associated with 105AD7 immunisation. This compares with murine monoclonal antibody administration, where flu-like symptoms, arthralgia, and myalgia have been described [Chapman PB 1992] [Mittelman A 1992]. In the latter paper, local toxicity was also observed, in

the form of erythema, induration and ulceration. This was thought to be associated with the use of BCG, as opposed to the milder aluminium hydroxide, as the adjuvant. The lack of toxicity may also equate to the use of allogeneic rather than xenogeneic monoclonal antibody.

A median survival of twelve months in those patients immunised with 105AD7 was 300% higher than that obtained in the control group. This approximates to the figure obtained by Erlichman (12.6 months) using 5-FU and Leucovorin in patients with liver metastases from a colorectal primary. A survival of four months was however slightly lower than historical data suggests [Zubrod CG 1960]. Despite this observation, it should be borne in mind that all patients were recruited concurrently from the same clinic, and randomised within a separate chemotherapy trial with identical eligibility criteria.

Nine of the thirteen patients showed either a blastogenetic response, or evidence of IL-2 production on ELISA. The blastogenesis experiments were carried out on a time-course set of lymphocytes. The proliferative response observed was not shown with lymphocytes from patients prior to immunisation. The response in patients 3,4 and 5 indicated that 105AD7 induced a specific recognition event.

Interleukin-2 is a marker of in-vivo T-cell activation, and clearly, raised levels support the fact that 105AD7 causes a significant immune response. Work has been done showing that if 3000 units/kg/hr of IL-2 is infused, it achieves a serum level of 5-10 units/ml [Lotze MT 1985]. Despite the fact that patients with advanced colorectal cancer have low levels of the lymphokine [Lissoni P 1990], IL-2 levels of this order of magnitude were achieved post-immunisation.

The kinetics of IL-2 production are variable, with peak levels seen after the first immunisation in five patients, the third cycle in one patient, and the fourth cycle in a further two patients. Generally however elevated

levels were seen 1-3 weeks post-immunisation. It is still unclear as to whether the intradermal dose given contributes significantly to the immune response observed, and also what the optimum dose of 105AD7 actually is.

No evidence of anti-anti-idiotype (Ab3), or anti-tumour antibody responses were observed following 105AD7 immunisation. These have however been shown by Chapman and Mittelman following administration of mouse monoclonal anti-idiotypic antibodies. The doses used however in these studies were higher, and the antibody was given in conjunction with carrier proteins, and/or BCG vaccine. Use of xenogeneic antibody may increase immunogenicity, due to antibody constant regions acting as carrier determinants. However the fact that 105AD7 is human derived may explain the lack of toxicity associated with its use.

Despite *in vitro* lymphocyte proliferative responses, no evidence of Type IV delayed hypersensitivity was observed after i.d. challenge with 105AD7. This probably relates to either the timing of administration, or the processing of the soluble monomeric IgG molecule.

The phase I study has confirmed most importantly that 105AD7 is not associated with toxicity. It also showed that immunisation caused a significant survival difference, T cell blastogenesis, and interleukin-2 production. The aim of this thesis is in part to test the survival results of the Phase I study in a randomised, placebo-controlled Phase II study.

Figure 1. Presentation of epitope in association with Class I and II MHC, to CD4+ and CD8+ lymphocytes.

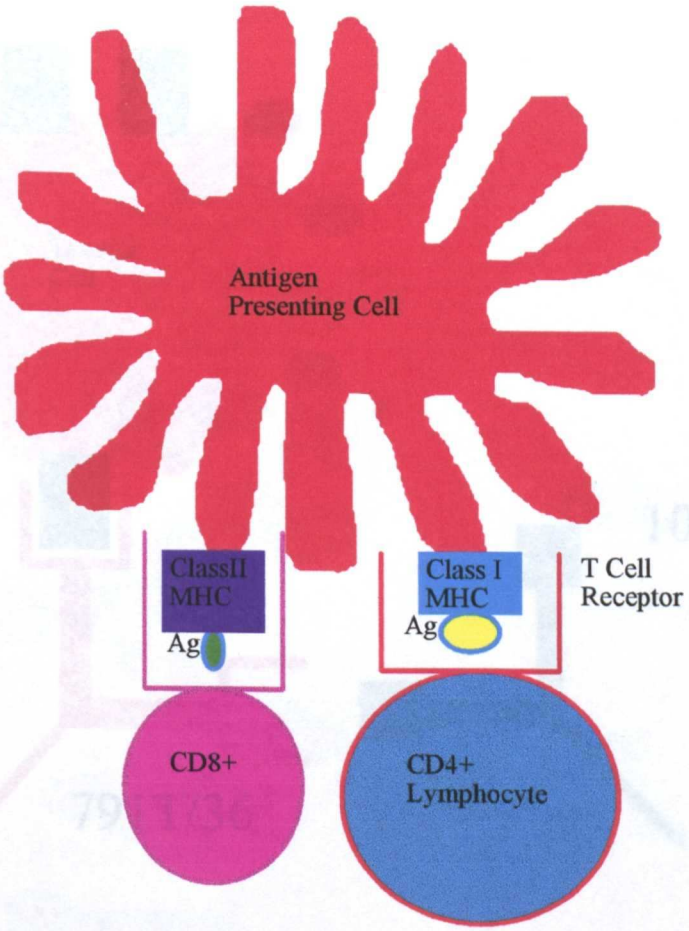


Figure 2. Concept of anti-idiotypic antibody immunisation, as applied to 105AD7.

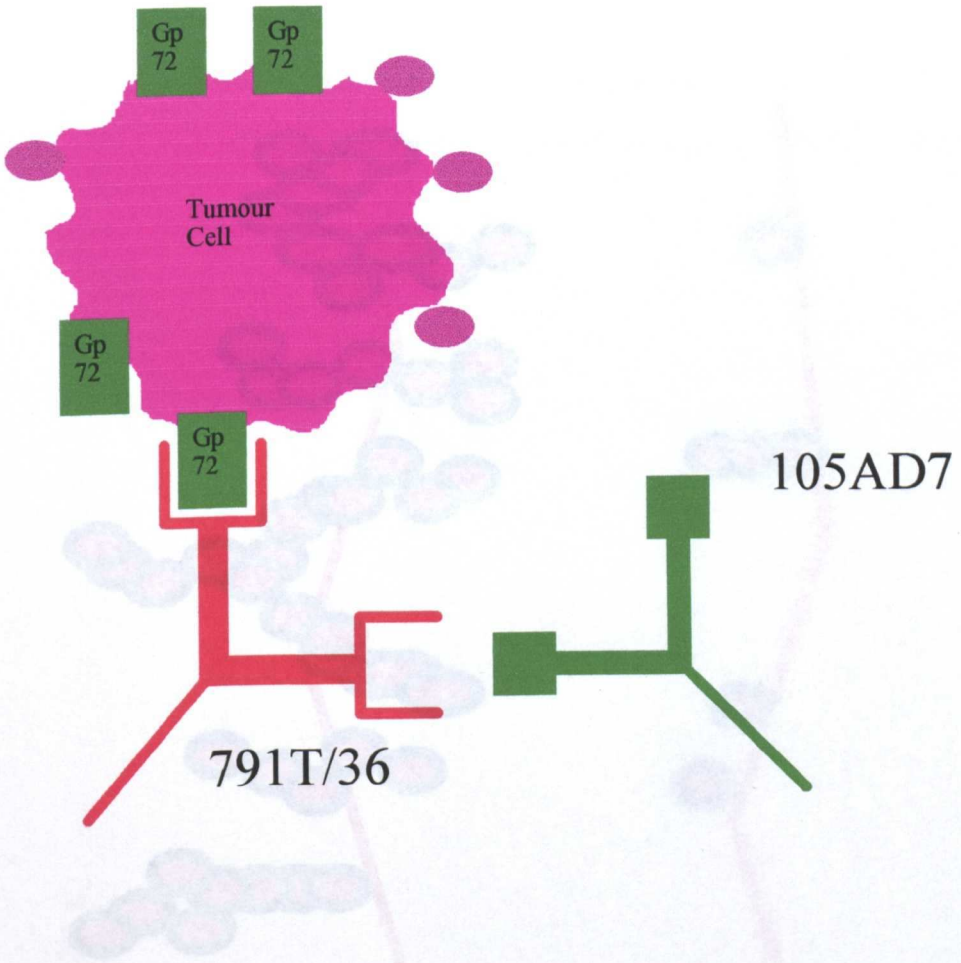
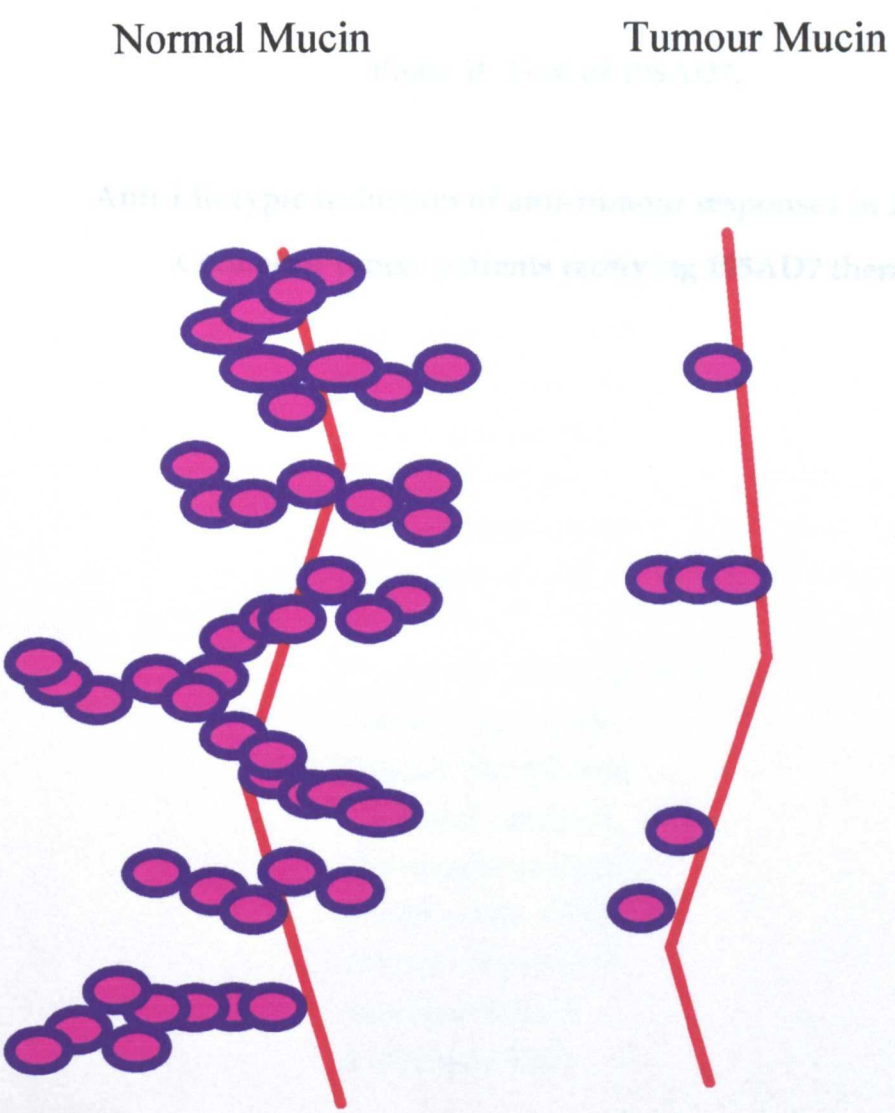


Figure 3. Aberrant glycosylation of tumour associated mucins.



Chapter 2

Phase II Trial of 105AD7.

Anti-idiotypic induction of anti-tumour responses in Advanced Colorectal cancer patients receiving 105AD7 therapy.

Contents.

Aim

This study aims to determine whether immunisation with the anti-idiotypic monoclonal antibody 105AD7 confers a survival advantage on patients with advanced colorectal cancer.

Materials and Methods.

Trial Design.

A randomised, double-blind, study comparing treatment with the human monoclonal antibody 105AD7 against supportive therapy in patients with advanced colorectal cancer. Patients were recruited in one of the following four centres - Nottingham, Hull, Newcastle and Leeds. For 105AD7 to be clinically effective, it would need to confer at least a survival advantage of 20% on immunised patients. Assuming a power of 90%, and significance at the 5% level, then statistically 162 patients would need to be recruited to the study.

Ethics Committee Aproval.

Approval by local ethics committees was obtained for this study.

Informed Consent.

The responsible physician informed the patient about the background and present knowledge of the anti-idiotypic monoclonal antibody 105AD7, with special reference to known activity and toxicity. The patient was informed that the treatment was experimental and that the exact degree of activity was unknown. They were also informed that their inclusion in the study would contribute to our knowledge further. Patients were made aware that this was a placebo controlled study, and that they had a 50% chance of receiving the vaccine. It was emphasised that the patient could refuse treatment, either before, or at any point during the study. Refusal to participate involved no penalty, or loss of

benefits to which the subject was entitled. An explanation of whom to contact for answers to pertinent questions about the research, and research subjects rights, and whom to contact in the event of a research-related injury was given to the subject. Prior to entry into the study, written informed consent was obtained.

Patients' General Practitioners were informed that their patient had been recruited to the study, and were given an information sheet, and contact telephone number, should they have further enquires relating to the trial.

Medicine-Induced Injury.

The trial was conducted under the auspices of the Cancer Research Campaign (Phase I/II Clinical Trial Committee), who would provide patients with compensation for adverse side-effects.

Labelling.

Individual ampoules were unlabelled, with vaccine and placebo indistinguishable to the naked eye. I.m. (1ml) and i.d. (0.1ml) doses were attached to each other by a label. This label carried a letter indicating the trial centre, and the number of the course/visit (1,2 or 3.). Each of the 3 'pairs' of ampoules were stored in a sealed envelope prior to use, and refrigerated at 4°C. No reconstitution was necessary. No known interaction existed between 105AD7/placebo and any other medication.

Drug Accountability.

Stocks of 105AD7 and placebo were stored at 4 °C in the Department of Clinical Oncology, City hospital, Nottingham. Randomised trial drug and placebo were stored at 4°C in a locked fridge in a locked room in the Department of Surgery, Queens Medical Centre,Nottingham, prior to administration. The drug/placebo was stored in the various pharmacy departments at the other trial centres.

Treatment.

The table below details the investigations performed at trial entry, and weeks 6 and 12.

Parameter	At trial entry	At week 6	At week 12
105AD7/placebo	X	X	X
Full blood count	X	X	X
U&E	X	X	X
LFT	X	X	X
CEA	X	X	X
CT/USS	X*		X *
CXR	X		X
Weight	X	X	X
WHO perf	X	X	X

* Investigation performed only if available at trial centre.

Study Population.

INCLUSION CRITERIA.

Histologically diagnosed primary colorectal carcinoma with at least one of the following :

- Histologically confirmed inoperable colorectal carcinoma.
- Metastatic colorectal carcinoma, either histologically confirmed, or a single lesion shown to be enlarging on serial investigations.
- Multiple lesions on hepatic Computerized Tomography (CT), Ultrasound (USS), or Chest X-ray (CXR), after resection of a colorectal adenocarcinoma.

Life expectancy greater than 3 months.

Written informed consent.

EXCLUSION CRITERIA.

- Acute intercurrent illness.
- Autoimmune or chronic haematological disorders.
- Other concomittant anti-cancer treatment within the last 3 months, excluding surgery, and radiotherapy within the last 1 month.

WHO performance grading 3 or 4.

Women of child bearing age, pregnancy test positive, or not taking reliable contraceptive precautions.

Subsequent Treatment.

No conditions existed whereby patients received more than the 3 courses of 105AD7/placebo. Once patients were 'off study', they were not routinely followed up. However contact was regularly made between General Practitioner and referring Clinician, in order to document if the patient receives any further treatment, and their date of death.

Concomitant Therapy.

Supportive therapy necessary for the general condition of the patient was allowed, and was recorded. Chemotherapy and radiotherapy did not exclude patients from analysis if given at least 4 weeks following the last dose of 105AD7/placebo. These were recorded on Case Report Forms (CRF), as well as any surgery or immunotherapy that the patient has received.

Clinical Procedures.

- Pre-Clinical assessment.

The following were carried out prior to the patient receiving 105AD7/placebo:

a. History and establishment of baseline disease.
b. Physical examination.
c. Height and Weight.
d. WHO performance status.

- During treatment assessment.

The investigations carried out while the patient was on-study are shown above.

- Post-treatment assessment.

The only investigations carried out once the patient had received the final dose were also documented.

Safety Evaluation.

Subject evaluability for toxicity was recorded on the CRF. Assessment of Minor and Serious Adverse Events was performed either by clinicians at the referring centre, Family Practitioners, or Investigators at Trial centres. These were recorded on CRFs. The following were defined as Serious Adverse Events (SAE):

a. Death occurring within 4 weeks after the last study drug administration.
b. Life-threatening events.
c. Events which are incapacitating, or permanently disabling.
d. Events which require, or prolong hospitalisation.
e. Clinical or laboratory events which lead to withdrawl of the drug.
f. Any event resulting from a drug overdose.
g. Any event that results in secondary cancer or congenital anomaly.

All serious adverse events were reported to the CRC Phase I/II Data Centre within 24 hours of them occurring. The Data Centre were then responsible for notifying the Protocol Chairman, and the other

investigators. An Adverse Event form was submitted to the Data Centre within 7 days of the event occurring.

Investigators, and others responsible for patient care instituted any supplementary investigations and treatment as was deemed clinically necessary. The findings of any post mortem performed was attached to the patients CRF.

Any other adverse drug reactions which were CTC grade 3 or 4, though did not fulfill the criteria for a serious adverse event, were reported to the Data Centre within 2 weeks, and noted on an Adverse Event form.

All minor adverse events were recorded on the CRF. In addition, time to onset, duration, toxicity grade , treatment and outcome were also noted. This information was obtained following discussion with the patient, review of the CRC Patient Diary Sheet, and from correspondence from General Practitioners and Referring Specialists. Diary sheets graded from 0-4 the presence or absence of the following symptoms.

1. Nausea and Vomiting.
2. Loss of appetite.
3. Pain.
4. Tiredness.
5. Constipation or diarrhoea.
6. Fever or sore throat.
7. Hair loss.

Data Collection.

Responsibility for completion of Case Report Forms (CRF) was with investigators at the individual trial centres. The study was monitored according to the Cancer Research Campaign Phase I/II Clinical Trials Committee Standard Operating Procedure DD/010.

The CRF was divided into the following sections:

Page	Contents.
1.	Patient demographics and eligibility check list.
2.	Treatment prior to study entry.
3.	Baseline disease at trial entry.
5.	Dates of immunisation and concomitant treatment.
6.	Adverse events noted during trial.
7.	Laboratory investigations at each visit. CXR and CT results.
8.	Overall response to therapy.
10.	Off study form.
12.	Post-study treatment for malignant disease.
13.	Survival follow up, and date of last contact.

Data Management.

Patients were registered at the Data Centre, according to the Cancer Research Campaign Standard Operating Procedure DD/013. The CRC Data Centre was located at 10, Cambridge Terrace, Regents Park, London.

Location of Study Data.

Investigators at the individual trial centres were responsible for the storage of patients CRFs. Details of the Trial centres involved in the study has been previously documented.

Deviations from protocol.

All patients recruited to the study were evaluable, and eligible. A number of patients received their course of 105AD7/placebo, either early or late. Thirty (48%) and 31 (53%) patients received their 6 week course of 105AD7 and placebo on time, with only 12 (19%) and 14 (23%) patients over 7 days early, or late. At 12 weeks, only 15 (32%) and 15 (33%) received 105AD7 or placebo respectively at the intended time, with 13 (28%) and 13 (29%) patients immunised either 1 week early, or late.

There are a number of reasons why patients did not receive a course on time. The most common were patient convenience, and immunisations falling on Public/University holidays. Some patients were too unwell to attend on a set day, and were thus seen when they were well enough.

Patient Population.

A total of 165 patients were recruited to the study. There were 93 men, and 72 women, with a mean age of 62 and 64 respectively. The analysis was performed on the first 162 patients, of whom 85 had received 105AD7, and 77 placebo, as defined in the protocol. These patients had a mean age of 63.2 and 62.3 respectively. Further demographic details are documented in Appendix 1, Table A1-1. The male: female ratio in the trial group was 52:34, as compared with 40:37 in the placebo arm. The mean time from diagnosis of advanced disease, and entry into the study was 277.3 days in the 105AD7 group, and 278.6 days in controls.

The primary tumour site was coded as either colonic, or rectal. The ratio of the two in 105AD7 patients was 49:35, and 45:32 in controls. In 1 patient, the site was not recorded. The Dukes classification for the primary tumours were 1 'A'(1.3%), 15 'B' (19.4%), 34 'C' (44.2%) and 27 'D' (35.1%) in patients receiving 105AD7, and where it was recorded. The figures for the four stages in control patients were 3 (4.8%), 12 (19.0%), 23 (36.5%) and 25 (39.7%) in the cases where it was recorded.

Originally patients were stratified into two arms - 'liver mets' and 'no liver mets'. The CRF however detailed disease state at the time of inclusion in the study, as shown in Table 1. Patients may also have had disease in more than one anatomical location. The number of sites involved by tumour is shown in Table 2.

Subject Compliance.

A number of patients withdrew from the study, and thus did not receive the full three courses. Number of doses received, and the percentage of the total, in each trial centre, per patient is outlined in Table 3. These figures relate to the total number of patients recruited ie 165. Compliance for the 162 patients used in the statistical analysis is shown in Table 4.

Table 1. Sites of tumour in trial and control patients at trial entry.

	Treatment	
	105AD7	Placebo
Primary tumour	10 (12%)	6 (8%)
Local recurrence	19 (22%)	30 (39%)
Regional nodes	13 (15%)	10 (13%)
Lung metastases	27 (32%)	20 (26%)
Liver metastases	61 (72%)	50 (65%)
Bone metastases	5 (6%)	1 (1%)
Skin metastases	2 (2%)	2 (3%)
Brain metastases	1 (1%)	1 (1%)
Malignant ascites	3 (4%)	3 (4%)
Soft tissue	0	4 (5%)
Peritoneal metastases	9 (11%)	7 (9%)
Other metastases	2 (2%)	3 (4%)

Table 2. Number of anatomical sites involved with tumour, in trial and control patients.

Total number of sites	105AD7	Placebo
1	41 (48%)	36 (47%)
2	26 (31%)	26 (34%)
3	10 (12%)	7 (9%)
4	2 (2%)	5 (6%)
5	4 (5%)	3 (4%)
6	2 (2%)	0

Table 3. Number of patients receiving 1,2 or 3 doses in each of the 4 trial centres.

	1. Dose	2. Doses	3. Doses	Total
Nottingham	32 (25.8%)	19 (15.3%)	73 (58.9%)	**124** (75.2%)
Leeds	1 (16.6%)	0	5 (83.4%)	**6** (3.6%)
Hull	2 (11.2%)	7 (38.8%)	9 (50.0%)	**18** (10.9%)
Newcastle	5 (29.4%)	4 (23.5%)	8 (47.1%)	**17** (10.3%)
	40 (24.2%)	**30 (18.2%)**	**95 (57.6%)**	**165**

Table 4. Compliance for the 162 patients considered in the statistical analysis.

	105AD7	Placebo
Week 0	85 (100%)	77 (100%)
Week 6	63 (74%)	59 (77%)
Week 12	47 (55%)	45 (58%)

Statistical Analysis.

SAS (version 6.0) was used for all data summary and analysis. All statistical tests were two-sided and carried out at the 5% level. As stated in the Protocol, survival time was measured in days from (1) randomisation and (2) diagnosis of advanced disease. The 21st February 1997 was taken as the censoring date for all patients in the study. Patients who were not known to have died by this date were assumed to be censored in the analysis.

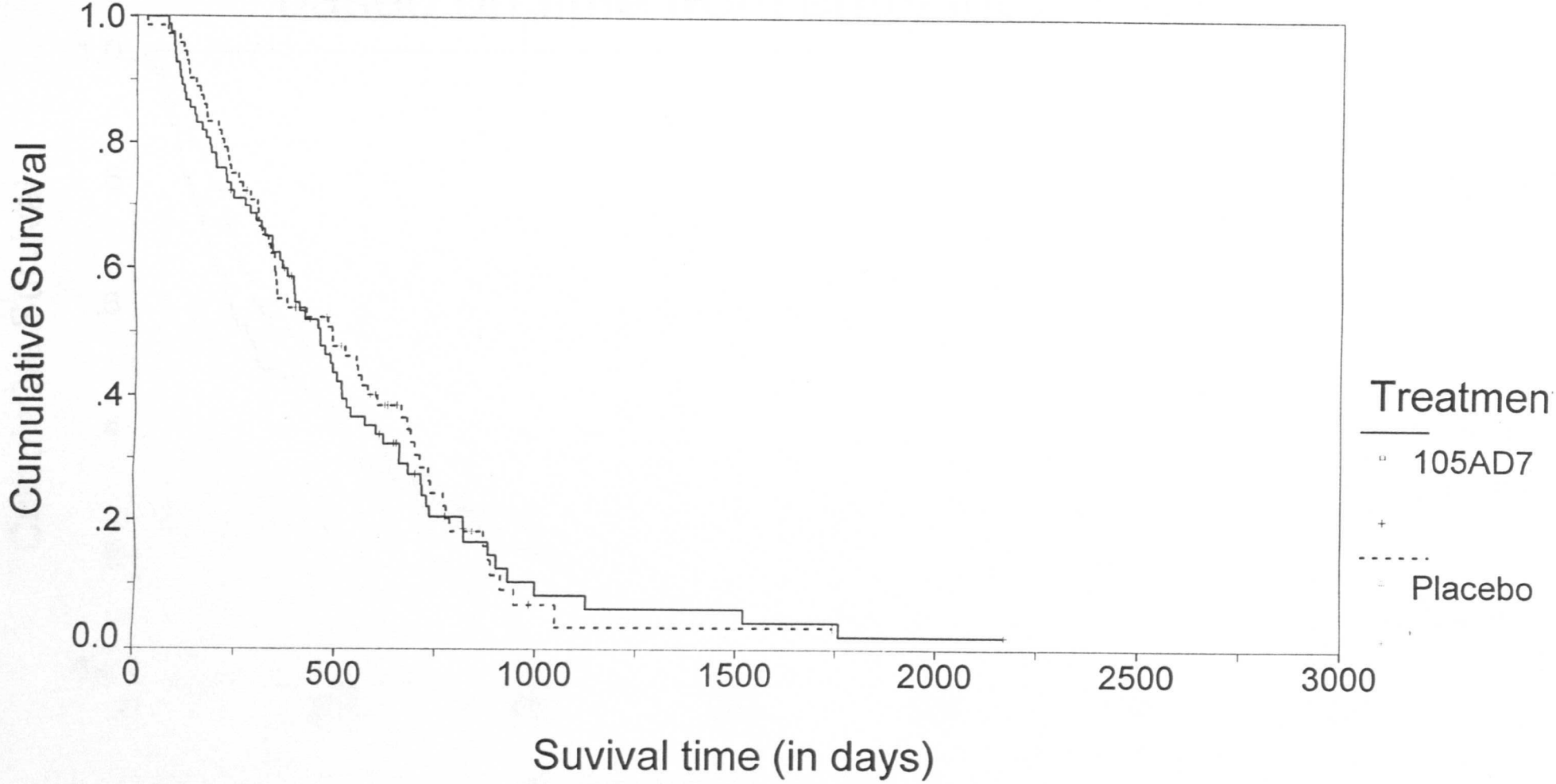
Univariate Analysis.

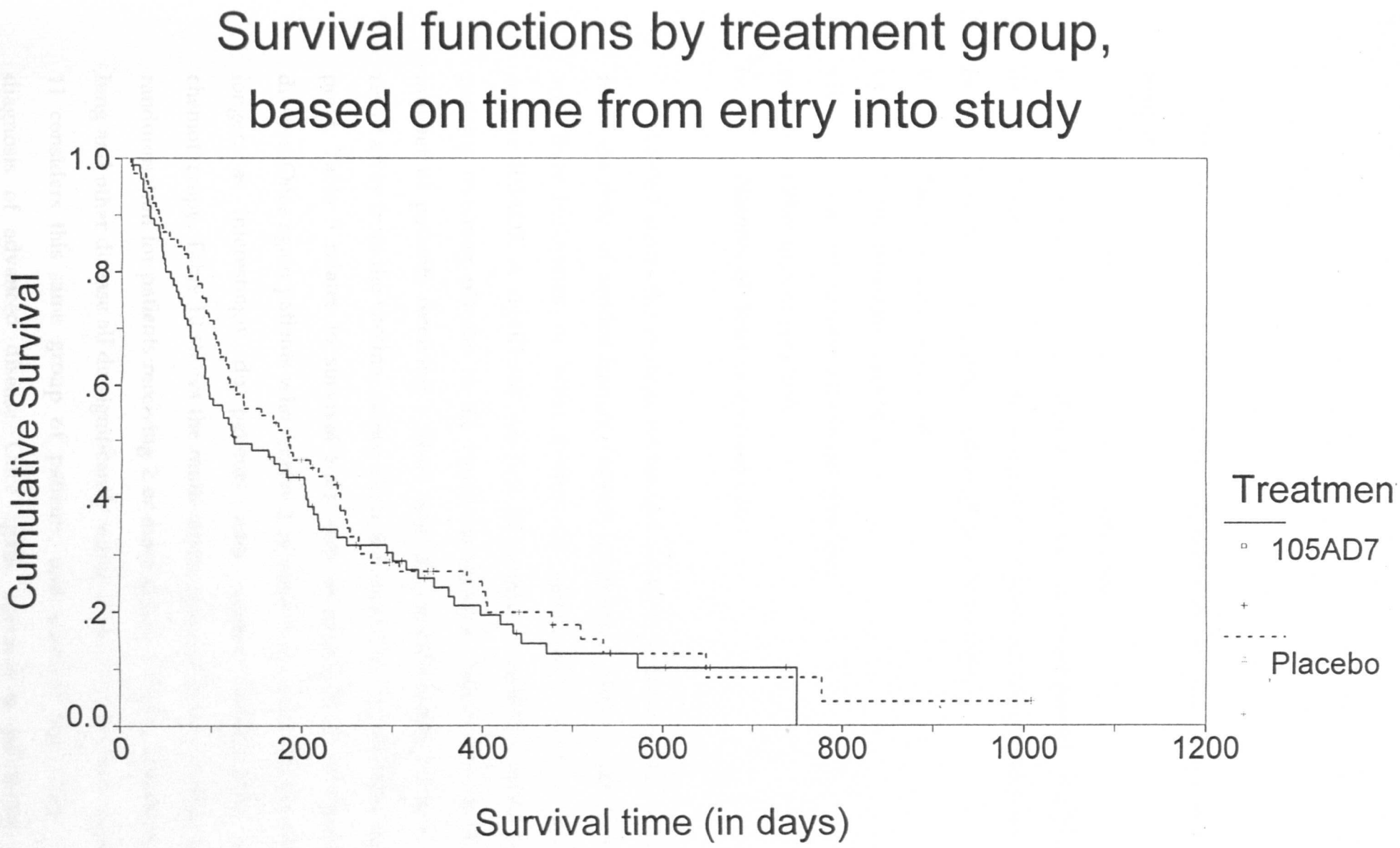
The log rank test was used to compare survival curves arising from the two treatment groups (105AD7 and placebo). Table 5 shows the results of a univariate analysis performed on an intention to treat basis. Median survival from date of randomisation was 124 and 184 days, in patients receiving 105AD7 and placebo, respectively (p=0.38), as shown in the Kaplan Meier graphs overleaf. Survival from date of diagnosis of advanced disease was 456 and 486 dyas for the two groups (p=0.82).

A univariate analysis was also performed restricted to those patients receiving 2 or more doses of 105AD7/placebo (Table 6). Median survival from the date of randomisation was 213 and 239 days for 105AD7 and placebo patients respectively (p=0.69). The median survivals from date of diagnosis of advanced disease were 511 and 486 days (p=0.60).

Univariate analyses were also performed for a number of subgroups of patients in the study. These included patients with lung metastases, liver metastases, those who had chemotherapy, and those who did not. Analyses were performed on an intention to treat basis from date of randomisation (Table 12), and from date of diagnosis of advanced disease (Table 13). There was no significant difference between patients receiving 105AD7 and placebo, for any of the subgroups considered.

Survival functions by treatment group, based on time from diagnosis of advanced disease




Survival functions by treatment group,
based on time from entry into study
Cumulative Survival
1.0
.8
.6
.4
.2
0.0
0
200
400
600
800
1000
1200
Survival time (in days)
Treatmen
105AD7
Placebo

Multivariate Analysis

The following variables were considered in the multivariate analysis:

i Treatment indicator (105AD7 v placebo).

ii Prior chemotherapy after diagnosis of advanced disease (yes/no).

iii Prior radiotherapy after diagnosis of advanced disease (yes/no).

iv Centre (Nottingham/Leeds,Hull and Newcastle).

v Lung metastases (yes/no).

vi Liver metastases (yes/no).

vii Other intraperitoneal disease (yes/no).

viii Other disease (yes/no).

ix Number of doses (≥ 2 versus <2).

Table 7 shows the analysis performed on an intention to treat basis, from the date of randomisation. Patients receiving 2 doses, and those with liver metastases, or 'other disease' do significantly worse if they receive 105AD7. A significant survival advantage is seen in favour of patients receiving placebo in the treatment variable. This relates to the number of patients receiving 1 dose, who disproportionately skew the result away from the vaccine. Hence Table 8 is included to illustrate this point. Table 9 relates to survival from date of diagnosis of advanced disease. Once again patients who receive 2 or more doses live significantly longer, as interestingly do patients who receive radiotherapy or chemotherapy. Table 10 shows the multivariate analysis from the time of randomisation for patients receiving 2 or more doses. Patients with liver, lung and other disease all do significantly worse in the 105AD7 arm. Table 11 considers this same group of patients, and survival from date of diagnosis of advanced disease. Once again, survival is enhanced if

patients receive chemotherapy or radiotherapy, and the presence of 'other disease' decreases survival.

Overall Experience.

There were very few adverse events deemed related to immunisation with the anti-idiotypic monoclonal antibody 105AD7, or placebo.

Adverse Events.

The number of deaths from the date of randomisation was 71 (84%) and 63 (82%) in 105AD7 and placebo groups respectively.

There were 3 Serious Adverse Events - 2 in placebo patients, and 1 in a patient who had received 105AD7. All were CTC grade 3, and were felt to be 'unlikely' to be due to the study drug. These are shown in Appendix 1, Table A1-26. A number of other SAEs were felt to be 'unrelated' to the study drug, and are thus not shown.

57 and 30 minor adverse events were documented in trial and control patients, respectively. The majority were CTC grade 1 or 2. Only 1 was felt to be due to 105AD7, while a further 9 and 8 in the two groups were classified as 'possibly' due to 105AD7. The remainder were graded as 'unlikely'. These are shown in Appendix 1, Table A1-25. The median times between trial entry and the occurrence of the event were similar in 105AD7 and placebo patients - 22 and 26 days respectively. Median durations of adverse events were markedly different in the 2 groups however - 2 days and 14 days.

Laboratory Data.

Chemical Pathology.

Results of patients' chemical pathology are tabulated in the Appendix 1, Table A1-24. Median figures for urea and electrolytes remain within normal limits in both trial and control patients for the duration of the study. As anticipated there is clear derangement of liver function tests and CEA. Alkaline phosphatase, Gamma glutaryl transferase, and CEA are all raised both at trial entry, and throughout the duration of the study. There is no obvious difference between the 2 groups for the alkaline phosphatase and γGT, though clearly CEA is higher in 105AD7 patients. This difference has not been statistically tested.

Haematology

White cell counts, platelets and neutrophil counts all remain within normal limits. Median haemoglobin scores show that patients are anaemic at trial entry, and throughout the study. Median lymphocyte scores are at the lower limit of normal. There is a trend towards an increased lymphocyte count in both groups. These are further detailed in Appendix 1, Table A1-23.

Table 5 Univariate analysis assuming intention to treat

	observed deaths	Median survival and 95% CI	RR and 95% CI	Log rank test (p-value)
From randomisation				0.38
105AD7	71 (84%)	124 (95, 206)	1.17 (0.83,1.64)	
Placebo	63 (82%)	184 (119, 242)	1	
From diagnosis of advanced disease[1]				0.82
105AD7	69 (83%)	456 (363, 522)	1.04 (0.73,1.48)	
Placebo	59 (82%)	486 (344, 659)	1	

1 Date of diagnosis of advanced disease known for 83 patients randomised to 105AD7 and 72 patients randomised to Placebo.

Table 6 Univariate analysis restricted to those patients who have received two or more doses

	observed deaths	Median survival and 95% CI[3]	RR and 95% CI[3]	Log rank test (p-value)
From randomisation[1]				0.69
105AD7	49 (77%)	213 (168, 316)	1.09 (0.72,1.63)	
Placebo	45 (76%)	239 (184, 265)	1	
From diagnosis of advanced disease[2]				0.60
105AD7	48 (77%)	511 (456, 656)	0.89 (0.59,1.36)	
Placebo	41 (76%)	486 (334, 674)	1	

1 Based on 63 patients randomised to 105AD7 and 59 patients randomised to Placebo

2 Date of diagnosis of advanced disease known for 62 patients randomised to 105AD7 and 54 patients randomised to Placebo

3 confidence interval (CI), risk ratio (RR)

Table 7 Multivariate analysis, assuming intention to treat, survival time from randomisation

Variable	Regression coefficient	RR[2]	95% CI for RR[2]	p-value
Treatment 105AD7 Placebo	0.473	1.61 1	1.12 to 2.31	0.010[1]
Centre Nottingham rest	-0.124	0.88 1	0.57 to 1.37	0.58
No. of doses ≥ 2 dose 1 dose	-2.048	0.13 1	0.08 to 0.20	<0.001[1]
Liver metastases Yes No	0.565	1.76 1	1.12 to 2.78	0.015
Other disease Yes No	0.599	1.82 1	1.20 to 2.75	0.005

1 see Table 8 for joint RRs for treatment and number of doses

2 confidence interval (CI), risk ratio (RR)

Table 8 Survival time from randomisation: joint RRs for treatment (105AD7 versus placebo) and number of doses (1 dose versus ≥2 doses)

Variable	Regression coefficient	RR
Placebo, 1 dose	0	1
Placebo, ≥2 doses	(0+(-2.0478))	0.13
105AD7, 1 dose	(0.473+0)	1.61
105AD7, ≥2 dose	(0.473+(-2.048))	0.21

Table 9 Multivariate analysis assuming intention to treat, survival time from date of diagnosis of advanced disease

Variable	Regression coefficient	RR[1]	95% CI for RR[1]	p-value
Treatment 105AD7 Placebo	0.189	1.21 1	0.84 to 1.73	0.30
Centre Nottingham Other centres.	0.326	1.38 1	0.90 to 2.13	0.14
No. of doses ≥ 2 dose 1 dose	-0.826	0.44 1	0.29 to 0.65	<0.001
Chemotherapy Yes No	-0.954	0.39 1	0.26 to 0.57	<0.001
Radiotherapy Yes No	-0.982	0.37 1	0.22 to 0.63	<0.001
Other disease Yes No	0.561	1.75 1	1.19 to 2.58	0.004

[1]confidence interval (CI), risk ratio (RR)

Table 10 Multivariate analysis, restricted to those patients who have received two or more doses, survival time from randomisation

Variable	Regression coefficient	RR[1]	95% CI for RR[1]	p-value
Treatment 105AD7 Placebo	0.213	1.24 1	0.80 to 1.90	0.33
Centre Nottingham Other centres	0.120	1.13 1	0.68 to 1.87	0.64
Liver metastases Yes No	0.658	1.93 1	1.15 to 3.25	0.013
Other disease Yes No	0.618	1.86 1	1.14 to 3.03	0.013
Lung metastases Yes No	0.463	1.59 1	1.00 to 2.52	0.050

1 confidence interval (CI), risk ratio (RR)

Table 11 Multivariate analysis, restricted to those patients who have received two or more doses, survival time from date of diagnosis of advanced disease

Variable	Regression coefficient	RR[1]	95% CI for RR[1]	p-value
Treatment				0.93
105AD7	0.020	1.02	0.67 to 1.57	
Placebo		1		
Centre				0.59
Nottingham	0.135	1.14	0.70 to 1.87	
Other centres.		1		
Chemotherapy				0.005
Yes	-0.660	0.52	0.33 to 0.82	
No		1		
Radiotherapy				0.011
Yes	-0.761	0.47	0.26 to 0.84	
No		1		
Other disease			1.04 to 2.56	0.03
Yes	0.492	1.64		
No		1		

1 confidence interval (CI), risk ratio (RR)

Table 12. Univariate subgroup analysis assuming intention to treat- survival measured from the date of randomisation.

	Number	Observed deaths	Median survival & 95% CI [2] (days)	RR and 95% CI [2]	p value by log rank test
Lung mets					
105AD7	27	24 (89%)	90 (65,168)	1.58 (0.82-3.04)	0.16
Placebo	20	15 (75%)	151 (100,277)	1	
Liver mets					
105AD7	61	52 (85%)	112 (90,202)	1.17 (0.77-1.77)	0.45
Placebo	50	40 (82%)	173 (102,242)	1	
Chemo [1]					
105AD7	30	27 (90%)	104 (71,183)	1.32 (0.70-1.43)	0.39
Placebo	21	15 (71%)	183 (68,400)	1	
No Chemo					
105AD7	55	44 (80%)	168 (97,250)	1.03 (0.68-1.56)	0.88
Placebo	56	48 (86%)	185 (117,242)	1	

1. Chemotherapy

2. Confidence Interval (CI), Relative Risk (RR).

Table 13. Univariate subgroup analysis assuming intention to treat- survival measured from the date of diagnosis of advanced disease.

	Number	Observed deaths	Median survival & 95% CI [2] (days)	RR and 95% CI [2]	p value by log rank test
Lung mets					
105AD7	26	23 (88%)	522 (339-656)	0.91 (0.46-1.82)	0.79
Placebo	19	14 (74%)	422 (344-422)	1	
Liver mets					
105AD7	60	51 (85%)	418 (337-511)	1.09 (0.72-1.67)	0.68
Placebo	49	39 (80%)	475 (173-334)	1	
Chemo [1]					
105AD7	30	27 (90%)	596 (456-679)	1.51 (0.80-2.87)	0.21
Placebo	21	15 (71%)	683 (560-909)	1	
No Chemo					
105AD7	53	42 (79%)	310 (194-488)	0.98 (0.64-1.50)	0.93
Placebo	51	44 (86%)	334 (250-475)	1	

1. Chemotherapy

2. Confidence Interval

Discussion.

Immunisation with the anti-idiotypic monoclonal antibody 105AD7 does not confer a survival advantage on patients with advanced colorectal cancer. This study therefore does not support the findings of the Phase I study [Denton GWL 1994]. A univariate analysis showed no significant difference between placebo and trial group, both in the intention-to-treat, and the analysis restricted to patients who had received 2 and 3 doses.

The study aimed to recruit 162 patients - 81 patients to both 105AD7 and placebo arms. In reality, eighty-five patients received 105AD7, and 77 placebo. The disparity between the two relates to the randomisation of the study drug, which was in 'blocks' of 6 (3 trial, 3 placebo), and stratified according to 'liver metastases' and 'no liver metastases' arms, in each of the 4 trial centres. None of the 'arms' of the study finished exactly at the end of a block of 6 in any of the centres, thus accounting for the difference seen in terms of patients recruited. In addition, a further 3 patients were recruited, making the total 165. These patients had agreed to be randomised, but were still within 1 or 3 months of previous courses of radiotherapy and chemotherapy. They were in effect waiting to become eligible for the study. They were not included in the statistical analysis, which was confined to the first 162 patients recruited, as defined in the protocol.

Patient characteristics were comparable in trial and placebo groups in terms of age, sex, site and Dukes stage of primary tumour. The time from diagnosis of advanced disease, and inclusion in the study were also very similar - medians of 172 and 179 days, in 105AD7 and placebo arms respectively. Inexplicably, the time between resection of the primary tumour and on study date was almost twice as long in the placebo arm (13.7 months v 22.8 months).

Therapy prior to entry into the study was comparable in the two groups. Identical numbers of patients received radiotherapy prior to, and following the date of diagnosis of advanced disease. No patient received any immunological, biological, or hormonal therapy prior to the diagnosis of advanced disease, reflecting how this treatment option has failed to become established as an adjuvant therapy. Two patients in the 105AD7 arm, and 4 in the control group however received Interferon, Interleukin-2 or an Investigational drug, following the diagnosis of advanced disease, but prior to entry into the study. Operations performed for removal of primary tumours were similar in the 2 groups. A number of patients also underwent further operations, as detailed in Appendix 1, Table A1-3.

Chemotherapy may be used in the treatment of colorectal cancer in two ways - either as adjuvant therapy immediately following surgery, or as treatment for advanced disease. Ten and fourteen patients receiving 105AD7 and placebo respectively fell into the former group. A number of regimes were used, in all but one case based on 5-Fluorouracil. Over a third of patients in both groups completed 6 courses. Regimes used for the treatment of advanced disease were more varied. In addition to schedules based on 5-Fluorouracil, Folinic Acid and leucovorin; cisplatin, doxorubicin, raltitrexed (Tomudex), Zilascorb, and Mitomycin C were also used. Partial responses were seen in only 2 patients. The median time between finishing chemotherapy, and trial entry was 161 and 174 days for 105AD7 and control patients respectively. It is possible that the immunosuppressive effects of chemotherapy may reduce the efficacy of immunotherapy, and if so 3-6 months may not be long enough for the immune system to recover. It is interesting to note the results of a sub-group analysis which showed that patients who had not received chemotherapy lived markedly longer than those who had. These results

suggest that chemotherapy and immunotherapy may not be synergistic, as has been suggested.

No significant survival advantage was seen in patients receiving 105AD7, either by univariate or multivariate analyses. The median survival from the date of diagnosis of advanced disease in patients receiving the vaccine was higher than that seen in the Phase I study - 456 days v 365 days. The reason for the lack of significance is that the placebo arm lived for a median of 487 days (16 months), markedly higher than that reported in the literature. It is important also to consider that only 34 out of the 77 placebo patients received chemotherapy prior to inclusion, potentially prolonging their survival. These results do not support those of the Phase I study for a number of reasons. It is likely that the control group in the Phase I study lived for a markedly shorter time than would be anticipated (4 months), and the non-randomised group of trial patients longer than would be expected for a group not receiving chemotherapy. This combined, with the small numbers involved, almost certainly accounted for the significant difference seen between the two groups. It is likely also with this unblinded work that some degree of selection bias could have taken place.

It has been shown that patients with large tumour burdens are immunosuppressed, and thus unlikely to mount effective immune responses when immunised with an anti-idiotypic antibody [Golub SH 1974][Eilber FR 1975]. In addition it has been shown that patients with advanced disease have decreased amounts of ζ chain in the TCR, and less expression of MHC Class I and II on tumour cells [Guilloux Y 1994][Mizoguchi H 1992]. IFN γ is a cytokine produced by activated T cells and Natural Killer cells, that enhances antigen presentation, and induces expression of ICAM-1 and LFA-1. Levels assayed from fresh colorectal cancer specimens were found to decrease as disease advanced [Numata A

1991]. Recent work has proposed that difficulties encountered generating CTL may be due to the inclusion of patients with advanced disease [Jacob L 1997]. It is also interesting to note that the lymphocyte counts of all patients in the study were at the lower end of normal. All of this suggests that patients with advanced disease have compromised immune systems, and may not be an ideal group for immunotherapy.

Recent work has shown that fas ligand is expressed on liver metastases from colorectal cancer, and it has been proposed that fas mediated destruction of hepatocytes may promote liver colonisation, and that expression of fasL on primary colorectal cancers may induce apoptosis in TIL [O'Connell J 1996][Shiraki K 1997]. It is possible that CTL stimulated by 105AD7 to destroy gp72 expressing liver metastases, may be apoptosed in the liver by fasL expressing tumour cells. This is further immunological evidence that may in part explain why patients receiving the vaccine did not live longer than controls.

The Phase I study recruited only patients with liver metastases, whereas this study included those with multiple sites of disease, and thus larger tumour burdens. It is likely that liver metastases will have a better blood supply than a large mass of pelvic recurrence - if primed T cells reach only a small proportion of disease, then it is unlikely that they will have a significant effect on tumour growth.

Forty-one patients in the study received various chemotherapy regimes prior to randomisation to the 105AD7 arm. In order to assess whether this had any bearing on survival, a sub-group analyses was performed for patients who had received either adjuvant, or chemotherapy for their advanced disease, and those patients who had not. Survival from the date of randomisation was markedly less in the former group, as compared with the latter (104v168 days), supporting the view that chemotherapy and immunotherapy may not be acting synergistically.

Both groups lived less than placebo, and those patients who received chemotherapy lived generally longer, as shown in the multivariate analysis.

The maximum number of doses of 105AD7 that an individual patient could receive in this study was 3. Some patients in the Phase I study recieved up to 7 doses, and it is possible that the reason no survival advantage was seen was that not enough courses of 105AD7 were given. 105AD7 is administered with alum. It may be that patients with such compromised immune systems require stronger adjuvants, such as BCG or GM-CSF, to augment the immune response generated.

Patients in the study were randomised to those with liver metastases, and those without. The actual baseline disease present at entry into the study was recorded in the Case Report Form. Clearly from Table 2 a number of patients had disease in more than one site, and with such large tumour burdens, would be expected to be immunologically suppressed. These patients would be unlikely to mount an immune response following immunisation with 105AD7, and this in part explains why no survival advantage was seen in the analysis. The other interesting point to note is that twice as many patients in the placebo arm had local recurrence. These patients live longer than those with liver metastasis, and it is possible that this might skew the result away from 105AD7. In view of this a multivariate analysis was performed, taking into account the site of baseline disease. This showed that patients with liver metastases, and 'other disease', such as bone and brain metastases fared worse in the placebo arm.

There was little difference between haematological and clinical chemistry results in trial and control patients. However it is clear from the results that mean and median lymphocyte counts fall below normal limits in both groups. This further supports the concept of malignancy equating

with immunosuppression, as it is likely that the low lymphocyte count reflects of the degree of metastatic disease present.

There was no toxicity associated with the use of 105AD7 in the Phase I study. This was confirmed in the Phase II study, where only 3 serious adverse events were documented as "unlikely" to be due to 105AD7, by the investigator who observed them Of the three, two occurred in the placebo arm. Of the minor adverse events, only one was felt to be definitely due to 105AD7.

The initial entry criteria for the study stated that eligible patients should have a life expectancy of 3 months, and should thus receive three immunisations. Clearly this was not seen in this study, with approximately 75% of patients receiving at least 2 doses, and only 55% receiving all three. The most likely cause of patients not completing the study was disease progression causing patients to die, or become too unwell to attend hospital. It was felt that this might have implications for the result of the study, as work done using 105AD7 suggests that the first immunisation may act as a priming dose, with further injections amplifying the response. An analysis was therefore performed considering only patients who had received 2 or more doses. This effectively reduces the number of patients in the study, and no significant difference was seen between 105AD7 and placebo groups.

The Phase II study has shown that 105AD7 does not confer a survival advantage on patients with advanced colorectal cancer. It is likely that this relates to tumour-induced immunosuppression, and further work will concentrate on use of the vaccine as adjuvant therapy.

Chapter 3.

Use of the anti-idiotypic monoclonal antibody 105AD7 as adjuvant therapy in patients with primary colorectal cancer.

Contents.

Aim.

The aim of this work is to assess the immunological changes that occur in the peripheral blood, and at the tumour site of patients who receive the anti-idiotypic monoclonal antibody 105AD7 prior to resection of their primary colorectal tumours. In addition an assessment will be made as to whether this confers a survival advantage on these patients.

Materials and Methods.

Human monoclonal anti-idiotypic antibody

Clinical grade monoclonal antibody was produced as previously described [Robins RA 1991] using the guidelines of the Cancer Research Campaign [Working Party on the Clinical use of antibodies 1986]. Samples of the seed lots passed testing for sterility and viral contamination. Antibody was prepared as 10μg in 0.1 ml of saline, and 100μg in 1ml of aluminium hydroxide. With a relative molecular mass of 150 KDa, the former dose of antibody, for example, contains 1.33×10^{13} molecules, and is a 6.6×10^{-6} M solution.

Patients.

Twenty-four patients were recruited prospectively from surgical out-patients by the author between August 1995 and December 1996 (Table 14). All had primary colorectal cancer, either diagnosed on biopsy, or double-contrast barium enema. The group consisted of 17 men, and 7 women, with a mean age of 71.3 years (range 56-87 years). There were 16 rectal tumours, 1 recto-sigmoid tumour, 3 sigmoid and 4 caecal cancers.

Twenty-three patients had already been recruited to the 105AD7 adjuvant study by the previous Cancer Research Campaign Fellow, between May 1993 and August 1995. These patients are considered in the survival analysis, as described in Part 4.

Trial patients were selected arbitrarily from the total number of patients obeying the inclusion and exclusion criteria outlined below. There was no selection bias, though due to the time constraints of

processing specimens, only a limited number of patients were "on-study" at any one time.

Inclusion Criteria

- Patients with primary colorectal cancer.

Exclusion Criteria

- Pre-operative radiotherapy
- Women of child bearing age not taking reliable contraception.
- Normal hepatic and renal function.
- Haemoglobin > 10g/dl, Platelets > $50x10^9/l$ and White blood count > $2x10^9/l$.
- Chronic haematological, autoimmune or intercurrent illness.

Clinical Protocol.

This study was performed under the auspices of the Cancer Research Campaign UK Phase I/II clinical trials committee, with local ethical committee approval. Informed consent was obtained in writing prior to treatment with 105AD7.

Eligible patients were registered with the Data Centre, at the Cancer Research Campaign prior to inclusion. Details of patients initials, date of birth, and hospital number were recorded.

Patients received varying doses of 105AD7, at different time-points prior to surgery, as detailed in Table 14. This was as part of ongoing work, aiming to optimise dose and route of administration. Seven patients received 10μg of antibody i.d. followed 48 hours later by 50μg i.m. One

patient (no. 5) received this regime twice. Ten patients were given 50μg i.m. followed by a further 50μg i.m. 1 week later. One patient was administered with 20μg i.m. followed 7 days later by another 20μg i.m. Three patients were given 10μg i.d. with a further 20μg i.m. 48 hours later, and two received two courses, 1 week apart, of 10μg i.d. with 50μg i.m. at the same time. All 23 patients immunised by the previous CRC fellow received 10μg i.d. followed by 100μg i.m.

In order to phenotype peripheral blood lymphocyte sub-sets, 20mls of venous blood was taken pre-immunisation, and on several occasions before surgery.

A mean of 16.8 days following immunisation, patients attended and underwent resection of their primary tumours. Two samples were taken from the resection specimen. One was snap frozen in liquid nitrogen, prior to immunohistochemical analysis. The other was suspended in RPMI, disaggregated, and TIL analysed by flow cytometry.

Six and twelve weeks following resection, patients attended the Department of Surgery, to receive booster doses of 105AD7. During the study, the protocol changed, and some received further doses. Six patients were referred for adjuvant chemotherapy, and were randomised into the QUASAR study. They were contacted three months after their last course, and offered 105AD7. Three patients underwent adjuvant post-operative radiotherapy. Dukes stage of the primary tumour, and further treatment is shown in Table 15.

Table 14. Demographics of patients recruited to the 105AD7 Adjuvant study, by the author.

No	Initials	Age [1]	Sex	Primary [2]	On-Study [3]	Dose [4]	Op [5]
1.	DT	75	F	Rectum	Aug '95	10 id + 50 im	18 days
2.	EN	79	F	Sigmoid	Sept '95	10 id + 50 im	14 days
3.	ER	82	M	Caecum	Sept '95	10 id + 50 im	11 days
4.	HT	86	F	Rectum	Oct '95	10 id + 50 im	14 days
5.	JG	64	M	Rectum	Oct '95	10 id + 50 im	31 days
6.	ET	87	M	Caecum	Oct '95	10 id + 50 im	20 days
7.	MF	63	F	Rectum	Nov '95	10 id + 50 im	19 days
8.	MB	75	M	Rectum	Nov '95	10 id + 50 im	26 days
9.	DH	56	M	Rectum	Nov '95	50 im + 50 im	9 days
10.	VMcC	63	M	Rectum	Dec '95	50 im + 50 im	21 days
11.	JK	76	M	Sigmoid	Jan '96	50 im + 50 im	14 days
12.	JGa	58	M	Rectum	Jan '96	50 im + 50 im	13 days
13.	TB	62	F	Rectum	Feb '96	50 im + 50 im	20 days
14.	CB	75	M	Caecum	Mar '96	50 im + 50 im	No op
15.	FCl	67	F	Rectum	Mar '96	50 im + 50 im	14 days
16.	JH	69	M	Rectum	Apr ' 96	50 im + 50 im	15 days
17.	FH	73	M	Rectum	May '96	50 im + 50 im	29 days
18.	FCh	70	M	Rectum	May '96	50 im + 50 im	13 days
19.	PT	72	M	Rectosig	May '96	20 im + 20 im	12 days
20.	GF	75	M	Rectum	Nov '96	10 id + 20 im	8 days
21.	DP	69	M	Rectum	Nov '96	10 id + 20 im	14 days
22.	AG	65	M	Sigmoid	Nov '96	10 id + 20 im	20 days
23.	JF	81	M	Caecum	Dec '96	10 id + 50 im x2	12 days
24.	M W	69	F	Rectum	Dec '96	10 id + 50 im x2	20 days

Key to Table 14.

1. Age at resection of primary tumour

2. Site of primary tumour.

3. Month that patient was recruited to the adjuvant study.

4. Dose of 105AD7 that patient received. Several dosing schedules were employed, as part of ongoing work aiming to establish the optimal regime. All intradermal (i.d.) doses were followed 48 hours later by an intramuscular (i.m.) dose, except for patients 23 and 24, who received i.d. and im doses at the same time. If an i.m. dose was administered at the outset (Nos 10-19), then the next i.m. dose was 7 days later.

5. Number of days between receiving first dose of 105AD7, and resection of the primary tumour.

Experimental Procedures involved in the adjuvant study.

In order to achieve the aim of this work, three scientific techniques were employed. These included immunohistochemical analysis of cryopreserved tumour sections, flow cytometric analysis of tumour infiltrating lymphocytes, and peripheral blood lymphocyte phenotyping. For logistical reasons, not all 24 patients recruited by the author had all 3 performed. The analyses performed on individual patients is shown in Table 16.

To assess whether 105AD7 conferrred a survival advantage, when used as adjuvant therapy, it was necessary to follow up the 23 patients recruited by the previous CRC fellow, and compare their survival with a contemporary group of patients undergoing surgery at the same time. This survival analysis is described in Part 4.

Part 1. Immunohistochemistry.

Analysis of tumour sections was performed at three different times throughout the two year period. Sections from thirteen trial patients immunised by the author, and a further three recruited by the previous CRC Fellow were labelled with MAb against CD4, CD8, CD56 and CD25 lymphocyte antigens.

At a later date specimens from twelve of the above patients were labelled with MAb against CD68, CD69, and CD3 ζ chain. Tumour from 8 patients was available for labelling with APO2.7 MAb, at a still later date.

Immunohistochemical analysis was also performed on cryopreserved tumour specimens from control patients who had not received 105AD7 pre-operatively. These were matched to trial patients according to tumour site, stage and differentiation, as well as sex, and

approximate age of patient. Such a close match was possible as specimens are taken from all colorectal tumours resected at the Queens Medical Centre, and cryopreserved.

Part 2. Venous blood phenotyping.

Venous blood was taken from 17 trial patients, pre-immunisation, and at various time points prior to surgery. Lymphocytes were separated out, labelled with MAb, and analysed by flow cytometry. Analysis was performed assessing whether peripheral blood lymphocytes were affected by immunisation with 105AD7.

Part 3. Tumour Infiltrating Lymphocytes.

Fresh tumour specimens from 19 immunised and 35 unimmunised patients were disaggregated, and separated lymphocytes labelled with MAb, and analysed by flow cytometry.

Part 4. Survival analysis.

Twenty-three patients were immunised by the previous CRC Fellow, between May 1993 and August 1995. Survival and recurrence data at 2 year follow up was collected, and compared with matched controls from the Trent Audit. The Trent Audit contains data relating to the management of 3520 patients with colorectal cancer. Each patient who had received 105AD7 was matched to between 2 and 5 controls, according to Dukes stage, site, differentiation, sex, age, ASA, and whether or not they had chemotherapy or radiotherapy.

Table 15. Pathological stage and outcome of recruited patients.

No.	Initials	Primary [1]	Stage [2]	Diff [3]	Chemo [4]	RTH [5]	105AD7 [6]
1.	DT	Rectum	C	Mod.	Yes x 4	Yes	3
2.	EN	Sigmoid	B	Mod.	Mort [7]		0
3.	ER	Caecum	C	Mod.	No	No	1
4.	HT	Rectum	-	-	No	Yes	2
5.	JGr	Rectum	D	Mod.	No	Yes	2
6.	ET	Caecum	B	Mod.	No	No	2
7.	MF	Rectum	B	Mod.	No	No	0
8.	MB	Rectum	A	Mod.	No	No	1
9.	DH	Rectum	B	Mod.	No	No	2
10.	VMcC	Rectum	C	Mod.	Yes	No	3
11.	JK	Sigmoid	A	Mod.	No	No	2
12.	JGa	Rectum	A	Mod.	No	No	1
13.	TB	Rectum	C	Poor	Yes	No	1
14.	CB	No op	-	-	No	No	0
15.	FCl	Rectum	A	Mod	No	No	3
16.	JH	Rectum	A	Mod.	No	No	2
17.	FH	Rectum	C	Mod.	No	No	0
18.	FCh	Rectum	B	Mod.	No	No	0
19.	PT	Rectosig	C	Mod.	Yes	No	1
20.	GF	Rectum	B	Mod.	No	No	0
21.	DP	Rectum	C	Mod.	No	No	0
22.	AG	Sigmoid	C	Mod.	Yes	No	0
23.	JF	Caecum	B	Mod.	No	No	0
24.	MW	Rectum	C	Mo/Po	Yes	No	0

Legend to Table 15.

1. Site of primary tumour.

2. Modified Dukes stage of primary tumour.

3. Differentiation of primary tumour.

4. Adjuvant chemotherapy administered post-operatively.

5 Adjuvant radiotherapy received by patient post-operatively.

6. Number of doses of 105AD7 received by patient post-operatively.

7. This patient died within 30 days of surgery.

Table 16. Experiments performed on individual patients.

No.	Initials.	Pheno [1]	CD4,8,56 & 25 [2]	CD68,69, & ζ [3]	APO2.7[4]	TILS [5]
1.	DT	Yes	Yes	No	No	Yes
2.	EN	Yes	Yes	Yes	Yes	Yes
3.	ER	Yes	Yes	Yes	Yes	Yes
4.	HT	Yes	No	No	No	No
5.	JG	No	Yes	Yes	No	Yes
6.	ET	Yes	Yes	Yes	Yes	Yes
7.	MF	Yes	No	No	No	Yes
8.	MB	Yes	Yes	Yes	Yes	Yes
9.	DH	Yes	Yes	Yes	Yes	Yes
10.	VMcC	Yes	No	No	No	Yes
11.	JK	Yes	Yes	Yes	Yes	Yes
12.	JG	Yes	Yes	No	No	Yes
13.	TB	Yes	Yes	Yes	Yes	Yes
14.	CB	Yes	No	No	No	No
15.	FCl	Yes	Yes	Yes	No	Yes
16.	JH	Yes	No	Yes	No	Yes
17.	FH	Yes	Yes	Yes	No	Yes
18.	FCh	Yes	Yes	Yes	Yes	No
19.	PT	No	No	No	No	Yes
20.	GF	No	No	No	No	Yes
21.	DP	No	No	No	No	Yes
22.	AG	No	No	No	No	No
23.	JF	No	No	No	No	No
24.	MW	No	No	No	No	No

Key to Table 16.

1. Phenotyping of lymphocyte subsets in venous blood.

2. Immunohistochemical analysis of section of tumour from trial patients using MAb against CD4, CD8, CD56 and CD25.

3. Immunohistochemical analysis of tumour sections from trial patients using MAb agaainst CD68, CD69, and ζ chain. Note this work was performed later than 2, and thus less tumour specimens were available from patients who had received 105AD7.

4. A number of sections from trial patients were available for labelling with the MAb APO2.7, directed against 7A6 antigen. This was the last immunohistochemical analysis performed, and thus specimens were only available on 8 trial patients.

5 TIL were assessed by disaggregating fresh tumour from immunised patients, labelling with MAb, and analysing by flow cytometry.

Part 1.

Lymphocytic Infiltration of primary colorectal tumours in patients receiving 105AD7, as measured by immunohistochemistry.

Contents.

Aim.

The aim of this work is to assess immunological changes occurring at the tumour site of patients with primary colorectal cancer who receive 105AD7 prior to surgery. In order to do this sections from trial patients, and their matched controls will be immunohistochemically labelled with a panel of antibodies against specific CD antigens expressed on tumour infiltrating lymphocytes. These include MAb against CD4, an antigen expressed on helper/inducer T cells, CD8 on cytotoxic T cells, and CD56 expressed on Natural Killer cells. In addition MAbs against the α subunit of the Interleukin-2 receptor (CD25), an antigen expressed on macrophages (CD68), and the 28/34 KDa transmembrane glycoprotein activation marker CD69 (Activation Inducer Molecule). Apoptosis at the tumour site was assessed using the MAb APO2.7 against 7A6, a mitochondrial antigen. Tumour samples were also labelled with an MAb against the ζ chain of the T cell receptor, in order to assess whether immunisation with 105AD7 up or down regulated its expression.

Materials and Methods.

Patients.

Twenty-four patients were recruited prospectively from surgical out-patients by the author, of whom 13 had tumour samples taken for immunohistochemical analysis. Tumour from 3 of the 23 patients immunised by the previous CRC Fellow, were also analysed.

The group consisted of 10 men, and 6 women, with a mean age of 71.3 years (range 56-87 years). The control group was composed of cryopreserved samples from patients, matched with trial patients according to site,stage and differentiation of tumour, as well as age and sex of patient. All patients had normal hepatic and renal function, and no patient received pre-operative radiotherapy. These details are summarised in Table 18.

The majority of the tumours were rectal (n=10), with the remainder either caecal (n=2), or from ascending (n=1), and sigmoid colon (n=3). All but two were classified on routine histopathology, as being moderately differentiated. There were equal numbers of Dukes stage A,B and C (n=5), with the remaining case being stage D.

Clinical Protocol.

Five patients received a test dose of 10μg of 105AD7 given intradermally. A further dose of 50μg intramuscularly was administered to those patients who showed no evidence of hypersensitivity on review of the initial injection site 24-72 hours later. One patient, in whom a pre-existing medical condition delayed operation, received this course twice - 10 and 30 days prior to surgery. A second dosing schedule consisted of 50μg of 105AD7 intramuscularly, followed 7 days later by a further 50μg

intramuscularly. Seven patients were recruited into this group. The 3 patients recruited by the previous CRC fellow received 10μg i.d. of 105AD7, followed 48 hours later by 100μg i.m. The mean number of days between first dose of 105AD7, and operation was 19.3 days for these patients.

Immunohistochemistry.

Immunohistochemical analysis of sections was performed on three occasions over two years. The initial analysis was performed using MAb against CD4, CD8, CD56 and CD25. The second used MAb against CD68, CD69 and ζ chain, and the third APO2.7, an MAb against the mitochondrial antigen 7A6. Due to the limitations of processing blocks of tumour tissue, less patients samples were available for each analysis (Table 19).

Resection specimens were retrieved, and samples taken from two edges, the centre of the tumour, and from normal bowel, at least 20cm from the lesion. Tumour tissue was stored in liquid nitrogen, prior to cutting into 5μm sections. Sections were mounted on Vectabond™ (Vector Laboratories) coated slides, air-dried for 5 minutes, and then placed in acetone for 10 minutes. They were air-dried again overnight, before bathing for 5 minutes in Tris buffered saline (TBS). Sections were blocked with 100μl of 20% rabbit serum, and left for 20 minutes. 100μl of mouse MAb, optimally diluted in TBS, were added to each section. MAbs used, source, and dilutions used are shown in Table 17. TBS and IgG_1 were added as negative controls. Specimens of human tonsil were used as a positive control, and labelled with identical dilutions of MAb. After 1hour, slides were washed for a further 5 minutes in TBS. One hundred microlitres of rabbit anti-mouse biotin (Dako), in 4% human serum, was then added for 30 minutes. Following further washing, as previously

described, 100μl of strep-avidin complex (Dako) was added for 30 minutes. This solution was washed off, and bound enzyme visualised using 1 ml of horseradish peroxidase in 3,3' diaminobenzidinetetrahydrocloride (DAB-Sigma Chemical Company). After 10 minutes, slides were transferred to a 0.5% copper sulphate bath for 10 minutes, and then stained with hematoxylin. Finally, slides were dehydrated in alcohol and mounted.

Table 17 Monoclonal Antibodies used for Immunohistochemical staining.

CD Antigen	Clone	Dilution	Manufacturer
CD4	SK3	1:40	Becton Dickinson [1]
CD8	RFT-8	1:20	Gift of Dr G King [2]
CD56	MY31	1:40	Becton Dickinson [1]
CD25	ACT-1	1:10	Dako [3]
CD68	Ki-M6	1:80	Serotec [4]
CD69	TPI/55.3.1	1:100	Serotec [4]
ζ Chain	TIA-2	1:20	Coulter [5]
APO 2.7	2.7A6A3	1:80	Immunotech [5]
IgG_1	W3/25	1:10	Serotec [4]

[1] Becton Dickinson, Between Towns Rd, Cowley, Oxford OX4 3LY

[2] Dr George King, Dept of Pathology, Aberdeen University, Scotland

[3] Dako A/S Produktionsvej 42.DK-2600 Glostrup, Denmark.

[4] Serotec Ltd. 22 Bankside, Station Approach, Kidlington, Oxford, OX5 1JE

[5] Immunotech (Coulter), BP 177-13276 Marseille Cedex 9- France

Image Analysis

Sections were viewed under x200 magnification, and the image digitised and transferred by camera to an Apple Macintosh Quadra 660AV computer. Using the NIH image programme it was possible to quantify the degree of brown staining relative to the blue Haematoxylin background, and express it as a pixel count. **This count is a reflection therefore of expression of that CD antigen analysed on invading lymphocytes.**

Each section was analysed in five different, randomly selected areas, and a mean pixel count obtained. All sections except those labelled with APO2.7 were analysed by the author. Analysis of sections labelled with APO2.7 was performed blindly by one observer (RQ), who analysed each section 15 times, producing 3 mean values. Pixel counts for individual patients are shown in Appendix 2, Tables 2-1 to 2-11.

In order to assess intra-observer variation, samples from 2 trial and 2 control patients analysed by the author, were reanalysed. This second analysis used an identical technique, and took place 9 months later.

An assessment of inter-observer variation was also performed. The sections from one patient were analysed by two observers (CMA and RM), using identical techniques. The results obtained for identical sections are shown in Appendix 2, Table 2-13.

Statistical analysis.

Median scores are given, and a two-tail Wilcoxon Signed Rank test was used to test for statistical significance, which was taken at the 5% level.

Table 18. Tumour sections from trial patients analysed immunohistochemically.

No.	Init.	Age [1]	Sex	Dose	Op [2]	Site [3]	Stage	Diff
1.	DT	75	F	10+50	18	Rectum	C	Mod
2.	EN	79	F	10+50	14	Sigmoid	B	Mod
3.	ER	82	M	10+50	11	Caecum	C	Mod
5.	JGr	64	M	10+50[4]	31	Rectum	D	Mod
6.	ET	87	M	10+50	20	Caecum	B	Mod
8.	MB	75	M	10+50	26	Rectum	A	Mod
9.	DH	56	M	50+50	9	Rectum	B	Mod
11.	JK	76	M	50+50	14	Sigmoid	A	Mod
12.	JGa	58	M	50+50	13	Rectum	A	Mod
13.	TB	62	F	50+50	20	Rectum	C	Poor
15.	FCl	67	F	50+50	14	Rectum	A	Mod
17.	FHa	73	M	50+50	29	Rectum	C	Mod
18.	FCh	70	M	50+50	13	Rectum	B	Mod
A [5]	AC	67	F	10+100	29	Asc.	B	Poor
B [5]	HS	67	M	10+100	17	Sigm	A	Mod
C [5]	AGe	83	F	10+100	30	Rectum	C	Mod

1. Age at time of removal of the primary tumour.

2. Number of days between first immunisation, and operation

3. Site of primary tumour

4. Patients received two courses of 10μg i.d. followed by 50μg i.m.

5 Three patients were recruited to the adjuvant study by the previous CRC fellow. Cryopreserved samples from these patients were also stained immunohistochemically.

Table 19. MAb labelling of tumour sections.

No.	Initials.	CD4,CD8, CD56 & CD25	CD68,CD69 CD3 ζ chain.	APO 2.7
1.	DT	Yes	No	No
2.	EN	Yes	Yes	Yes
3.	ER	Yes	Yes	Yes
5.	JGr	Yes	Yes	No
6.	ET	Yes	Yes	Yes
8.	MB	Yes	Yes	Yes
9.	DH	Yes	Yes	Yes
11.	JK	Yes	Yes	Yes
12.	JGa	Yes	No	No
13.	TB	Yes	Yes	Yes
15.	FCl	Yes	Yes	No
17.	FHa	Yes	Yes	No
18.	FCh	Yes	Yes	Yes
A [1]	AC	Yes	No	No
B [1]	HS	Yes	No	No
C [1]	AGe	Yes	No	No

[1] Patients recruited by the previous CRC fellow.

Results.

Patients were reviewed on several occasions between first immunisation, and operation. No side-effects related to the use of 105AD7 were observed. This finding confirms the results of the Phase I and Phase II studies, in a cohort of patients with primary colorectal cancer.

Samples of normal bowel, tumour centre, and two tumour edges, from sixteen trial patients, and their controls, were analysed immunohistochemically. Each section was analysed randomly 5 times, and a mean pixel count obtained. This raw data is shown in Appendix 2, Table 2-1 to 2-11. In practice, however, not all patients had two edges analysed. If the original tumours were small then only one sample would be taken from the tumour edge, in order to allow sufficient tissue for routine histopathological analysis.

Insufficient numbers of patients were present in the individual dosing regimes, to allow comparison, so results were combined to give figures for all patients who had received 105AD7. The analysis was perfomed to assess infiltration into the centre and edge of the tumour, and also for the tumour edges alone. In addition, a ratio of tumour infiltration to that of normal bowel infiltration was calculated. This is expressed as Tumour/Normal (T/N) ratios. No assumptions were made about the distribution of the data, and medians and interquartile ranges for the two dosage schedules, and combined results are shown.

Human tonsil was used as a positive control, and the results obtained shown in Table 22. Lymphocytic infiltration was markedly higher than in tumour tissue, as would be expected, and the findings confirm that the MAbs label lymphocytes at the dilutions used.

Infiltration at the tumour centre and edge is shown in Table 20 for all patients results combined. Median infiltration of CD4, CD8 and CD56

expressing lymphocytes was higher in trial patients than controls, when results were combined (0.92v0.65: p=0.021). Infiltration by CD4 expressing lymphocytes was similarly significantly higher in the 16 trial patients (1.17v0.81: $p<0.05$). This is shown graphically in Figure 16. Median scores for trial patients sections labelled with MAb against CD8, CD56, CD25, CD69, CD68, and CD3ζ chain were 0.81, 0.82, 0.75, 0.51, 1.10, and 0.56 respectively. The corresponding median infiltrations for control patients were 0.75, 0.44, 0.50, 0.79, 1.02, and 0.62. There was no difference between pixel scores for trial and control sections labelled with the negative control antibody IgG_1 (0.20v0.18) or TBS (0.23v0.18). Median pixel score for APO2.7 labelling was significantly higher in trial patients than controls (2.86v1.77: $p<0.005$). The IgG_1 negative control used for these sections was 0.37 for both trial and control patients.

Median pixel scores were also calculated for figures from the edge of the tumour alone. These are also shown in Table 20. When results of CD4, CD8 and CD56 were combined, scores were significantly higher in trial patients when compared with controls (0.85v0.58: p=0.028). Expression of the α subunit of the Interleukin-2 receptor was also significantly higher in trial patients (0.76v0.33: $p<0.025$). Median scores for trial patients labelled with MAb against CD8, CD4, CD56, CD69, CD68, and CD3ζ chain were 0.81, 1.02, 0.85, 0.51, 0.99, and 0.56. There was no significant difference between these figures and the controls, whose scores were 0.75, 0.81, 0.43, 0.83, 1.01, and 0.80, respectively. Pixel counts for trial patients labelled with IgG_1 and TBS were 0.24 and 0.29 respectively. The corresponding scores for their controls was 0.18 and 0.20. Neither of these differences was statistically significant.

Ratios of pixel scores obtained from the edge and centre of the tumour, and normal bowel, in trial and control patients are shown in Table 21. The first column considers results of both centre and edge of the

tumour. The ratio for the combined results of CD4, CD8, and CD56 was once again significantly higher in trial patients (0.93v0.78: p=0.04), as was expression of CD25 (1.12v0.42: p<0.05). There was no significant difference between trail and control ratios in sections labelled with CD8, CD4, CD56, CD69, CD68, CD3ζ and APO2.7. Scores in trial patients were 0.91, 1.04, 0.62, 0.59, 0.86, 0.55 and 0.56 for each respective MAb. Corresponding scores in controls were 0.64, 0.90, 0.40, 0.82, 0.81, 0.48 and 0.55. Values for IgG_1 and TBS were higher in trial patients than controls, though not significantly so (0.61v0.50 and 0.90v0.46).

The ratio of tumour edge to that of normal bowel was also calculated, and also shown in Table 21. A significant difference was seen in favour of trial patients for the combination of CD4, CD8 and CD56 (1.00v0.80: p=0.04). Pixel counts for CD8 were also significantly higher in trial patients (1.03v0.65: p<0.05), as was CD25 expression (1.08v0.41: p<0.05). The former result is shown graphically in Figure 17. There was no significant difference in ratios of edge to normal bowel when sections were labelled with CD4, CD56, CD69, CD68, CD3ζ chain. Figures for trial patients were 1.04, 0.67, 0.59, 0.76, and 0.51, compared with 0.91, 0.37, 0.88, 0.82, and 0.51. There was no difference for scores for IgG_1 and TBS. These were 0.65 and 0.65 in trial patients, and 0.49 and 0.48 in controls, respectively.

Median pixel counts are also shown, when figures from the tumour edge alone were considered. Significantly higher values were once again seen for overall infiltration, and T/N ratios for CD25 (p<0.025 and p=0.05), and when results from CD56, CD4, and CD8 were combined (p=0.028 and p=0.041). In addition the T/N ratio for CD8 was significantly higher in immunised patients (p<0.05).

Sections from 2 trial and 2 control patients were analysed twice by the same observer, to test for intraobserver variation. The concordance between individual mean pixel scores is shown in Appendix 2, Table 2-12.

Median pixel counts were 0.62 and 0.50 for first and second assessments respectively. Interquartile ranges were comparable, 0.19-1.02 and 0.23-0.88, and there was no significant difference between the two groups. The correlation coefficient was +0.36 (covariance 0.11). Figures for the tumour:normal ratios showed a median of 0.71 and 0.62 for first and second analyses respectively. Relative IQR were 0.42-1.15 and 0.40-1.23, with a correlation co-efficient of +0.27, and covariance of 0.14.

Slides from one patient were analysed blindly by a further observer, in order to assess the degree of interobserver variation. Mean scores for edge and centre of the tumour, for CD25, CD8, CD56, and CD4 MAbs, and TBS control, are shown in Table 13, Appendix 2. Mean and median pixel counts of all the results were 0.34 and 0.14 (IQR 0.07-0.60), and 0.71 and 0.56 (IQR 0.95-1.56), respectively for Observer 1 and Observer 2. This difference was statistically significant ($p<0.005$), and the correlation coefficient was +0.69 (covariance 0.13). The ratio of tumour:normal bowel pixel counts were also calculated. Mean and median scores for Observer 1 were 0.47 and 0.40 (IQR 0.14-0.49), as compared with 0.76 and 0.71 (0.51-1.03) for Observer 2. This difference was once again statistically significant ($p<0.05$), with a comparable correlation coefficient (+0.65).

Statistical analysis was performed in order to assess whether the effect of 105AD7 was the same across all four Dukes stages, for CD4, CD8 and CD56 expression combined (Table 23). Of the 16 patients, 5 were Dukes A,B and C, with the remaining 1 a stage D tumour. Clearly the number of patients is small, so the results need interpreting with a degree of caution. Median pixel counts were 0.99, 1.13, 0.79 and 0.24 for stages A-D in trial patients, and 1.04, 0.60, 0.60 and 0.75 in controls. The only significant differences were between stage B ($p<0.005$) and stage D ($p<0.025$). When tumour infiltration was compared with that of normal bowel, the ratios

obtained were 1.12, 1.04, 0.66, and 0.33 in trial patients, and 1.04, 0.77, 0.51 and 0.29 in controls. Only stage B was significant ($p<0.01$).

A further analysis was performed to assess whether CD3ζ chain expression varied with Dukes stage. Pixel counts in trial patients were 0.42, 0.59 and 0.53 for stages A-C, as compared with figures of 0.35, 0.54 and 0.99 in controls. Insufficient numbers precluded any analysis of the patient with a Dukes D tumour. The tumour : normal bowel ratios were 0.41, 0.51, and 0.44 for the three stages respectively in patients who had received 105AD7. Figures for controls were 0.24, 0.54 and 0.83.

Eight tumour specimens from immunised and unimmunised patients were analysed immunohistochemically for APO2.7 expression. Individual pixel scores for edges, and tumour centres are shown in the Appendix, Table 2-11. Median, and mean scores are shown in Table 20 for both APO2.7, and the negative control. Median scores for the negative control (IgG_1) are very similar in both trial and control patients, both for absolute labelling of 7A6 antigen by APO2.7, (0.37v0.37), and the tumour : normal bowel ratios (0.70v0.89). Median pixel scores were higher in trial sections stained with APO2.7 than controls (2.86 v 1.77: $p<0.005$). The median tumour: normal ratios were however essentially the same (0.56v0.55).

In order to assess in which part of the tumour apoptosis was highest, median figures for tumour edge and centre were calculated. Absolute labelling by APO2.7 in trial patients was 2.36 (1.82-3.68) at the centre, and 3.42 (2.07-4.41) at the edge. This compared with figures of 1.94 (0.94-2.59) and 1.77 (1.64-2.00) respectively, in controls. The ratios of tumour:normal bowel labelling were also calculated, for centre and edge. The figures in trial patients were 0.57 (0.50-1.09) and 0.52 (0.35-0.95), as compared with 0.59 (0.46-0.78) and 0.46 (0.44-0.60). None of the differences between centre and edge were statistically significant.

Table 20. Lymphocytic infiltration at tumour edge & centre [1], and at edge [2] alone.

	Trial [1].	Control [1]	Trial [2].	Control [2]
CD56, 4 & 8.	0.92 (0.59-1.41)	0.65 ¶ (0.24-1.35)	0.85 (0.60-1.49)	0.58 ♦ (0.25-1.27
CD8	0.81 (0.49-1.16)	0.75 (0.24-1.36)	0.81 (0.60-1.22)	0.75 (0.24-1.36)
CD4	1.17 (0.76-1.87)	0.81 * (0.32-1.41)	1.02 (0.59-1.87)	0.81 (0.27-1.43)
CD56	0.82 (0.51-1.27)	0.44 (0.18-1.29)	0.85 (0.62-1.30)	0.43 (0.20-0.97)
CD25	0.75 (0.40-1.27)	0.50 (0.15-0.94)	0.76 (0.46-1.11)	0.33 § (0.14-0.78)
CD69	0.51 (0.43-0.95)	0.79 (0.52-1.19)	0.51 (0.44-0.92)	0.83 (0.54-1.18)
CD68	1.10 (0.79-1.31)	1.02 (0.83-1.56)	0.99 (0.57-1.24)	1.01 (0.65-1.39)
ζ chain	0.56 (0.37-0.87)	0.62 (0.39-0.95)	0.56 (0.44-0.93)	0.80 (0.36-0.92)
APO2.7	2.86 (1.89-3.99)	1.77 Σ (1.54-2.21)	-	-
IgG_1 [3]	0.20 (0.12-0.40)	0.18 (0.11-0.27)	0.24 (0.15-0.40)	0.18 (0.13-0.25)
IgG_1 [4]	0.37 (0.02-0.92)	0.37 (0.08-0.85)	-	-
TBS	0.23 (0.11-0.62)	0.18 (0.06-0.48)	0.29 (0.13-0.58)	0.20 (0.09-0.96)

Key to Table 20.

1. Lymphocyte infiltration at the tumour edge and centre.

2. Lymphocyte infiltration at the tumour edge only.

3. Negative control for sections labelled with CD69, CD68, and CD3ζ chain.

4. Negative control for sections stained with APO2.7.

5. TBS as negative control for labelling with MAb against CD4, CD56, CD8 and CD25.

Figures in parentheses denote Interquartile Ranges

* $p<0.05$; ¶ $p=0.021$; ♦ $p=0.028$; § $p<0.025$; Σ $p<0.005$

Table 21. Ratios of tumour edge and centre [1] to normal bowel, and of edge alone to normal bowel [2].

CD Ag	Trial [1].	Control [1]	Trial [2].	Control [2]
CD56, 4 & 8.	0.93 (0.48-1.48)	0.78 Δ (0.36-1.18)	1.00 (0.59-1.48)	0.80 Δ (0.33-1.13)
CD8	0.91 (0.50-1.90)	0.64 (0.30-1.47)	1.03 (0.64-2.14)	0.65 © (0.27-1.11)
CD4	1.04 (0.69-1.46)	0.90 (0.49-1.13)	1.04 (0.69-1.37)	0.91 (0.39-1.13)
CD56	0.62 (0.38-1.39)	0.40 (0.20-1.30)	0.67 (0.49-1.36)	0.37 (0.28-1.36)
CD25	1.12 (0.67-1.82)	0.42 © (0.21-1.10)	1.08 (0.61-1.39)	0.41 © (0.16-0.97)
CD69	0.59 (0.38-0.91)	0.82 (0.56-1.30)	0.59 (0.45-0.80)	0.88 (0.55-1.29)
CD68	0.86 (0.54-1.00)	0.81 (0.64-1.10)	0.76 (0.53-0.86)	0.82 (0.69-1.05)
ζ chain	0.55 (0.39-0.80)	0.48 (0.25-0.63)	0.51 (0.42-0.76)	0.51 (0.26-0.76)
APO2.7	0.56 (0.45-0.99)	0.55 (0.44-0.77)	-	-
IgG_1 [3]	0.61 (0.43-1.79)	0.50 (0.26-0.82)	0.65 (0.41-2.06)	0.49 (0.26-0.94)
IgG_1 [4]	0.70 (0.53-1.20)	0.89 (0.60-1.57)	-	-
TBS	0.90 (0.38-1.82)	0.46 (0.21-1.44)	0.65 (0.38-1.10)	0.48 (0.17-1.58)

Key to table 21.

1. Lymphocyte infiltration at the tumour edge and centre.

2. Lymphocyte infiltration at the tumour edge only.

3. Negative control for sections labelled with CD69, CD68, and CD3ζ chain.

4. Negative control for sections stained with APO2.7.

5. TBS as negative control for labelling with MAb against CD4, CD56, CD8 and CD25.

© $p<0.05$; Δ $p=0.04$

Table 22. Infiltration of Tonsil by lymphocytes expressing various CD antigens - Positive control.

CD Ag	1.	2.	3.	4.	5.	Mean
TBS	0	0.01	0	0	0	0.005
IgG	0.66	0.01	0	0	0.02	0.14
CD4	3.03	3.52	6.73	2.34	6.77	4.48
CD8	2.24	1.68	1.28	1.43	1.22	1.57
CD25	1.36	1.36	1.27	1.27	2.45	1.54
CD68	1.29	1.09	2.12	1.96	0.25	1.34
CD69	1.44	2.51	0.85	0.60	3.31	1.74
ζ	0.89	0.20	0.98	3.13	0.57	2.89
CD56	1.43	0.01	2.07	0.64	5.32	1.89

Table 23. Variation in pixel count with Dukes stage of trial and control patient. Combined results of CD4, CD8 and CD56 MAb labelling.

Stage	Trial patient Pixel count	Control patient Pixel count	Trial patient T/N ratio [1]	Trial patient T/N ratio [1]
A n=5	0.99 (0.73-1.56)	1.04 (0.27-1.73)	1.12 (0.75-2.08)	1.04 (0.41-1.42)
B n=5	1.13 (0.78-1.78)	0.60 **p<0.005** (0.19-1.24)	1.04 (0.72-1.66)	0.77 **p<0.01** (0.38-1.19)
C n=5	0.79 (0.57-1.26)	0.60 (0.23-1.20)	0.66 (0.43-1.09)	0.51 (0.30-0.85)
D n=1	0.24 (0.19-0.33)	0.75 **p<0.03** (0.37-0.85)	0.33 (0.25-0.47)	0.29 (0.18-0.63)

1. Ratio of tumour to normal bowel infiltration.

Figure 4. Immunohistochemical staining of human tonsil with IgG_1 negative control monoclonal antibody (x200). Lymphoid follicles are clearly visible within the photomicrograph. The section is blue due to the background hematoxylin counter-stain.

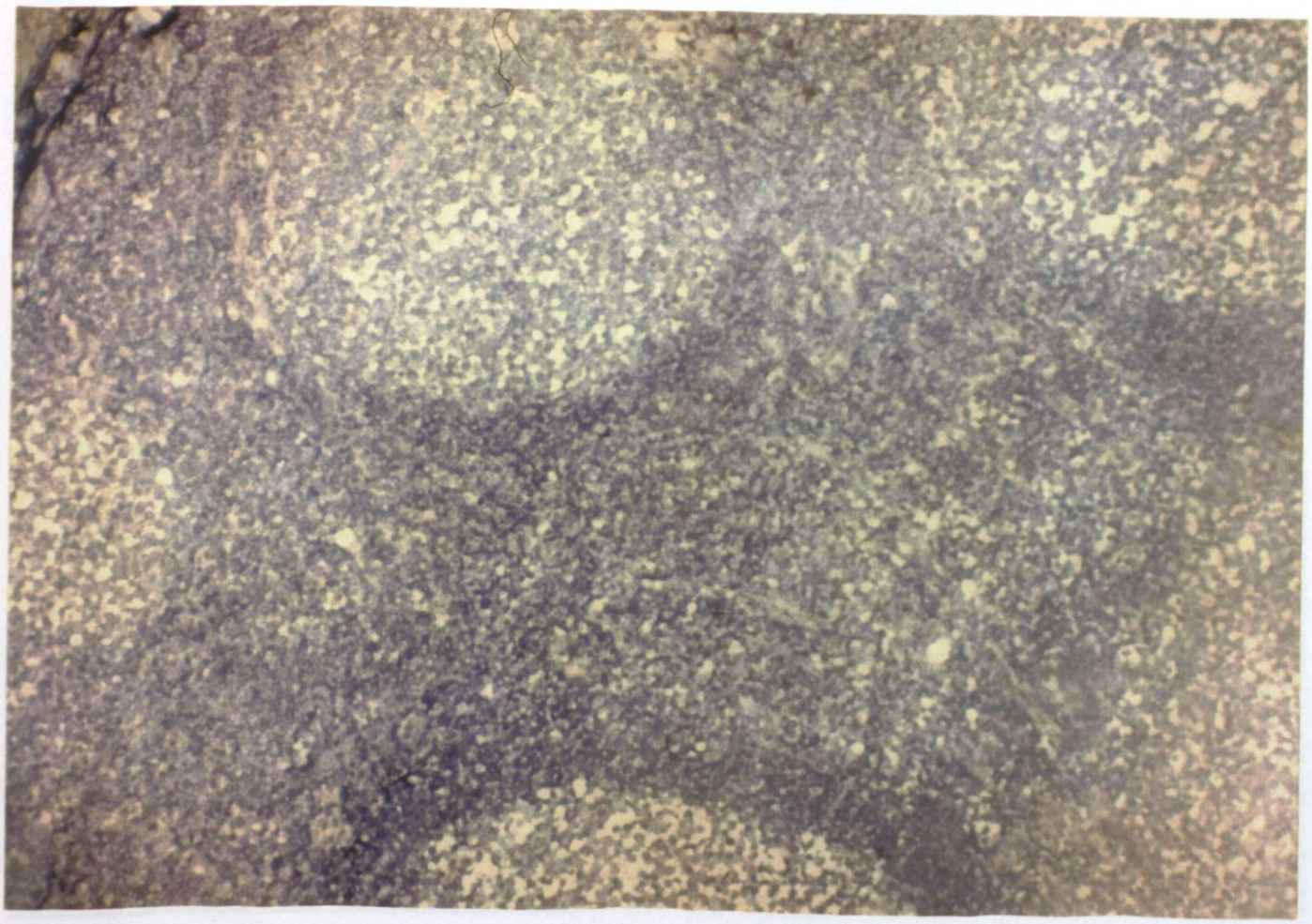


Figure 5. Immunohistochemical staining of sections of human tonsil by CD4 monoclonal antibody (x200). The brown stain within the follicles labels lymphocytes expressing the CD4 surface antigen. As would be expected this is greater than that of the negative control (Figure 4).

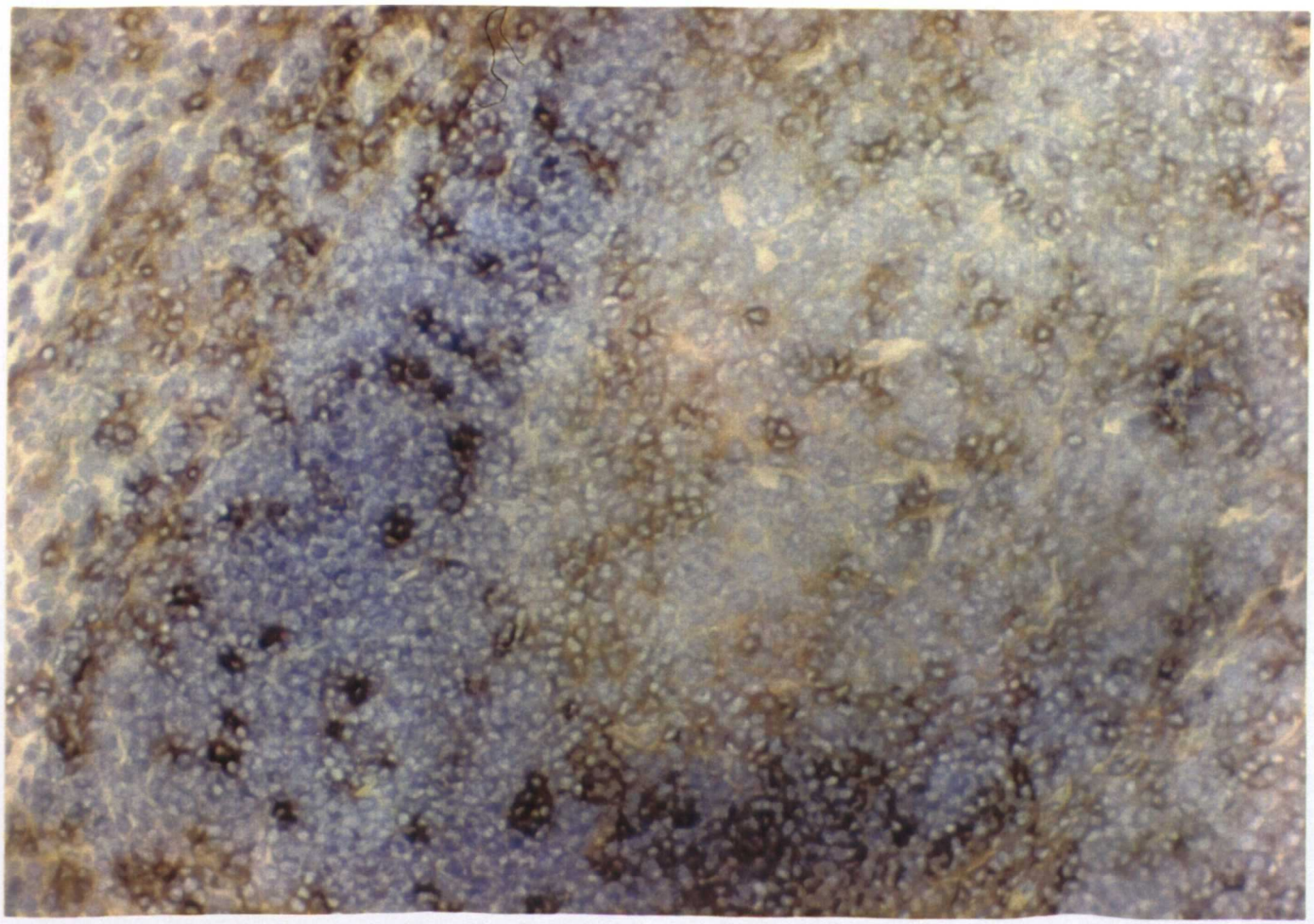


Figure 6. Immunohistochemical staining of tumour centre by CD4 monoclonal antibody, in a patient who received 105AD7 pre-operatively (x200). The brown stain represents stromal infiltration of lymphocytes expressing CD4 surface antigen. Tumour cells are stained blue with counter-stain.

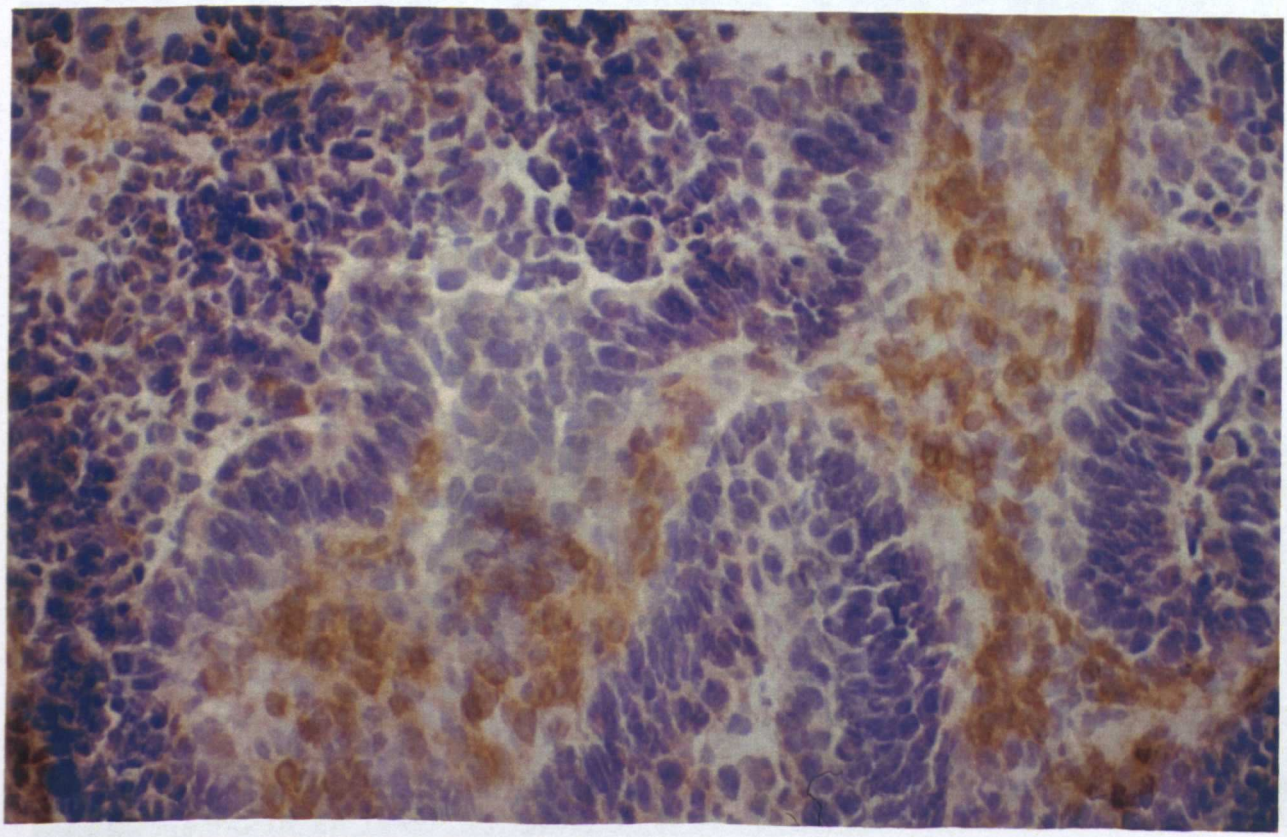


Figure 7. Immunohistochemical staining of tumour edge by CD4 monoclonal antibody, in a patient who received 105AD7 pre-operatively (x200). Brown stain can clearly be seen at the interface between normal bowel and the leading edge of the tumour. Once again there is stromal infiltration of lymphocytes expressing CD4.

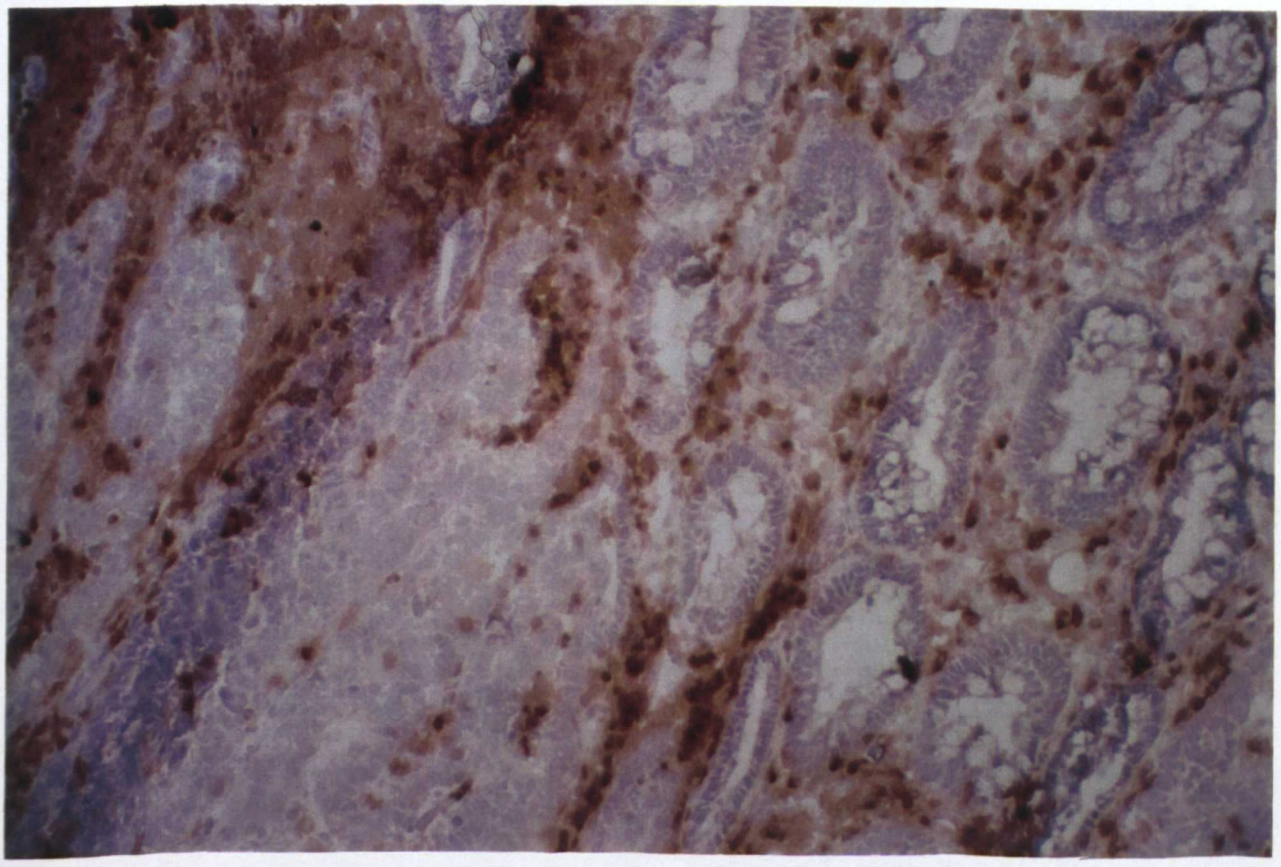


Figure 8. Immunohistochemical staining of tumour centre by CD8 monoclonal antibody, in a patient who received 105AD7 pre-operatively (x200). Once again infiltration is predominantly stromal.

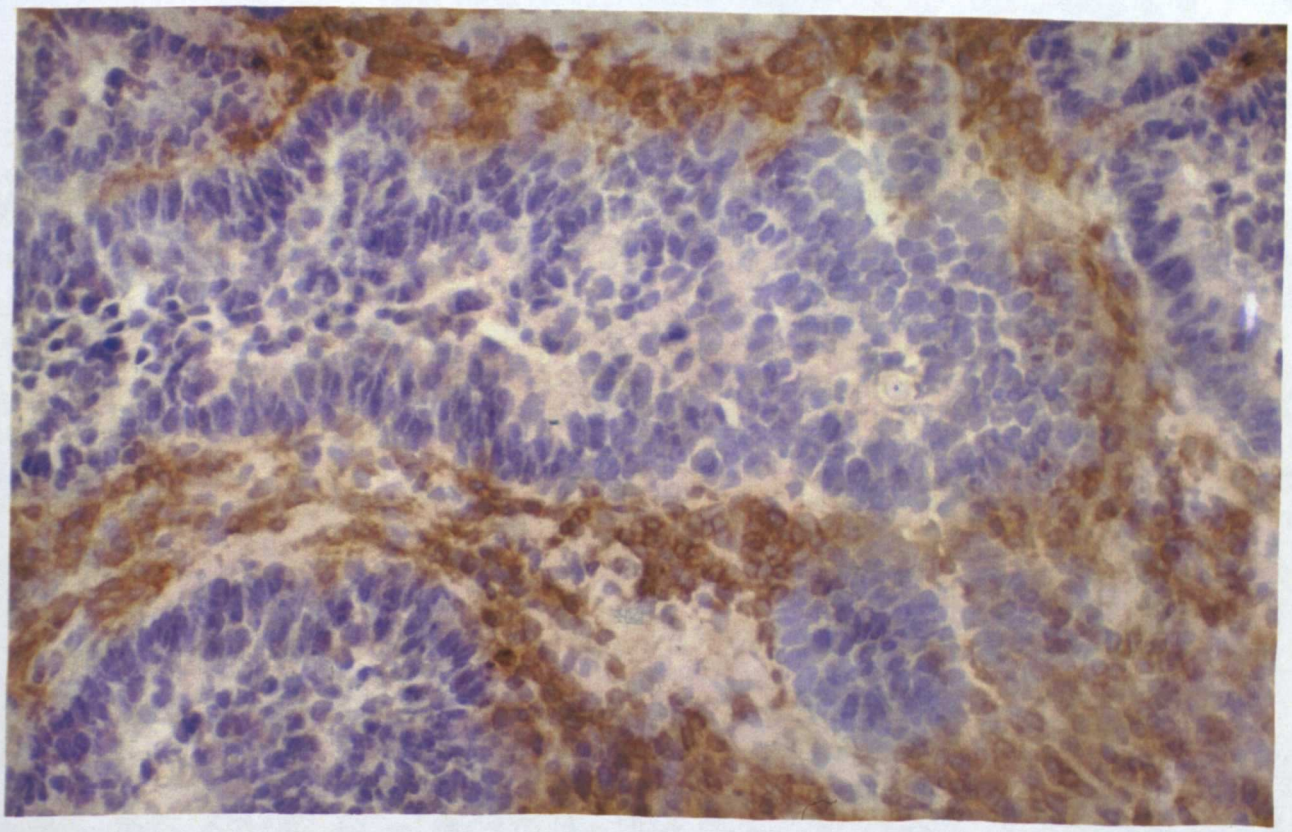


Figure 9. Immunohistochemical staining of tumour edge by CD8 monoclonal antibody, in a patient who received 105AD7 pre-operatively (x300). Higher magnification view showing the leading edge of the tumour, with normal villi visible at the bottom-right hand corner of the photomicrograph.

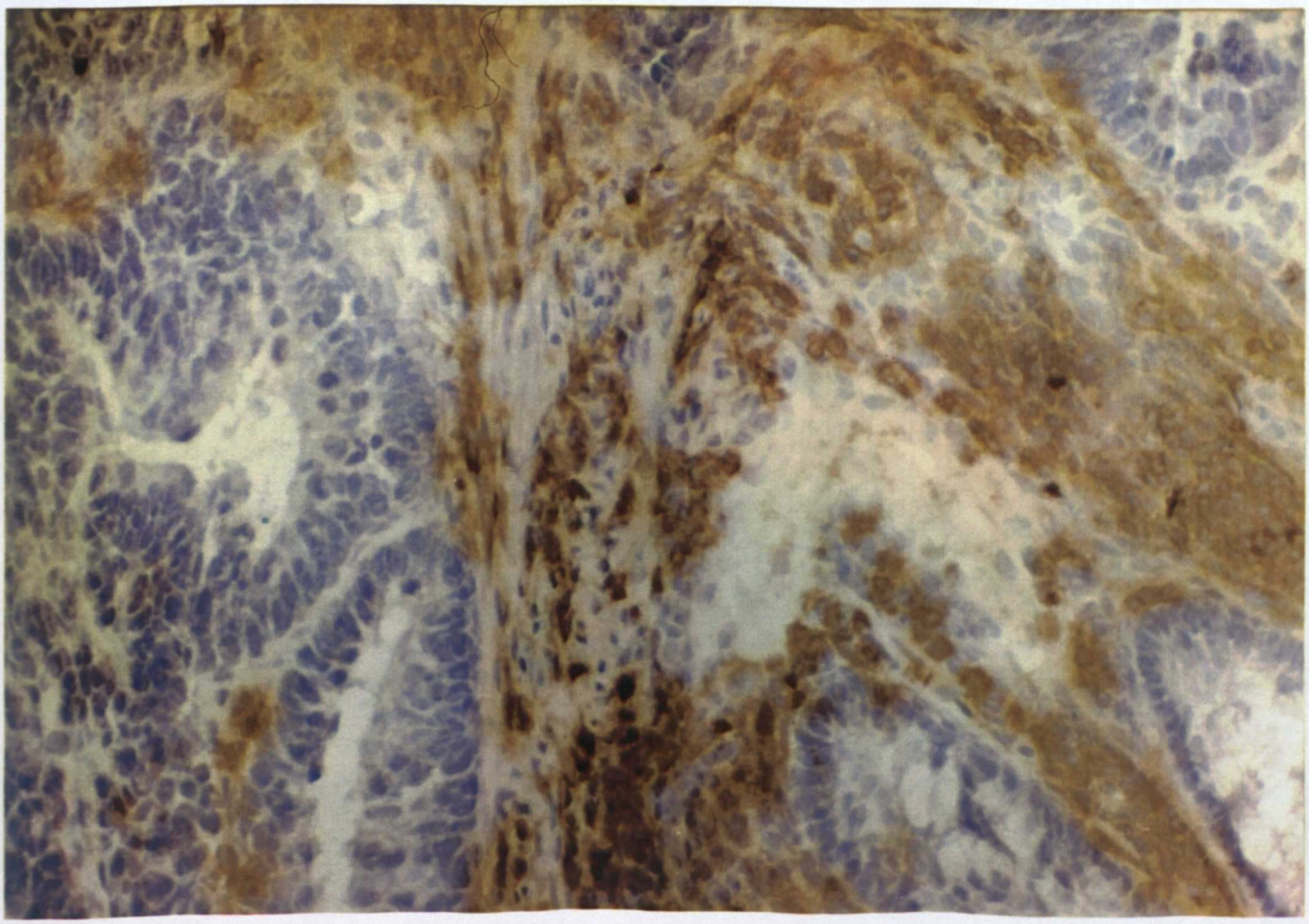


Figure 10. Immunohistochemical staining of tumour centre by CD56 monoclonal antibody, in a patient who received 105AD7 pre-operatively (x200). Stromal infiltration of NK cells between nests of tumor. White areas represent artefact introduced on cutting sections.

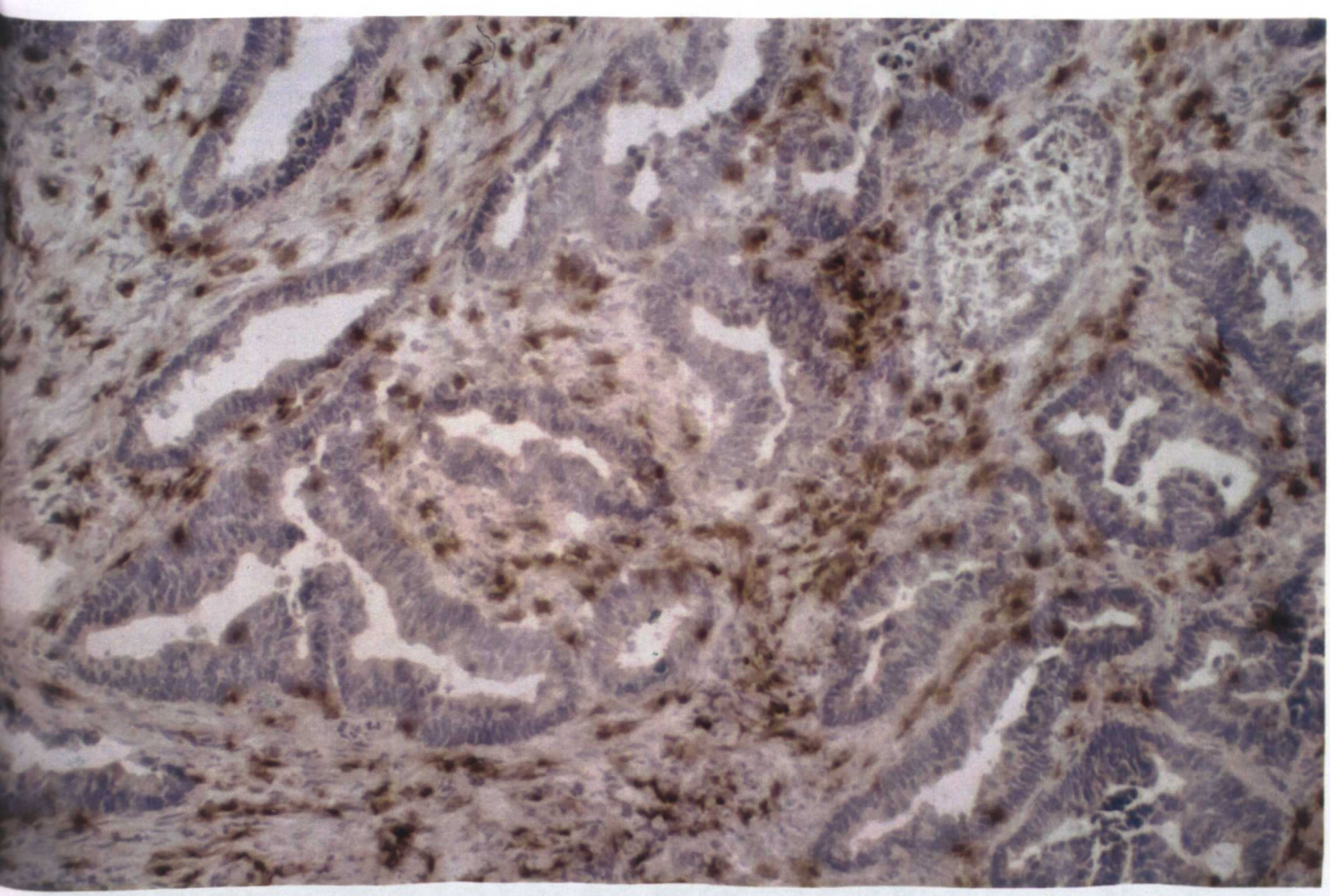


Figure 11. Immunohistochemical staining of tumour edge by CD56 monoclonal antibody, in a patient who received 105AD7 pre-operatively (x300). Photomicrograph shows predominantly normal villi, with leading edge of tumour to the left of the picture. Clearly this is where lymphocytic labelling predominates.

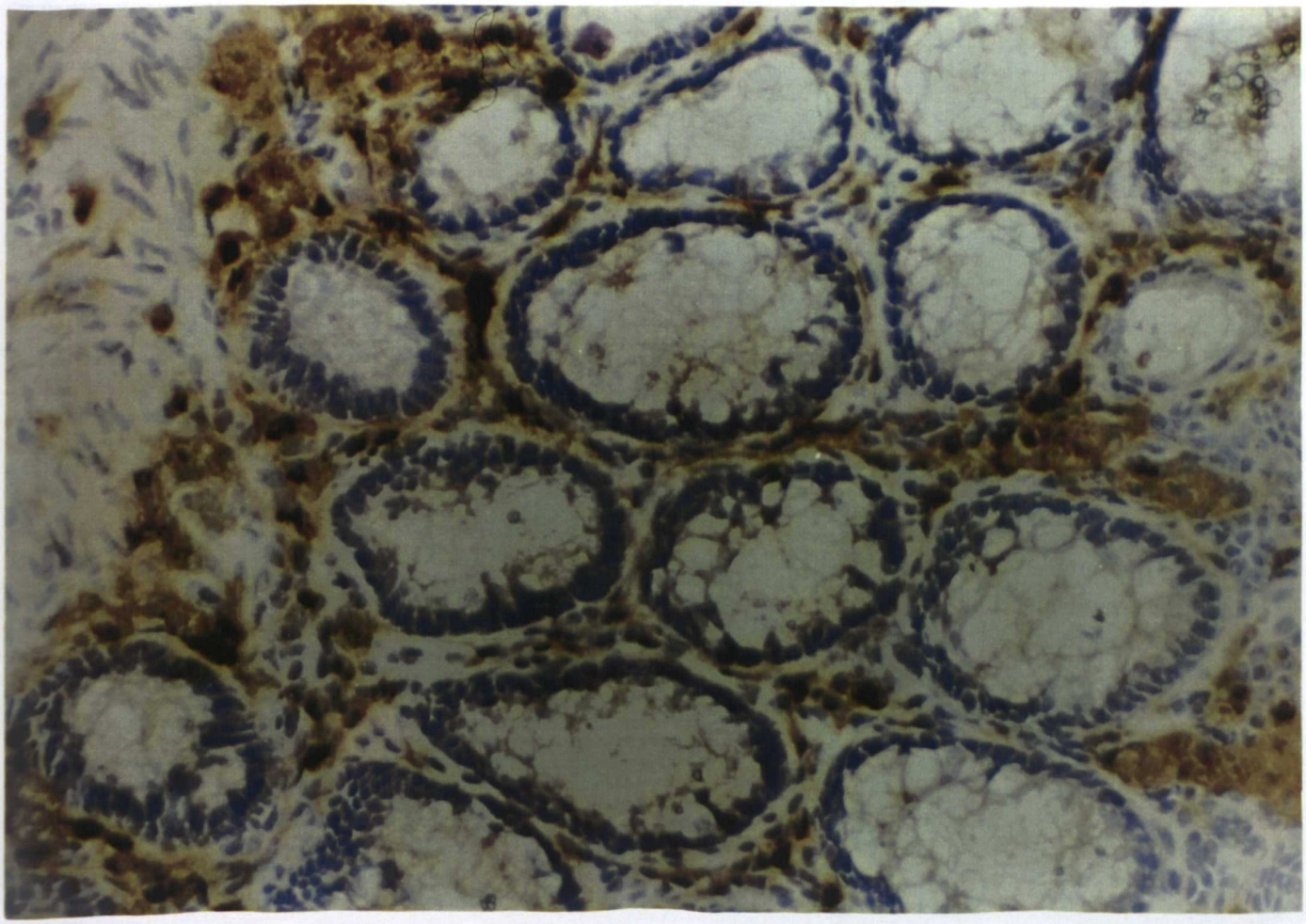


Figure 12. Immunohistochemical staining of tumour centre by CD25 monoclonal antibody, in a patient who received 105AD7 pre-operatively (x300). Stromal infiltration of activated lymphocytes.

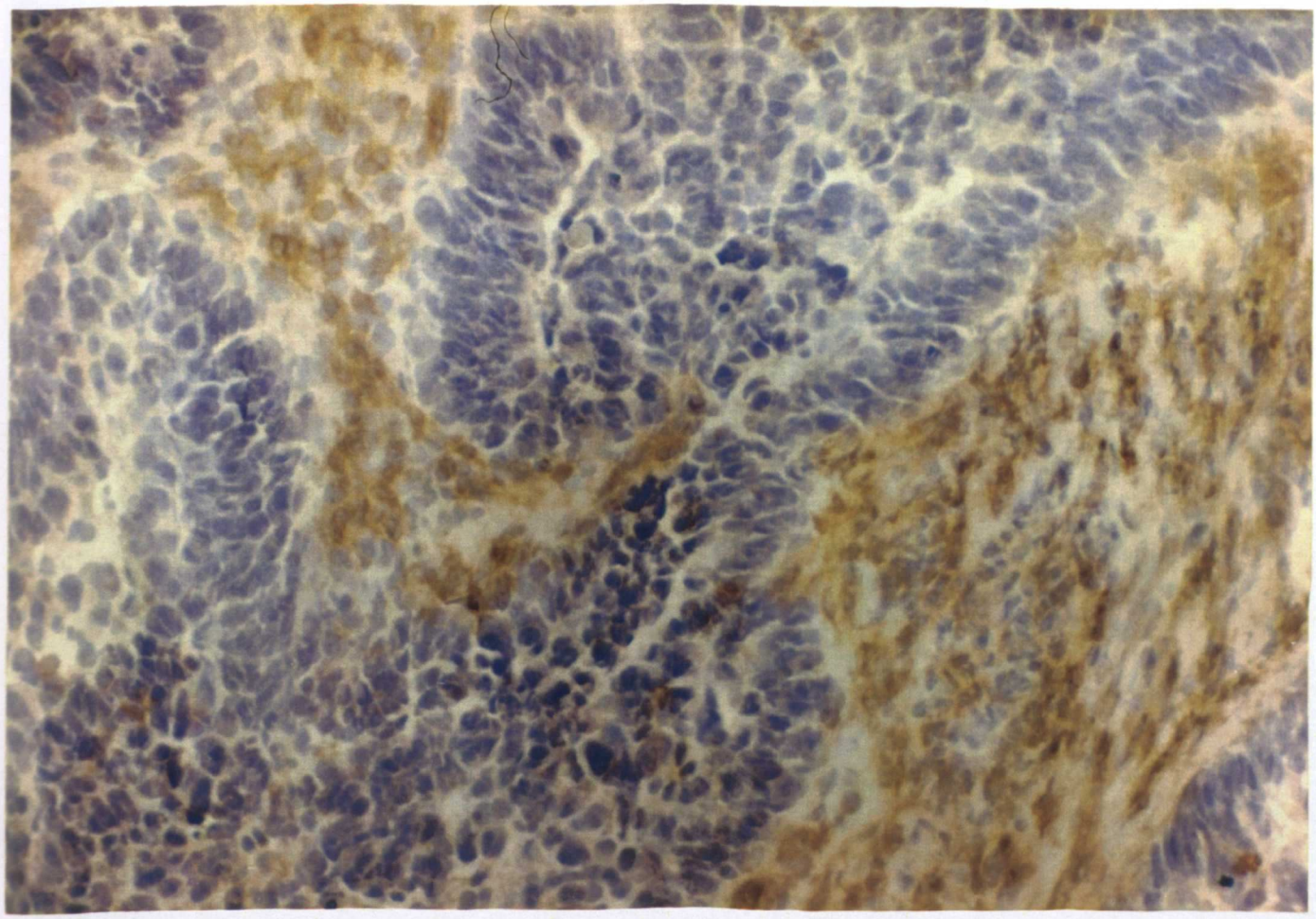


Figure 13. Photomicrograph of tumour centre labelled with CD25. Section taken from matched unimmunised control patient for patients shown in Figure 12. Clearly less brown stain seen, suggesting fewer activated lymphocytes at the tumour site of the patient who did not receive 105AD7 pre-operatively.

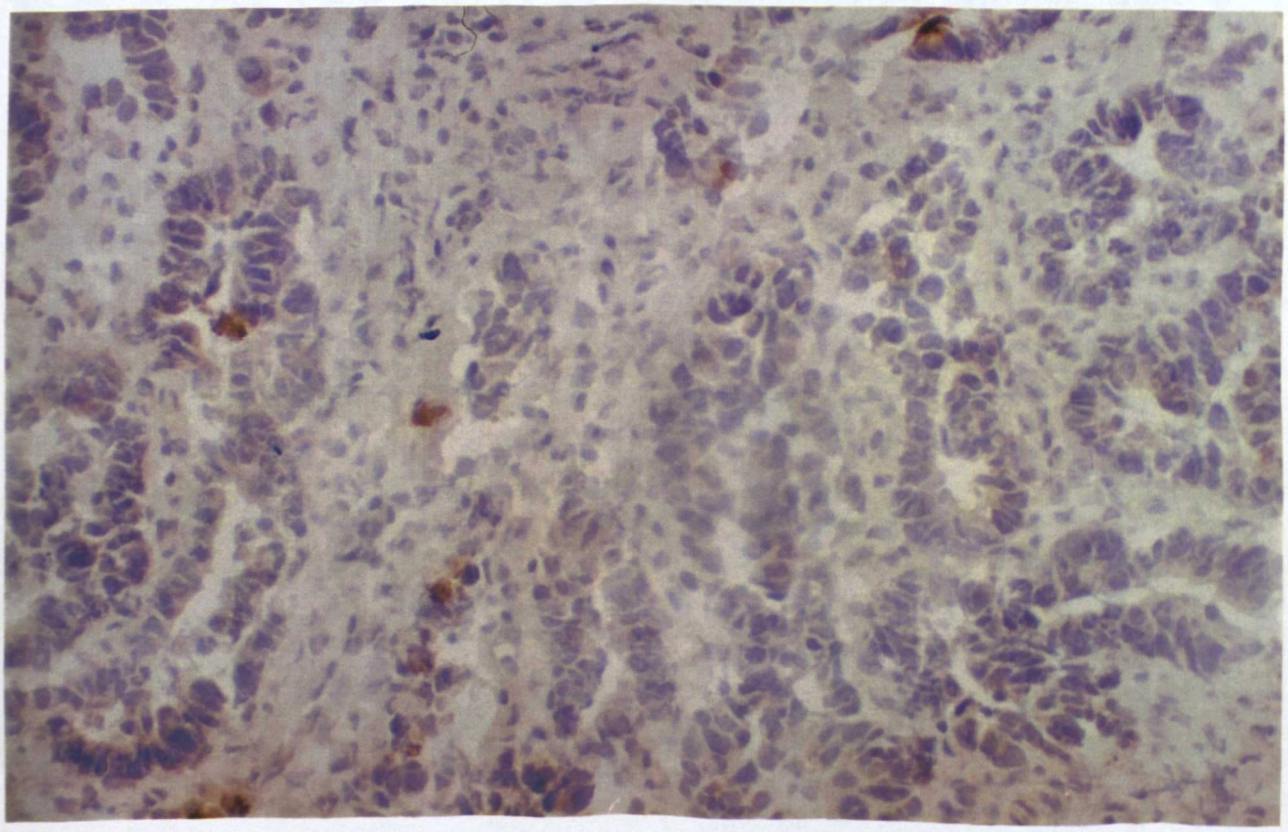


Figure 14. Immunohistochemical staining of tumour centre by IgG_1 monoclonal antibody, in a patient who received 105AD7 pre-operatively (x300). Blue staining is indicative of the background hematoxylin.

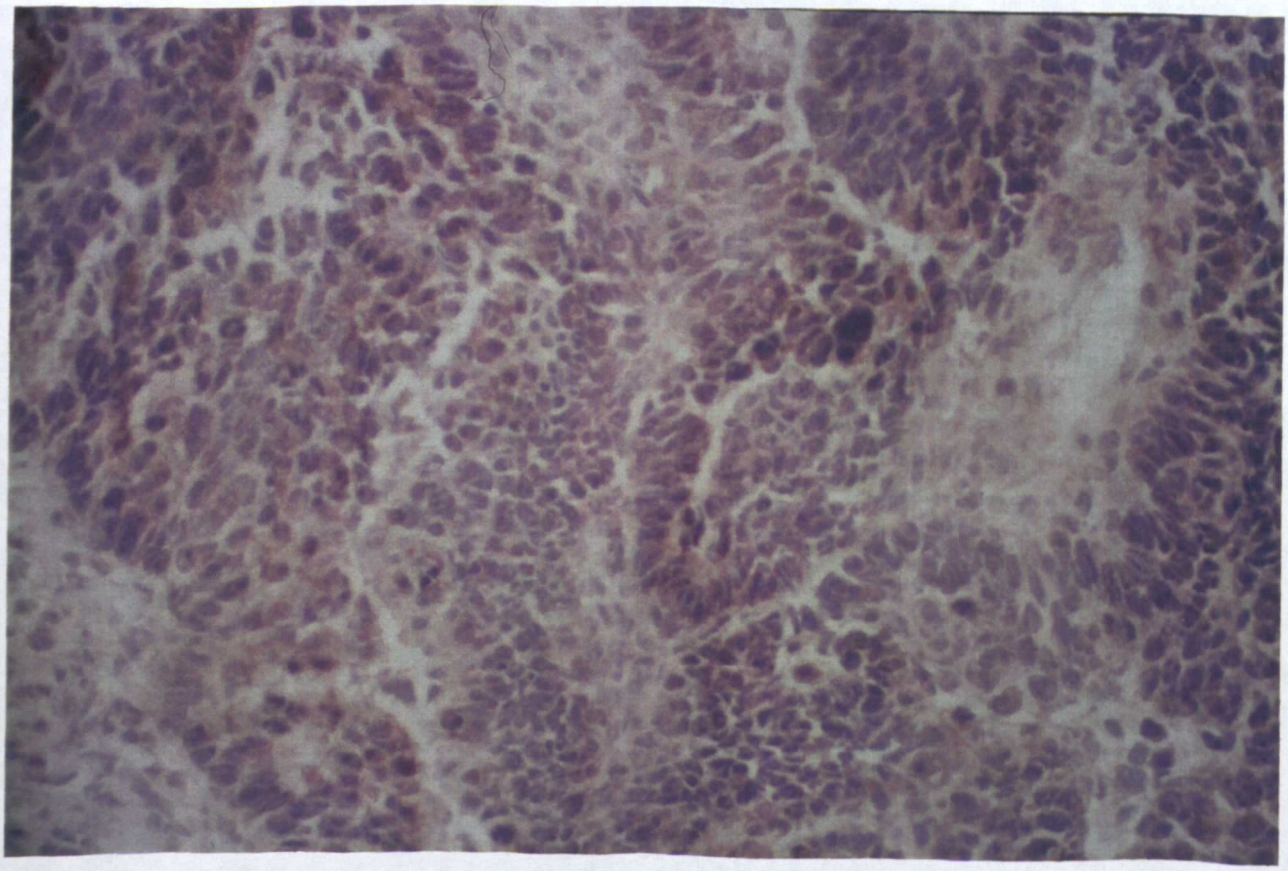


Figure 15. Photomicrograph of normal bowel immunohistochemically labelled by CD4 monoclonal antibody, in a patient who received 105AD7 pre-operatively (x300). The brown stain predominates in the connective tissue between the villi.

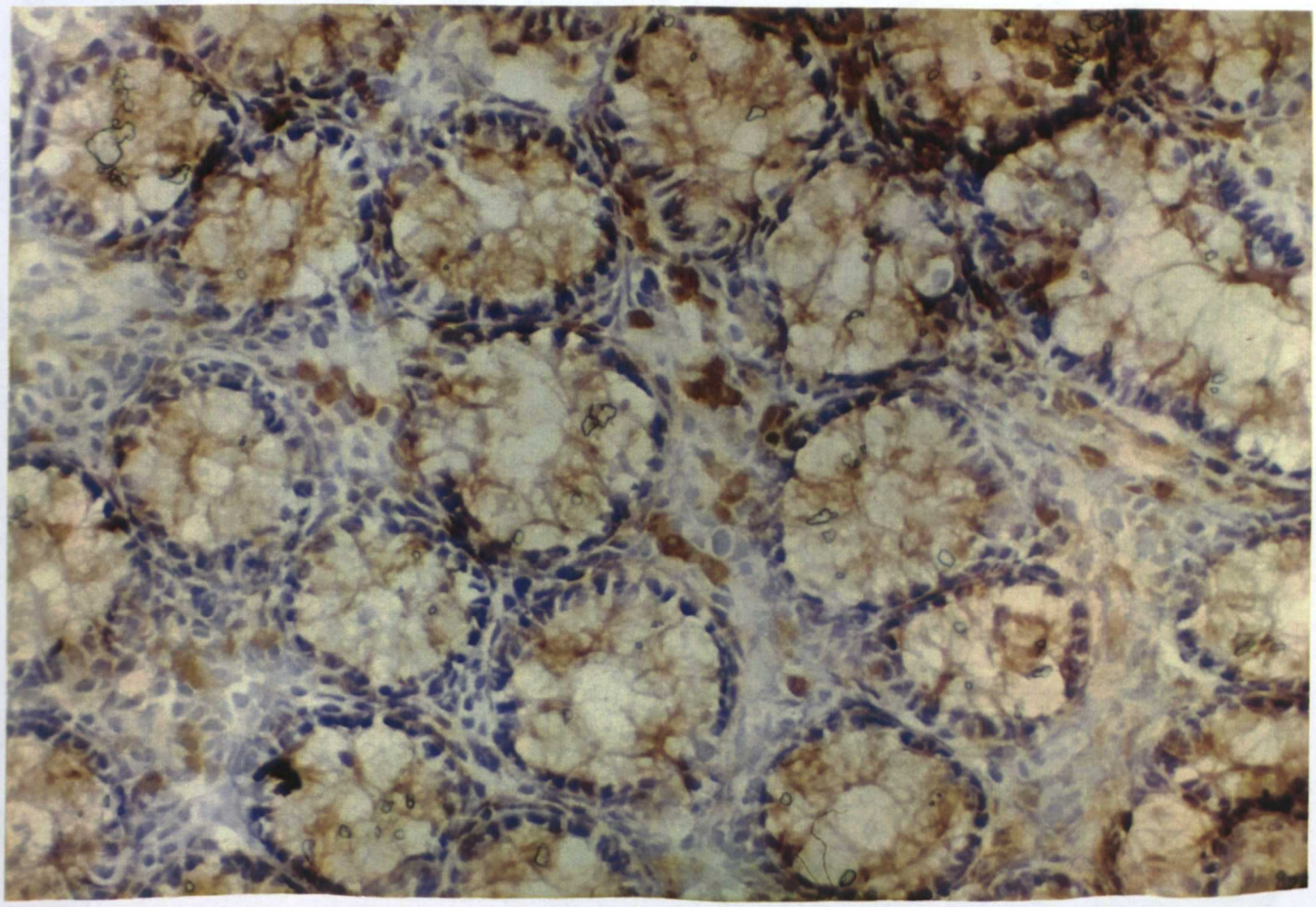


Figure 16. Immunohistochemical staining of normal bowel by IgG_1 monoclonal antibody, in a patient who received 105AD7 pre-operatively (x300). Photomicrograph shows normal colonic villi stained blue by the negative control.

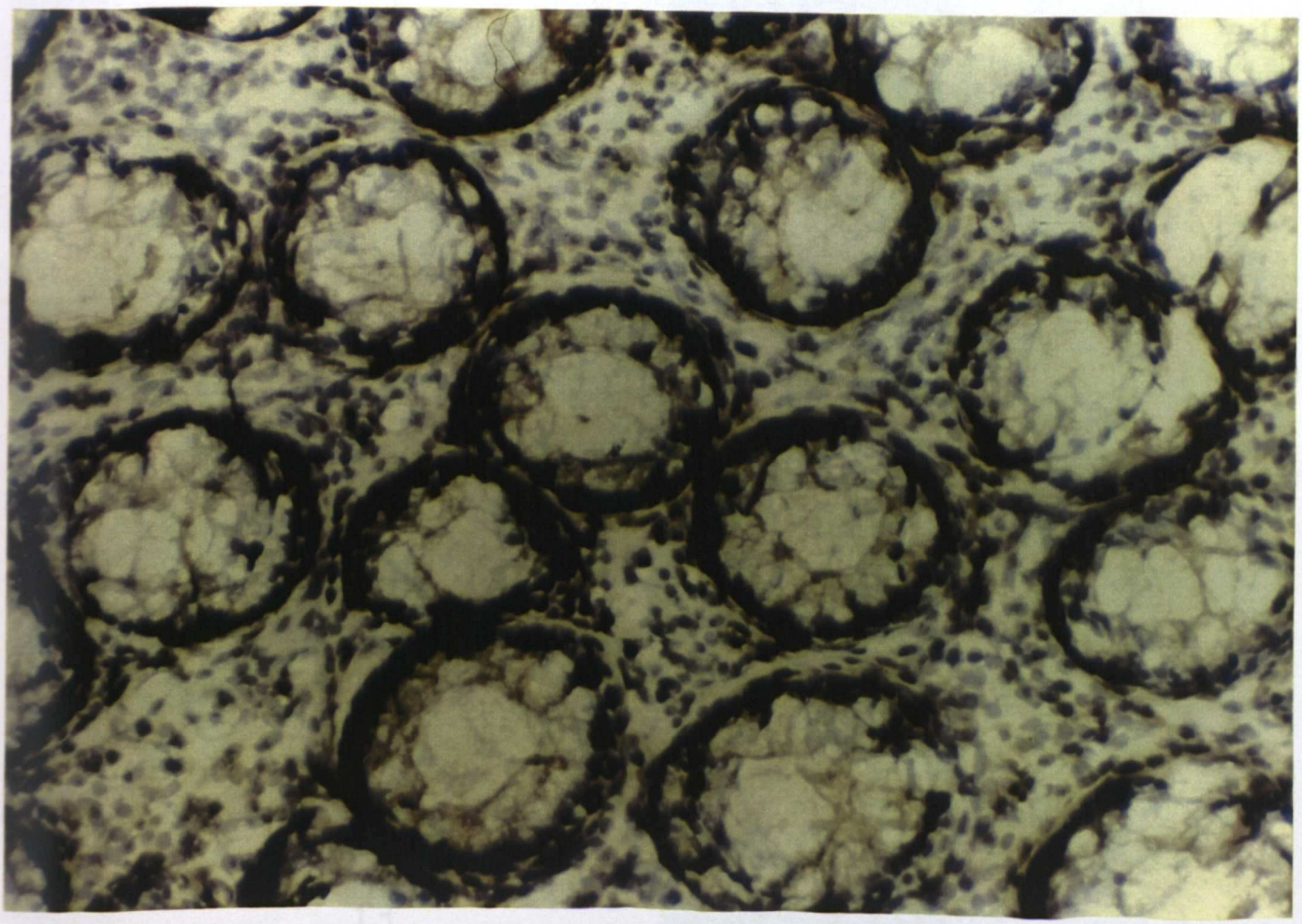


Figure 16. Infiltration of centre and edge of tumour from trial patients and their matched controls, by CD4+ lymphocytes.

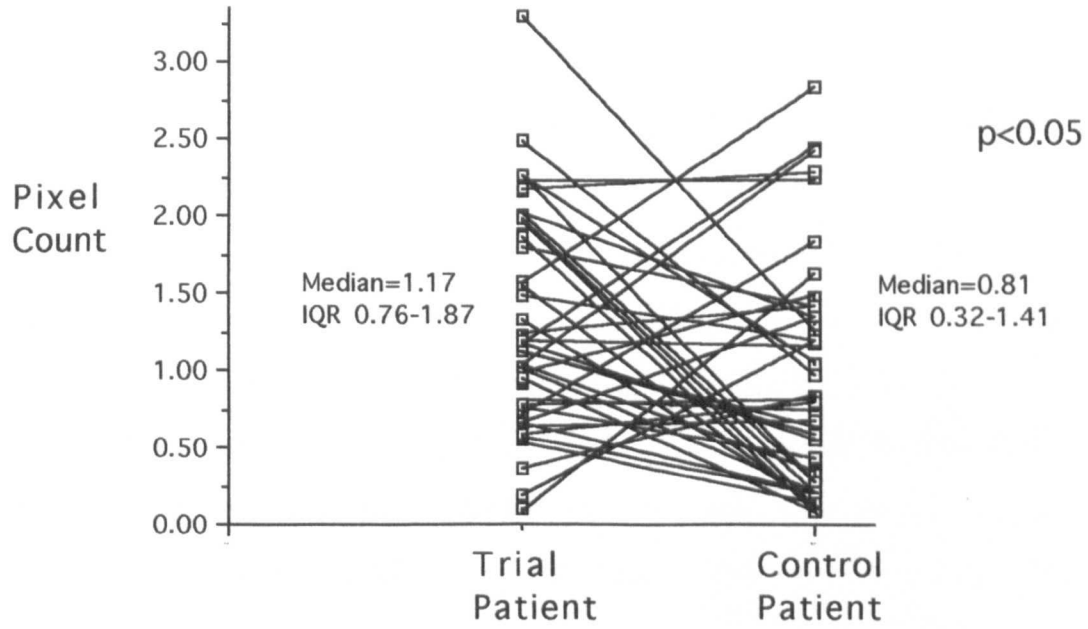
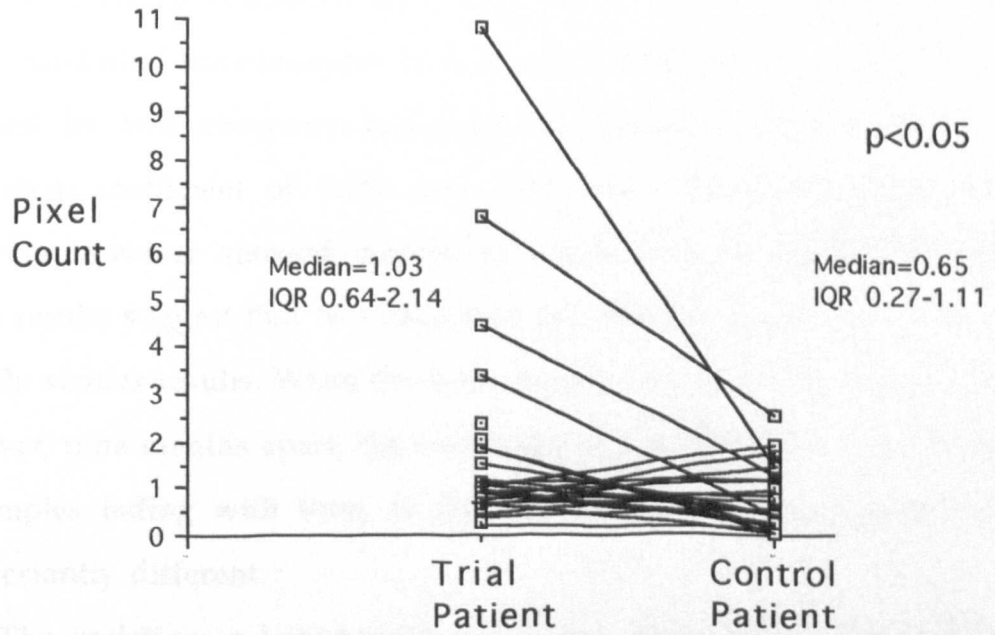


Figure 17. Ratio of CD8 expression between tumour edge and normal bowel, in trial patients and their matched controls.



Immunohistochemical analysis of tumour sections from immunised and unimmunised patients showed a significant infiltration of CD4+ and CD8+ lymphocytes in the former group. CD4, CD8 and CD56 subsets were combined to reflect overall lymphocytic infiltration. A statistically significant difference in favour of patients who received 105AD7 prior to surgery was seen. The benefit of lymphocytic infiltration of colorectal cancer was first noted in 1931 by WC MacCarthy, and has subsequently been shown in a number of other papers [Murray D 1975][Zhou X-G 1983][Carlon CA 1985][Svennevig JL 1984][Jass JR 1986 a][Jass JR 1986 b[Jass JR 1987][Jass JR 1996][Kubota Y 1992][Secco GB 1990]. This work suggests that vaccination with 105AD7 is capable of inducing changes at the tumour site.

A computer-assisted method was used to analyse individual sections. The results are semi-quantitative, as the computer in effect 'counts' areas of brown staining consistent with lymphocytic labelling, relative to the background blue haematoxylin counter stain. Interobserver variation was assessed by two observers analysing one patient's sections twice. A correlation coefficient of +0.65 and +0.69 was obtained. Intraobserver variation however showed correlation coefficients of +0.36 and +0.27. These results suggest that two observers can analyse the sections, and get broadly similar results. When the same samples are analysed twice by one observer, nine months apart, the correlation is not high. This may be due to samples fading with time, or the settings on the microscope being inadvertantly different.

The variation in lymphocytic infiltration with Dukes stage has also been assessed (Table 23). These results should be interpreted with caution in view of the small numbers. Comparable infiltration was seen between

trial and control for Dukes stage A. Median scores obtained for Dukes B and C were however higher in trial patients, significantly so in the former. With only 1 stage D tumour, it is dificult to draw firm conclusions. Ratios of tumour to normal bowel were higher in trial patients for all four stages. In addition infiltration was clearly higher in early stage disease, an observation that has been borne out by other authors [Watt AG 1978][Jackson PA 1996][Hakansson L 1997].

Infiltration of CD4 and CD8 lymphocytes.

A number of lymphocyte subsets were independently raised in trial patients as compared with controls. Infiltration of the tumour centre and edge by CD4+ lymphocytes was significantly higher, as was the ratio of tumour edge to normal bowel, for CD8+ cells. This is consistent with other studies, which have shown that tumours are infiltrated predominantly by T-cells [Werkmeister J 1979][Hutchinson GH 1981]. The results also suggest that 105AD7 is inducing a cellular response, and that stimulated lymphocytes may be targetting gp72 expressing cells at the tumour site.

Previous work has shown that 105AD7 can stimulate both helper and cytotoxic T-cell responses, leading to autologous tumour cell killing [Durrant LG 1994]. These results add further support to these findings. Pre-clinical research has shown induction of delayed-type hypersensitivity responses in mice, rats and rabbits immunised with anti-idiotypic antibodies against gp72 and CEA [Irvine K 1993], and evidence of T cell responses have also been seen in patients with advanced colorectal cancer receiving anti-idiotypic monoclonal antibodies, as described in Chapter 1 [Herlyn D 1991][Samonnigg H 1992]. Anti-idiotypic antibodies mimicking CEA have been shown to stimulate T cell responses [Foon KA 1995][Pervin S 1997]. In the former study, twelve patients with advanced colorectal

cancer were immunised with the Ab2, 3H1. Seven showed idiotype specific proliferative T cell responses, and 4 patients had evidence of T cell proliferation to CEA. The same group in the latter study showed that cellular anti-CEA immunity could be induced by 3H1, in mice previously injected with the colorectal cancer cell line MC-38. Importantly, immunised mice were protected against a challenge with lethal doses of MC-38. A goat polyclonal anti-idiotypic antibody mimicking the TAA GA733 has been administered as adjuvant therapy to 13 patients with colon cancer. Two patients developed CD4+ MHC class II dependent T cells, that specifically proliferated in culture in response to stimulation with either anti-Id, or GA733 antigen. A further two patients had evidence of lymphocytes that suppressed the proliferative responses of cultured pre-therapy lymphocytes to stimulation with anti-Id or GA-733 antigen [Somasundaram R 1995]. There is evidence suggesting that anti-idiotypic antibodies are capable of eliciting T cell responses. The immunohistochemistry results shown suggest that T cells stimulated in the peripheral blood are capable of targetting tumour cells expressing gp72.

A number of mechanisms have been proposed for the ways in which TIL might control tumour growth. These have included cytokine release, cytotoxicity, and helper/suppressor activity [Itoh K 1986][Topalian SL 1989]. Conflicting reports exist regarding the ability of freshly isolated TIL to exhibit cytotoxicity, however. Some workers have, for example, barely detected CTL using conventional assays [Rabinowich H 1987][Yoo Young-Kul 1990]. Werkmeister et al however showed evidence of *in vitro* CTL responses against autologous tumour cells in 18 of the 60 cases of colorectal cancer studied [Werkmeister J 1979]. Ostenstad et al develped a system for testing T cell capacity to induce DNA fragmentation, one of the hallmarks of apoptosis. They found that TIL were highly active effectors, killing the target cells at least as efficiently as control lymphocytes derived

from the peripheral blood of the same patients [Ostenstad B 1994]. Work from the same group also demonstrated a limited heterogeneity in the V-gene usage of TIL from seven patients with colorectal cancer, suggesting a local antigen-driven immune response at the tumour site. There is therfore evidence suggesting that TIL may be capable of destroying tumour cells.

Infiltration of Natural Killer Cells.

Natural Killer cells have been shown to infiltrate tumours such as malignant melanomas, squamous cell carcinomas, keratocarcinomas, and malignant breast lesions. The proportion of invading cells may however be as low as 2% [Kernohan NM 1990][Markey AC 1989][Teisa A 1987][Itoh K 1986]. Immunohistochemistry results confirm labelling of lymphocytes bearing CD56, in both trial and control patients, as pixel counts obtained in both are higher than sections stained with the negative control IgG_1 MAb. Tumour from patients who had received 105AD7 had consistently higher scores than their matched unimmunised controls, though the differences do not reach statistical significance.

Killer Inhibitory Receptors (KIR), such as p58, p70/NKB1, and CD94, interact with HLA molecules, sending inhibitory signals to the NK cell [Brooks AG 1997]. Unlike CTL, NK cells are capable therefore of lysing virally-infected, and malignant cells that down-regulate expression of Class I and II MHC molecules. Such killing may occur by a direct cytolytic effect, be cytokine or antibody mediated, or by interaction between the cells and macrophages/T-cells/B-cells.

Murine and rat studies have shown that NK activity may be augmented by innoculation with certain viruses, or immune adjuvants such as BCG or *C parvum*. Increased infiltration of primary colorectal

tumours has also been seen in humans receiving pharmacological doses of L-Arginine [Heys SD 1997]. These results show increased levels of CD56 on TIL in patients immunised with 105AD7 prior to surgery. The implications of this will be discussed in a further section.

Expression of the activation markers CD25 and CD69.

This work demonstrates that the α subunit of the IL-2 receptor is increased in patients who receive 105AD7, suggesting that the enhanced numbers of TIL seen, are activated, and potentially capable of tumour cell killing. Expression of the early activation marker CD69 was not however significantly increased in trial patients.

Interleukin-2 is a cytokine that plays a key role in T-cell proliferation and differentiation, mediating its various effects by binding to specific receptors [Robb 1984][Waldmann TA 1991]. CD25 is the alpha chain, present in both high and low affinity receptors on CD4 and CD8 cells [Wang HM 1987]. Contact of naive T lymphocytes with epitope induces synthesis of IL-2, and its receptor. Released IL-2 has been shown to promote activation, growth and differentiation of T lymphocytes, B cells, NK cells and monocytes, and in addition further promote receptor formation [Henney CS 1981][Trinchieri G 1984] [Waldmann TA 1984]. The autocrine/paracrine expression of this receptor therefore suggests lymphocyte activation [Harel-Bellan A 1986][Smith KA 1989]. This work shows that the lymphocytes previously seen at the tumour site of patients who received 105AD7 pre-operatively are expressing CD25, and are thus activated. Immunohistochemical labelling of tumour sections for the α subunit of the IL-2 receptor is consistently higher in trial patients, relative to controls, when tumour edge and centre were considered together, and when pixel scores for edge were analysed independently. Ratios of tumour

expression to that of normal bowel are similarly higher in patients who receive 105AD7 prior to surgery.

Interleukin-2 receptor expression has however been demonstrated on human solid tumour cells *in situ*. Weidmann et al (1992) showed that human squamous cell carcinomas of the head and neck expressed the β subunit, as did gastric and renal carcinomas [Weidmann E 1992][Yasumura S 1994]. Presence of the α subunit (CD25) was not however seen. A recent immunohistochemical analysis of 52 cryopreserved solid tumour specimens has confirmed that while the β subunit is seen on tumour cells, the α subunit is not [McMillan DN 1995]. These results suggest therefore that CD25 MAb is labelling IL-2 receptor present only on infiltrating lymphocytes. This is consistent with other authors who have confirmed the presence of CD25 immunohistochemically on lymphocytes in cryopreserved tumour sections [Hakansson L 1997][Allen C 1987].

The advantage of immunohistochemistry is that it enables a topographical assessment of the tumour, allowing a comparison between edge and centre. In our department we have cryopreserved a large number of tumour samples from unimmunised patients, enabling us to select a well matched control group for patients who have received 105AD7. A trend towards increased infiltration of CD25 expressing lymphocytes with earlier stage disease was seen. The numbers are however small, and the differences not statistically significant. It is also possible that the increased CD25 expression with early stage disease may be due to increased activation, associated with less immunosuppression. Trial patients showed increased infiltration of the tumour edge relative to the avascular centre, though numbers were once again too small to allow statistical analysis. The converse was true for control patients, suggesting that 105AD7 may be enhancing infiltration of activated lymphocytes at the leading edge of the tumour.

Thirteen patients with advanced colorectal cancer have been immunised with 105AD7, as part of a Phase I study. Of these, 6 had evidence of IL-2 production in their peripheral blood [Denton GWL 1994]. Immunohistochemical analysis of tumour sections from 6 patients immunised with 105AD7 pre-operatively has also shown increased expression of CD25 in all samples relative to stage-matched controls [Buckley TJD 1995]. Results outlined here in a different cohort of patients, confirm our original results, and show that patients immunised with 105AD7 prior to resection of their primary tumours have a higher tumour : normal ratio, and an increased number of CD25 expressing lymphocytes relative to controls. This suggests a higher proportion of activated effector T-cells at the tumour site in patients who have received the vaccine.

In addition to CD25, tumour sections were labelled with an MAb against CD69 (Activation Inducer Molecule), another activation marker. This is a 28/34 kDa type II transmembrane glycoprotein expressed on Natural Killer cells and other lymphocytes [Ullman KS 1990]. It is one of the earliest cell surface surface antigens to be induced on T cells by cross-linking of the TCR/ CD3 complex. Levels peak well before CD25, at between 30 and 60 mins post-stimulation. They return to normal, as soon as the stimulus is withdrawn. The exact function of CD69 is not known, though it has been suggested it serves as a signalling receptor for T-cell activation. It is absent from resting lymphocytes, but has been shown on *in vivo* activated cells infiltrating sites of chronic inflammation, and recent evidence suggests that ligation of CD69 by MAb may be sufficient to induce apotosis in GM-CSF cultured eosinophils [Walsh GM 1996].

Attempts have been made to measure the presence of CD69 on lymphocytes following administration of various forms of immunotherapy. Peripheral blood lymphocyte expression of CD69 in cancer patients was increased following administration of anti-CD3

monoclonal antibodies, without changes in CD25 levels [Urba WJ 1992]. Further work has confirmed this in patients with metastatic ovarian and breast cancer receiving TF-KLH and STn-KLH respectively, and shown that >50% increased expression on PBL correlates with prolonged survival [Bowen Yacyshyn MB 1995]. Activation markers on TIL have similarly been measured using immunohistochemistry. Expression of CD69 and HLA-Dr is increased on lymphocytes infiltrating melanomas, following immunisation with autologous vaccine modified by the hapten DNP (Dinitrophenyl) [Berd D 1994]. There was however no increase in CD25 expression, which, the authors suggest, was a consequence of recruiting patients with advanced disease, who have been shown to have defective lymphokine production, reduced ζ chain and MHC molecule expression, as well as decreased CTL activity [Jacob L 1997]. They are not therefore an ideal group for immunotherapy, as highlighted in the discussion of the Phase II study. Of the 16 patients receiving 105AD7 prior to surgery, all but 1 underwent a curative resection. These patients were potentially less immunsuppressed than the group recruited by Bird. This may explain why our results show an increase in CD25 expression, whereas his do not.

Immunohistochemistry results suggest that there is no significant difference in expression of CD69 betweeen patients receiving 105AD7, and their controls. It should be noted however that pixel scores are consistently higher in the immunised group. The fact that CD25 is significantly higher in trial patients, and that CD69 is not, may be due to levels of the latter returning to normal at the tumour site, as described. The autocrine nature of CD25 expression may act in a positive feedback loop promoting further expression, as discussed. This phenomenon has not been described for CD69.

The aim of this work was to assess infiltration of macrophages at the tumour site of trial and control patients, using an MAb against CD68. Macrophages are monocyte-derived cells, with stimulatory, suppressive and phagocytic potential [Tormey VJ 1997]. They are strongly positive for CD68, as well as CD14, acid phosphatase, and occasionally MHC class II markers. They are morphologically large, rounded cells lacking cytoplasmic protrusions [Kerrebijn JD 1994].

The role of macrophages at the tumour site is contentious. Despite their cytotoxic effect when activated by lymphokines, or bacterial products, work in head and neck malignancy has shown that 50% of the patients studied had macrophages that were not cytotoxic towards tumour cells *in vitro* [Kerrebijn JD 1994][Cameron DJ 1984].This has been confirmed in colorectal cancers, and it has been hypothesised that tumour cells become insensitive to cytolysis *in vivo* by macrophages, by building up resistance to tumour necrosis factors [Allen C 1987]. Other authors have suggested in a rat model that macrophages may be assisting, rather than preventing tumour growth [Evans 1979]. In addition, lysosomal enzymes of stimulated macrophages might promote metastasis by causing detachment of cells from the tumour mass [Fulton AM 1984].

Immunohistochemical analysis of tumour sections for macrophages has produced conflicting results. Some authors report very little infiltration, while others suggest it is widespread, increasing from normal bowel to tumour, and further as disease advances [Allen C 1987][Ebert EC 1989]. Recent work has confirmed the presence of CD11c+ macrophages in large numbers at the tumour site [Hakansson L 1997]. Conversely, there were more present with earlier stage disease, and they tended to be present round areas of cytodestruction, suggesting a cytotoxic role.

Analysis of sections showed higher pixel counts for tumour samples stained for CD68, than for the negative control. This suggests the presence of macrophages at the tumour site, in part confirming some of the results above. There was however no difference between trial and control patients, and the ratios of tumour: normal bowel infiltration were less than 1 for all the analyses performed, refuting the work of Allen and Hogg. CD68 is highly expressed on macrophages. It is likely that these results are more reflective of the true picture at the tumour site, as other authors use MAb against antigens such as CD11c, which is also present on monocytes and granulocytes [Hakansson L 1997]. It has further been suggested that the reason early work failed to show infiltration of tumour by macrophages was that the surface antigens being assessed were lost during the preparation process [Hakansson L 1997].

A balance is thought to exist between three macrophage subsets (stimulatory, suppressive and phagocytic), in normal tissues, and certain pathological conditions [Hutter 1992][Poulter LW 1994]. Recent work has shown that cytokines released by T cells may influence which macrophage phenotype predominates. IL-4 and IFN-γ has been shown to induce stimulatory macrophages, while IL-10 promotes suppressor cells [Tormey VJ 1997]. Immunohistochemical analysis clearly shows there is no difference in terms of absolute infiltration of macrophages between immunised and unimmunised patients, using an MAb against CD68. It would have been interesting to use this MAb in conjunction with RFD1 and RFD7 MAb, which may be used to distinguish the three macrophage subsets. In this way it might have been possible to assess if stimulatory macrophages, for example, predominated at the tumour site of patients who had received 105AD7. Having shown enhanced infiltration of CD4 expressing lymphocytes we might have expected to have seen increased

infiltration of macrophages as a result of cytokine release by activated Helper T cells. This was clearly not seen.

CD3 ζ chain expression

Signal-transducing molecules associated with the CD3/TCR complex include the ζ chain- a 16KDa disulphide linked homodimer [Weissman AM 1988]. The cytoplasmic domain of the CD3 ζ subunit is involved in signal transduction, and the subsequent activation of T-cells [Irving 1991]. Work has shown a decrease in CD3 ζ chain levels on T cells from mice bearing an experimental colon carcinoma [Mizoguchi H 1992]. NK cells and TIL from patients with colorectal carcinomas have similarly been found to have significantly fewer ζ chains than peripheral blood lymphocytes (PBL), which themselves had less than normal [Nakagomi H 1993]. Expression on PBL has also been found to decrease as disease becomes more advanced, though our immunohistochemisty results do not suggest any decrease in ζ chain with increasing stage, and if anything show the opposite [Matsuda M 1995]. It is likely that this reflects the small numbers involved. Matsuda also showed that levels of ζ chain were higher in normal bowel, with a direct relationship between amount of signal-transducing molecule, and distance from the tumour. This observation is consistent with this work in that tumour : normal bowel ratios of ζ chain were less than 1 in both trial and control patients.

Immunohistochemical analysis of tumour sections failed to show any difference between trial patients who had received 105AD7, and their matched controls, suggesting that the vaccine is having no effect on ζ chain expression. This is at variance with work by Finn's group, who have shown that ζ chain levels are increased following immunisation with a

synthetic peptide containing five mucin antigenic epitopes [Finn OJ 1996]. Patients received 3 doses of vaccine, 3 weeks apart - markedly more vaccine, over a longer time course than in this study. It is possible that increased doses of 105AD7 prior to surgery might have an effect on ζ chain expression, thus reducing the inhibitory effect of the tumour on signal transduction.

APO2.7 MAb labelling of tumour sections.

Programmed cell death, or apoptosis, is a selective process of physiological cell deletion. It has a number of roles including destruction of tumour cells by CTL and NK cells [Apasov S 1993]. It has been shown to be accompanied by certain characteristic morphological changes, and the degradation of internucleosomal DNA [Zhang C 1996]. Before death occurs apoptotic cells undergo alterations in phenotype, and various functional properties. These include activation of endogenous endonucleases, molecular marker expression, and a loss of protein expression. Apoptotic cells also have abnormal mitochondria, and reductions in their membrane potentials [Castedo M 1995]. Recently workers have observed that a 38kDa mitochondrial antigen is expressed on cells undergoing apoptosis, against which an MAb (APO2.7) has been raised [Zhang C 1996]. It has been shown to be expressed early in apoptosis, rather than as a final product of dead cells, and it has been suggested that it may be present on the cellular membrane in addition to that of the mitochondria.

This work uses the MAb APO2.7 to assess degree of apoptosis immunohistochemically, in patients receiving 105AD7, and their controls. Previous work has shown increased infiltration of CD4, CD8, and CD56 expressing lymphocytes at the tumour site of trial patients, and up-regulation of CD25. These results suggest that apoptosis is increased

significantly at the tumour site of patients who receive 105AD7 prior to surgery, possibly reflecting tumour cell killing by infiltrating lymphocytes.

A comparison was made between trial and control patients, in terms of APO2.7 expression at the edge and centre of the tumour. No significant difference exists between the two areas. The results need interpreting with caution in view of the small numbers involved, and the fact that statistical analysis is based on relatively few pairs.

There is evidence that colorectal cancer cells express FasL, and may be capable of killing TIL by apoptosis, thus evading the patients immune system [Hahne M 1996]. These results should be interpreted with caution, as it is not as yet clear whether the increased labelling with APO2.7 is a reflection of an increased numbers of lymphocytes undergoing apoptosis as a result of FasL expression on the tumour cells, or whether invading TIL are causing death of tumour cells by apoptosis.

Work is currently ongoing aiming to address this issue. Using 2-colour flow cytometric analysis of disaggregated tumour specimens labelled with BerEP4 and CD3, in conjunction with APO2.7 MAb, we hope to assess tumour cell and lymphocyte apoptosis, separately. In addition we are using Annexin V, a calcium and phospholipid binding protein, to bind phosphatidylserine, a negatively charged phopholipid expressed on the surface of apoptotic cells. This change precedes DNA fragmentation [Martin SJ 1995]. Using these techniques it will be possible to determine which cells are undergoing apoptosis, and hopefully show that the apoptosis seen at the tumour site immunohistochemically is due to increased cancer cell killing in patients who receive 105AD7. This work is currently being performed by an MSc student in the University

Conclusion.

Infiltration of CD4, CD8 and CD56 expressing lymphocytes is significantly increased in patients receiving 105AD7 prior to surgery, when results of the three subsets were combined. Retrospective pathological assessments of tumour sections would suggest that this might confer a survival advantage on immunised patients. Infiltration of CD4+ and CD8+ lymphocytes are independently higher in trial patients relative to well matched controls, supporting the premise that 105AD7 is stimulating cytotoxic and helper T cell responses. Increased expression of the α subunit of the Interleukin-2 receptor (CD25) implies that these TIL are activated, and potentially capable of releasing cytokines such as IFNγ, TNFβ, IL-4, IL-5, IL-6 and IL-10. These are potentially chemotactic and tumoricidal. In addition they could cause an alteration in expression of cell-surface adhesion molecules, such as L-selectin, ICAM-1, VCAM-1 and LFA-1, all of which might promote NK cell trafficking through the vascular endothelium [Rothlein R 1988]. This might account for the observation that CD56 is increased in trial patients.

If the hypothesis outlined above were true, then apoptosis at the tumour site would be higher in immunised patients, as tumour cells were lysed by the increased, infiltrating, activated lymphocytes. A significant increase in apoptosis at the tumour site in patients who received 105AD7, as denoted by APO2.7 labelling might suggest that this were true. However it is not as yet clear whether activated infiltrating lymphocytes are causing tumour cell apoptosis, or whether the TIL themselves are being apoptosed by FasL bearing malignant cells. Work is currently ongoing using flow cytometry to address this issue.

Part 2.

Assessment of peripheral blood lymphocyte subset changes following immunization with 105AD7

Contents.

Aim.

The aim of this chapter is to assess whether immunological changes seen at the tumour site of patients receiving 105AD7, can be seen in the peripheral blood.

Materials and Methods.

Patients.

A total of 17 patients with primary colorectal cancer were recruited prospectively from surgical out-patients. All had had their diagnosis made either at endoscopy and biopsy, or on double-contrast barium enema. Patient demographics are shown at Table 25.

Clinical Protocol.

Patients attended the Department of Surgery, where consent for enrollment in the study was obtained, as described. Thirty millilitres of venous blood was then taken prior to immunization with the vaccine. Patients received varying doses of 105AD7, as outlined in Table 25. Further blood samples were taken following immunisation, and prior to resection of primary tumour.

Phenotyping.

Thirty microlitres of blood were added to labelled Falcon (FACS) tubes containing 5μl of FITC, PE or PE-Cy5 directly labelled monoclonal antibody (MAb). The MAb's used are detailed in Table 24. Samples were then incubated at room temperature, in the dark, for 15 minutes. One millilitre of FACS lysing solution ® (Becton Dickinson) was added to each tube, and left for 10 minutes. Vials were then centrifuged for 5 minutes at 200g (1200rpm), and the ensuing supernatant removed. Cells were resuspended by vortexing the tubes. One millilitre of PBS/Azide was added and the sample centrifuged for a further 5 minutes at the aforementioned speed. A further 1 ml of PBS/Azide was added, and the

centrifugation repeated. Three hundred microlitres of FACS Fix® (Sysmex, Tarpen 15A, Hamburg, Germany) were added, and the sample introduced into the FACScan (Becton Dickinson). Lymphocytes were gated, and checked for non-lymphocyte contamination. The absolute counts were therefore obtainable from the haematological white cell count differential.

Table 24. Monoclonal antibodies used to phenotype whole blood lymphocyte subsets, by flow cytometry.

Cluster Designation	Clone.	Manufacturer
CD45/CD14 [1]	2DI / MθP9	Becton Dickinson [2]
Ig G_1 / Ig G_{2a}	-	Becton Dickinson [2]
CD3/CD4	SK7 / SK3	Becton Dickinson [2]
CD3/CD8	SK7 / SK1	Becton Dickinson [2]
CD3/HLA DR	SK7 / L243	Becton Dickinson [2]
CD3/CD25	SK7 / ACT/1	Becton Dickinson [2] and Dako [3]
CD4/CD25	MT310 / ACT/1	Dako [3]
CD8/CD25	DK 25 / ACT/1	Dako [3]
CD8/CD56	DK 25 / MOC-1	Dako [3]
CD3/CD16+56	SK7 / B73.1+MY31	Becton Dickinson [2]
CD3/CD19	SK7 / 4G7	Becton Dickinson [2]
CD3/CD69	UCH 31 / T1L78	Dako [3]

[1] All MAbs were used undiluted. The first Cluster Designation is directly conjugated with fluoroscein isothiocyanate, and the second with phycoerythrin.

[2] Becton Dickinson, Between Towns Road, Cowley, Oxford. UK

[3] Dako, 16 Manor Courtyard, Hughendon Avenue, High Wycombe, UK.

Table 25. Demographics of patients whose venous blood was phenotyped following immunisation with 105AD7.

Pt no.	Sex	Age	Site [1]	Stage [2]	Diff [3]	Samples [4.]	Time [5.]
1. DT	F	75	Rectum	C	Mod	1,6,13,18	18
2. EN	F	79	Sigmoid	B	Mod	1,4,14	14
3. ER	M	82	Caecal	C	Mod	1,4,6,11	11
4. HT	F	86	Sigmoid	-	Mod	1,10,14	14
6. ET	M	87	Caecum	B	Mod	1,8,13,20	20
7. MF	F	63	Rectum	B	Mod	1,9,14,19	19
8. MB	M	75	Rectum	A	Mod	1,9,14,20,26	26
9. DH	M	56	Rectum	B	Mod	1,5,9	9
10. VMcC	M	63	Rectum	C	Mod	1,8,11,15,20	20
11. JK	M	76	Sigmoid	A	Mod	1,9,14	14
12. JGa	M	58	Rectum	A	Mod	1,8,12	12
13. TB	F	62	Rectum	C	Poor	1,10,20	20
14. CB	M	75	Caecum	-	Mod	1,8,12 *	-
15. FCl	F	67	Rectum	A	Mod	1,7,13	13
16. JH	M	69	Rectum	A	Mod	1,8,12,15.	15
17. FH	M	73	Rectum	C	Mod	1,7,14,19,28	28
18. FCh	M	70	Rectum	B	Mod	1,6,13	13

[1] Site of primary tumour.

[2] Modified Dukes stage of tumour (Turnbull RB, 1967).

[3] Differentiation of primary tumour.

[4] Days on which venous blood samples were taken. Day 1 denotes the pre-immunisation sample.

[5] Number of days between first immunisation and surgery. Patient 14 refused further sampling, and ultimately did not have tumour resected.

Statistical analysis.

This data presents several analytical difficulties. The number of venous blood samples, and the duration of time over which they taken vary for individual patients, making comparison difficult. In the first instance, a Wilcoxon signed rank test was performed comparing values for each phenotype prior to immunisation with those at day 7, and immediately pre-operatively.

A new approach to this problem of serial data involved calculating the area under the curve per unit time [Matthews et al 1990]. This is achieved by adding the areas under the graph between each pair of consecutive observations. For measurements y_1 and y_2 at times t_1 and t_2, then the area under the curve between these two times is the product of the time difference and the average of the two measurements. Thus we get $(t_2-t_1)(y_1+y_2)/2$. This is known as the trapezium rule, because of the shape of each segment of the area under the curve. Therefore if we have n+1 measurements y_i at times t_i ($_i$ = 0,1,2...n), then the area under the curve (AUC) is calculated as:

$$AUC = 1/2 \sum_{i=0}^{n-1} (t_{i+1} - t_i)(y_i + y_{i+1}).$$

The AUC score obtained is then standardised by dividing the figure obtained by the number of days between first and last measurements. If there is no response to immunisation, then the overall AUC/day will be 0. Clearly a positive score indicates a response. The way in which this test was applied to the phenotyping data is shown in Figure 19. A paired t-test

was then performed comparing AUC/day for trial patients against scores of 0, the theroretical AUC that would be obtained from an unimmunised group, for each leucocyte phenotype.

Results.

A total of 17 patients were recruited to this arm of the adjuvant study. There were 6 women and 11 men, with a mean age of 71.5 years (range 56-87). Eleven had rectal tumours, 3 caecal, and 3 sigmoidal. All patients had pre-immunisation venous blood samples taken, and a mean of 3.65 samples (range 3-5) were taken overall prior to surgery. The mean time between first immunisation and pre-operative blood sample/surgery was 16.63 days (range 9-28). Of the 17 patients, 15 had resection of their primary tumours. In all but one case they were moderately differentiated. Five tumours were Dukes A, five Dukes B and 5 Dukes C. Patient numbers 4 and 14 did not have their tumours removed. In the first case the tumour was unresectable, while the second refused operation, when he was found to have lung metastases.

Table 26 shows mean and median values for all the lymphocyte subsets analysed. Statistical analysis was performed, comparing the pre-immunisation scores with those obtained at approximately 7 days, and samples taken immediately before surgery. Figure 18 shows graphically for patient number 1, how phenotypes varied with time between immunisation, and surgery.

Mean peripheral blood phenotypes higher at day 7, than pre-immunisation included CD3/4, CD3, B cells, CD3/HLA Dr, CD4/25 and CD3/69. Those lower included CD3/8, NK cells, CD3/25, CD56, CD8/56, and CD69 with lymphocytes, CD3/8, CD8/25, CD3/16+56 phenotypes being unaffected by immunisation. None of these differences reached statistical significance on a two-tailed Wilcoxon Signed rank test, assuming significance at the 5% level. Mean, median, SD, IQR, and range for all phenotypes are shown in Table 26.

A similar comparison was performed between phenotypes pre-immunisation and those taken immediately before surgery, approximately 17 days later. Those phenotypes showing an increase after receiving 105AD7 included lymphocytes, CD3, CD3/4, CD3/8, CD3/16+56, CD69 and CD3/69. No difference was observed between B cells and CD8/25. Those phenotypes showing a decrease included CD3/HLA Dr, CD3/25, CD4/25, CD56, and CD8/56. Once again there was no significant difference between the two groups.

Paired analysis is one way to answer the question of whether a particular lymphocyte subset is influenced by immunisation with 105AD7. Another statistical technique employed involved calculating the Area under the Curve (AUC) per unit time. This principle is shown graphically in Figure 19, and described in the preceeding section. Patients showed either positive or negative scores- the former denoting an overall response to immunisation, the latter no response. The number of patients showing positive scores for the various phenotypes is shown below, with figures for individual patients shown in Table 27.

Lymphocytes	11/17 (65%)	**B cells**	11/15 (73%)	**CD3**	9/15 (60%)
CD3/CD4	10/15 (67%)	**CD3/CD8**	8/15 (53%)	**NK cells**	9/15 (60%)
CD56	10/14 (71%)	**CD3/HLA Dr**	8/15 (53%)	**CD3/16+56**	7/15 (47%)
CD4/25	8/14 (57%)	**CD8/25**	10/14 (71%)	**CD3/25**	7/14 (50%)
CD8/56	7/14 (50%)	**CD3/69**	6/12 (50%)	**CD69**	7/12 (58%)

Table 27 shows the analysis of the area under the curve data for all patients, and for the two dosing schedules used. The only lymphocyte subsets when all patients were grouped together to show an overall negative response to immunisation were NK cells, CD3/25, CD56, and CD8/56. The others were all positive. If there was no overall response then

the mean score would be 0 ie no effect of immunisation. Paired analysis was therefore performed comparing scores obtained with 0. No significant difference was however seen using a Wilcoxon Signed Rank test.

Figure 18. Venous blood phenotypes measured pre-immunisation, and on three further occasions before surgery.

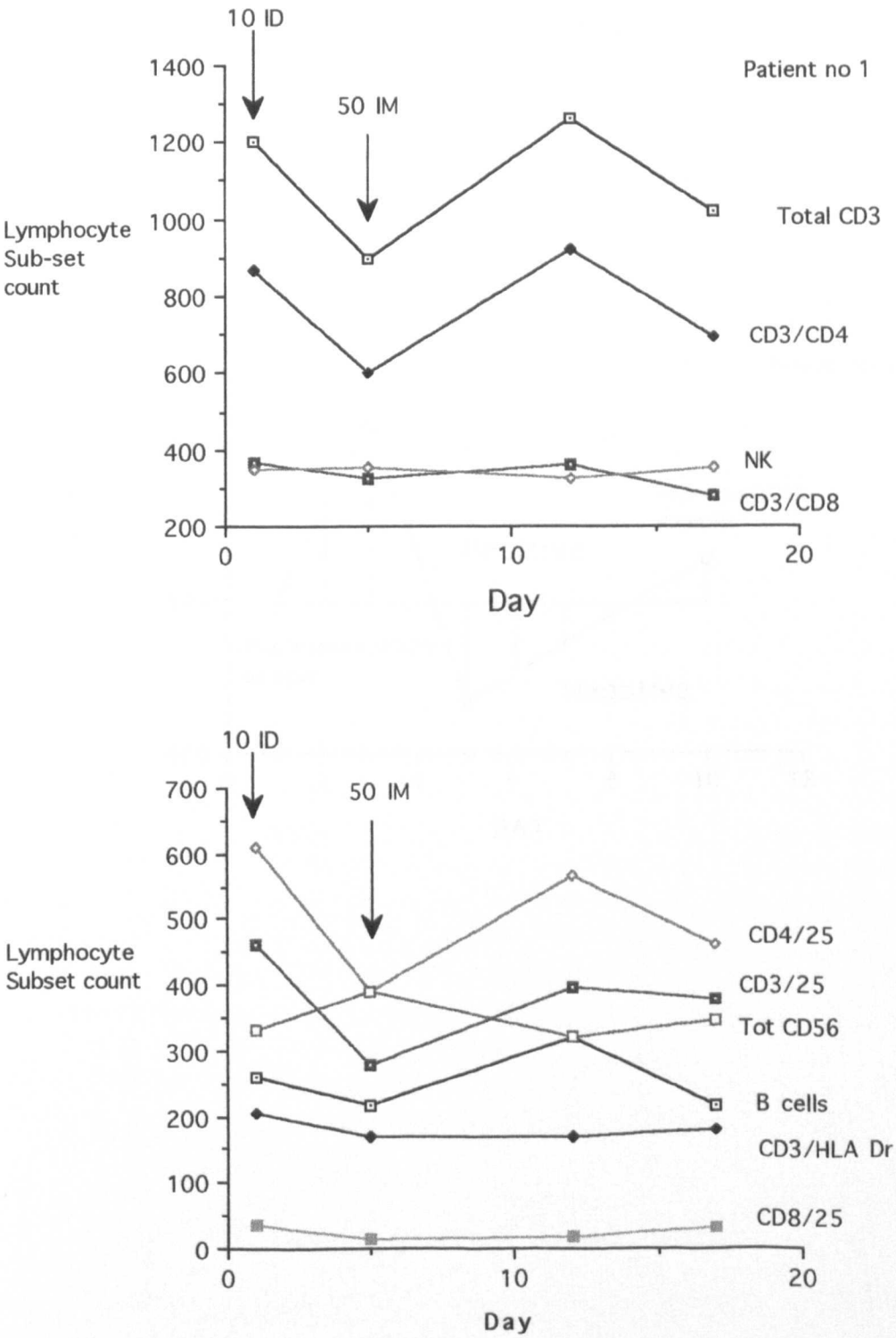


Figure 19. Venous blood phenotyped for CD3/CD8 lymphocytes, prior to immunisation, and on three further occasions before surgery. Graph indicates how the area above and below a line drawn across from the pre-immunisation value can be calculated. An average value per day can then be calculated.

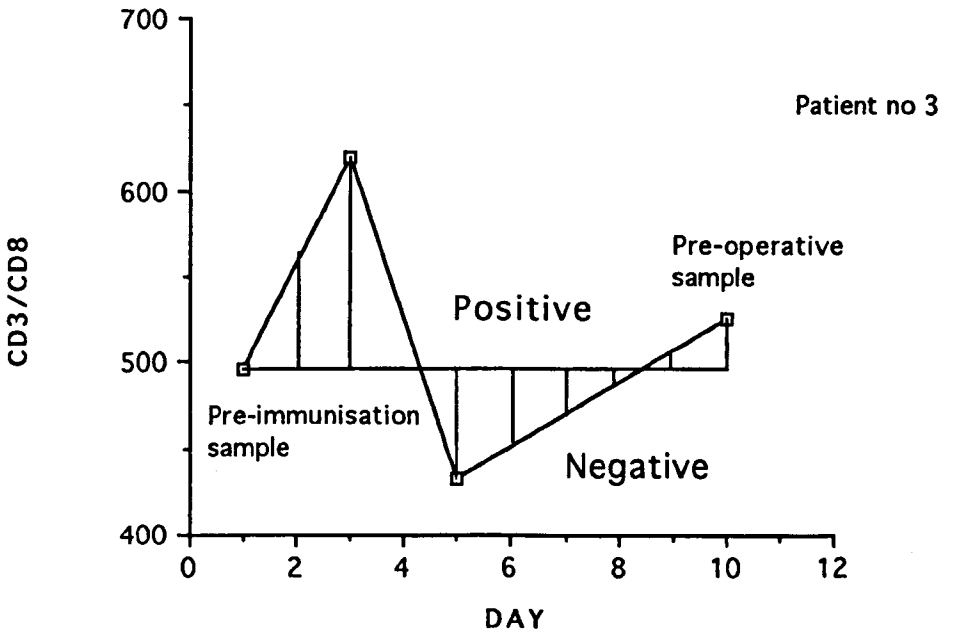


Table 26. Pre and post immunisation peripheral blood phenotypes.

Lymphocytes (1500-4500 x 10^6/l)		Pre-im[2]	Post-im[3] T=7.41		Pre-im[2]	Pre-op[4] T=16.35
n=17	mean	1510	1520	n=17	1510	1530
	median	1460	1500		1460	1600
	SD	360	340		360	320
	IQR	1250-1740	1300-1680		1250-1740	1300-1810
	Range	940-2210	1060-2350		940-2210	860-1970

CD3 (1000-2100 x 10^6/l)		Pre-im[2]	Post-im[3] T=7.53		Pre-im	Pre-op[4] T=16.13
n=15	mean	982	997	n=16	1002	1030
	median	967	972		1011	998
	SD	243	214		248	306
	IQR	835-1121	878-1090		847-1133	834-1191
	Range	573-1525	649-1485		573-1525	516-1756

CD3/CD4 (500-1700 x 10^6/l) [1]		Pre-im[2]	Post-im[3] T=7.53		Pre-im	Pre-op[4] T=16.13
n=15	mean	545	567	n=16	552	572
	median	544	515		550	544
	SD	156	129		154	150
	IQR	423-634	499-648		423-657	476-666
	Range	332-870	389-883		332-870	353-888

Table 26. Pre and post immunisation peripheral blood phenotypes.

CD3/CD8 (200-1000 x 10^6/l) [1]		Pre-im[2]	Post-im[3] T=7.53		Pre-im	Pre-op[4] T=16.13
n=15	mean	437	434	n=16	455	462
	median	470	420		473	371
	SD	189	193		196	248
	IQR	278-562	298-511		282-620	309-613
	Range	169-796	209-941		169-796	146-1119

CD16 + 56 (60-240 x 10^6/l) [1]		Pre-im[2]	Post-im[3] T=7.43		Pre-im	Pre-op[4] T=15.93
n=14	mean	265	240	n=13	222	213
	median	267	209		207	211
	SD	127	117		128	120
	IQR	168-370	161-357		116-335	126-288
	Range	69-465	74-442		12-420	39-471

CD19 (40-400 x 10^6/l) [1]		Pre-im[2]	Post-im[3] T=7.53		Pre-im	Pre-op[4] T=16.13
n=15	mean	160	171	n=16	159	161
	median	171	170		161	140
	SD	84	101		81	92
	IQR	85-237	95-231		96-237	97-213
	Range	25-262	23-389		25-262	36-389

Table 26. Pre and post immunisation peripheral blood phenotypes.

CD56 x 10^6/l		Pre-im[2]	Post-im[3] T=7.43		Pre-im	Pre-op[4] T=16.80
n=14	mean	346	317	n=15	364	349
	median	333	295		351	312
	SD	140	101		153	153
	IQR	261-420	247-405		241-442	264-482
	Range	122-622	154-461		122-622	77-698

CD8/ CD56 x 10^6/l		Pre-im[2]	Post-im[3] T=7.43		Pre-im	Pre-op[4] T=16.80
n=14	mean	182	165	n=15	195	189
	median	175	178		208	187
	SD	81.8	75.7		88.3	101
	IQR	122-247	101-220		123-265	110-264
	Range	47-319	32-279		47-332	32-388

CD3/16 + 56 x 10^6/l		Pre-im[2]	Post-im[3] T=7.50		Pre-im	Pre-op[4] T=16.50
n=14	mean	80.2	79.8	n=15	70.4	80.9
	median	73	57		70.0	58.5
	SD	59.6	75.6		47.6	86.4
	IQR	24-112	21-123		20-99	23-105
	Range	16-218	11-281		16-175	13-347

Table 26. Pre and post immunisation peripheral blood phenotypes.

CD3/CD25 x 10^6/l		Pre-im[2]	Post-im[3] T=7.43		Pre-im	Pre-op[4] T=17.14
n=14	mean	269	252	n=14	269	243
	median	241	248		241	281
	SD	162	140		162	105
	IQR	150-388	138-351		150-388	136-312
	Range	74-641	56-495		74-641	79-403

CD4/CD25 x 10^6/l		Pre-im[2]	Post-im[3] T=7.43		Pre-im	Pre-op[4] T=16.80
n=14	mean	306	315	n=15	301	295
	median	303	291		262	324
	SD	126	134		123	107
	IQR	220-354	223-340		220-354	205-360
	Range	123-611	127-614		123-611	98-462

CD8/CD25 x 10^6/l		Pre-im[2]	Post-im[3] T=7.43		Pre-im	Pre-op[4] T=16.8
n=14	mean	47.8	47.6	n=15	45.4	45.4
	median	33.5	46.5		33.5	32.5
	SD	41.7	34.0		41.1	32.1
	IQR	14-67	16-70		14-62	19-70
	Range	9-155	11-120		9-155	9-99

Table 26. Pre and post immunisation peripheral blood phenotypes.

CD3/HLA Dr x 10^6/l		Pre-im[2]	Post-im[3] T=7.53		Pre-im	Pre-op[4] T=16.60
n=15	mean	293	314	n=16	309	295
	median	234	255		285	238
	SD	171	208		177	204
	IQR	145-415	139-458		153-437	174-373
	Range	94-663	99-759		94-663	80-888

CD69 x 10^6/l		Pre-im[2]	Post-im[3] T=8.45		Pre-im	Pre-op[4] T=17.15
n=11	mean	160	154	n=13	159	205
	median	142	141		142	177
	SD	73.6	97.1		67.7	111
	IQR	104-237	81-196		108-206	139-253
	Range	66-264	74-396		66-264	92-502
	WSR	NS			p=0.0549	1 tail

CD69/CD3 x 10^6/l		Pre-im[2]	Post-im[3] T=7.75		Pre-im	Pre-op[4] T=17.15
n=12	mean	80.1	83.5	n=13	80.5	104
	median	69	58		77	80
	SD	62.2	95.3		56.8	113
	IQR	50.3-91.8	35-81		50.8-89.3	51-96
	Range	19-250	27-363		19-250	26-463

Key to Table 26.

1 Normal range for lymphocyte sub-set, on FACScan used for phenotypic analysis. In a number of cases this range has not as yet been established.

2 Sample taken prior to immunisation.

3. First sample taken after immunisation. T denotes mean number of days since immunisation.

4. Sample taken before surgery. T denotes time since immunisation.

5. Two-tailed WSR test performed at the 5% level. NS denotes Not Significant.

Table 27. Area under the curve data for phenotyped patients.

Patient number	Lym 10^6/l/day	CD3 10^6/l/day	CD3/4 10^6/l/day	CD3/8 10^6/l/day	CD16/56 10^6/l/day	CD56 10^6/l/day	CD8/56 10^6/l/day	CD19 10^6/l/day
1.DT	-110	-105	-94.3	-30.7	-7.3	10.3	15.9	0.56
2. EN	100	55.8	50.5	23	-4.6	-22.1	-19.3	40.7
3. ER	90	97.9	69.7	14.7	-30.8	20.8	13	8.5
4. HT	100	61.8	39.1	38	13.9	0.07	-9.25	41.1
6. ET	80	60	72.5	-0.70	6.6	-227.6	-26	14.4
7. MF	260	223.7	129	54.8	54.6	152.5	21.7	7.3
8. MB	-60	-8.10	99.1	-73.5	-106.7	-155.7	-82.8	2.0
9. DH	-130	-	-	-	-	-	-	-
10. VG	120	-	-	-	-	-	-	-
11. JK	260	288.2	133.7	194.4	14.6	-	-	7.2
12. JGa	-100	-92.7	-108.6	-8.6	11.5	8.6	1.0	-24.8
13. TB	70	32.6	39.1	8.7	20.1	12.4	-6.8	15.2
14. CB	20	-219	-8.3	-14	6.4	4.8	1.3	-2.7
15. FCl	-200	111.9	-45.2	-76.2	-77.1	-82.4	-20.5	-12
16. JH	140	65.8	15.9	31	27.4	42.8	27	28.4
17. FH	100	111.9	74.2	28.1	-31.2	88.1	10.5	9.0
18. FC	-260	-219	-125	-115	3.1	28.3	-2.04	-32.8

Table 27. Area under the curve data for phenotyped patients.

Patient number	CD3/25 10^6/l/day	CD4/25 10^6/l/day	CD8/25 10^6/l/day	CD3/16+56 10^6/l/day	CD69 10^6/l/day	CD3/69 10^6/l/day	CD3/HLADr 10^6/l/day
1.DT	-94.9	-96.7	-15.6	-1.2	-	-	-25.4
2. EN	-2.96	24	0.70	-14.3	-	-	-8.21
3. ER	50.8	93.2	21.6	6.9	-	-	11.4
4. HT	55.9	41.3	10.6	17.5	-15	-3.6	15.6
6. ET	36.0	71.7	6.6	-3.6	16.1	1.45	42.2
7. MF	38.3	103.2	19.2	3.9	2.5	-2.97	10.8
8. MB	-23	-10.4	-22.8	-47.6	-105.6	-36.2	-51.3
9. DH	-	-	-	-	-	-	-
10. VG	-	-	-	-	-	-	-
11. JK	-	-	-	80.6	104.4	90.5	280.1
12. JGa	-29.8	-56.2	4.3	-5.6	-10.8	-12.5	-7.7
13. TB	10.8	26.3	13.6	0.73	5.85	4.5	26.4
14. CB	-21.5	-35.9	-7.9	1.2	42.7	28.7	-4.96
15. FCl	-22.2	-71.8	1.8	-0.92	-8.77	-4.2	-99.7
16. JH	9.3	37	1.67	-1.5	33.7	10.5	10.6
17. FH	67.1	36.9	21.2	4.38	35.8	19.2	25.3
18. FC	-180.8	-23.7	-44.9	-7.4	-6.5	-15.7	-114.5

Table 28. Statistical analysis of Area under the Curve data.

	Mean	Median	IQR	SD	WSR [1]
Lymphocytes [2]	28.0	80	-100 to 110	149	NS
CD3	29.96	60	-71.6 to 108.4	130.6	NS
CD3/CD4	22.8	39.1	-36.0 to 73.8	83.0	NS
CD3/CD8	4.93	8.7	-26.5 to 30.3	70.8	NS
CD 16 + 56	-6.63	6.4	-24.9 to 14.4	40.9	NS
CD19	6.80	7.3	-1.9 to 15.0	20.7	NS
CD3/HLA Dr	7.38	10.6	-21.1 to 22.9	22.7	NS
CD3/CD25	-7.64	3.17	-23.0 to 38.3	65.9	NS
CD4/CD25	9.92	25.2	-35.9 to 41.3	61.0	NS
CD8/CD25	0.72	3.05	-7.9 to 13.6	18.6	NS
CD56	-8.1	9.5	-22.0 to 28.3	94.9	NS
CD8/56	-5.5	-0.52	-19.3 to 13	27.4	NS
CD3/16+56	2.21	-0.92	-5.1 to 4.26	25.9	NS
CD69	9.0	4.18	-9.8 to 34.8	48.5	NS
CD3/CD69	6.64	-0.76	-8.4 to 14.9	31.2	NS

1. Two-tailed Wilcoxon signed rank test comparing AUC/ unit time against values of 0, the theoretical mean AUC for a control group. Significance was taken at the 5% level. NS denotes Not Significant.

[2] All lymphocyte subsets in units x 10^6 l^{-1} day^{-1}.

Discussion

These results demonstrate that 105AD7 has no measurable effect on peripheral blood lymphocyte subset levels. There are several explanations for this observation. The easiest conclusion to draw is that the anti-idiotypic antibody does not elicit an immune response. This is clearly at variance with the results of the Phase I study, and work done using the vaccine as adjuvant therapy. It is more likely that other factors are contributory towards the lack of any change in lymphocyte subset levels, with immunisation.

Flow cytometry is a technique whereby a large number of cells can be analyzed, and divided into functional subsets based on expression of surface antigens. A number of lymphocyte subsets were increased following immunisation, though differences were not statistically significant. It seems likely that this technique is not sensitive enough to detect the small changes in lymphocyte subsets that would occur following immunisation.

Statistical analysis of the results used a two-tailed Wilcoxon signed rank test, thus not assuming any particular frequency distribution, or direction of presumed effect. While unimpeachable, this is not as powerful as a students t test, and is therefore less likely to show any significant difference should there be one.

Venous blood samples were taken at various time points following immunisation. It is possible that the paired analysis performed between pre-immunisation phenotypes, and those at days 7 and 16 failed to detect a rise occurring at day 3, or day 12 for example. Previous work has also shown blastogenesis responses, and increased plasma IL-2 levels not occurring until 2-3 weeks after immunisation [Robins RA 1991]. Clearly it would not be ethical to delay a patients operation in order to take more blood samples to confirm or refute these findings. In order to compensate

for this, the area under the curve per unit time was calculated. NK cells (CD16+56), CD3/25, CD56 and CD8/56 were the only lymphocyte subsets that showed overall negative mean AUC scores, with all others showing a positive response.

Parallels can be drawn between this work, and that done in the field of transplant immunology. Unsuccessful attempts have previously been made to measure a variety of peripheral blood immunological parameters, such as T cell subsets and activation markers, in order to predict organ rejection. If a blood test can show rejection is occurring, then the graft need not be biopsied [Coles M 1987]. Expansion of peripheral blood CTL with IL-2 proved similarly fruitless, even though CTL were found to be present at the site of the organ undergoing rejection [Vaessen LMB 1992]. Immunohistochemical analysis of tumour sections from patients who also had venous blood phenotyped, has shown increased infiltration of CD4,CD8 and CD56 positive lymphocytes, with enhanced expression of the Interleukin-2 receptor. Based on these observations in transplant patients, it is possible that lymphocytes primed by 105AD7 are localising to the tumour site, and therefore no changes are seen when peripheral blood is phenotyped.

Two colour MAb labelling has also been performed, assessing the number of cells expressing CD16 and CD56 receptors. There is evidence that peripheral blood levels of this phenotype are higher in patients with colorectal cancer, as compared with controls [Takii Y 1994]. Our results are consistent with this, in so much as peripheral blood NK cell levels are just above the normal range. There was however no significant increase in this phenotype with 105AD7 administration.

Expression of the activation markers CD69 and HLA DR has been assessed on PBL following administration of active specific immunotherapy, in patients with metastatic breast and ovarian cancer.

There was no increase in levels of the latter, though CD69 levels were found to be raised [Bowen Yacyshyn MB 1995]. A separate study has also shown that CD25 levels are not increased following administration of anti-CD3 MAb [Urba WJ 1992]. It seems therefore that flow cytometry is not sensitive enough a technique to measure subtle changes in activation markers on lymphocytes, and that our negative findings are consistent with those of other authors.

It is likely that 105AD7 is stimulating a very small population of the total number of T cells in the peripheral blood. As such it is unlikely that flow cytometry is sensitive enough to detect any slight increase. Peripheral blood phenotyping is therefore not a sensitive, or viable tool for assessing responses to the vaccine. In retrospect it might have been more appropriate to measure IL-2 or TNFα, for example. Work has shown a correlation between clinical response and levels of the latter in patients receiving active non-specific immunotherapy, and one could hypothesise that these might be expected to increase in a patients receiving an immunotherapy agent, rather than anticipating a significant increase in specific T cell subsets [Blay JY 1990].

Part 3.

Flow Cytometric analysis of Tumour Lymphocytic Infiltration in Patients receiving 105AD7 and their controls.

Contents.

Aim.

The aim of this work is to use flow cytometry to assess the phenotypes of lymphocytes at the tumour site of patients receiving 105AD7, and their controls. Using a panel of monoclonal antibodies it is possible to quantify the percentage of lymphocytes expressing different surface antigens, and thus determine if certain subsets are stimulated by the vaccine, in preference to others.

Materials and Methods.

Patients.

A total of 19 patients with primary colorectal cancer were recruited prospectively from surgical out-patients, as described. Patient demographics are shown in Table 29.

Clinical Protocol.

Patients attended the Department of Surgery, where consent for enrollment in the study was obtained. They received varying doses of 105AD7, as previously outlined, and underwent resection of their primary tumours, a mean of 16.9 days (range 9-31 days) following initial immunisation. Samples were taken from the specimen, immediately following removal, and stored in RPMI, at 4°C. Disaggregation was performed within 12 hours of removal, as outlined below.

Control Group.

Tumour disaggregation and flow cytometry were also performed on fresh samples from 35 unimmunised patients. Patient demographics for this control group are also shown in Table 29.

Table 29. Trial and control patients undergoing tumour disaggregation.

No	Initials	Hosp no	105AD7 [1]	Date [2]	Sex	Age	Site [4]	Stage [5]	Diff [6]
1.	RS	S825068	Control	Feb 95	M	64	Sigmoid	B	Mod
2.	KG	S263116	Control	Mar 95	F	58	Caecum	B	Mod
3.	GE	S270734	Control	Mar 95	F	80	Ascend	B	Mod
4.	JH	S999126	Control	Mar 95	M	75	Caecum	C	Mod
5.	IB	S362759	Yes [3]	Mar 95	F	71	Caecum	B	Mod
6.	RS	S56716A	Yes [3]	Apr 95	M	71	Rectum	C	Mod
7.	GB	S819486	Control	May 95	M	64	Sigmoid	B	Mod
8.	MS	S975702	Control	May 95	F	66	Caecum	B	Mod
9.	HS	S856235	Control	May 95	F	82	Caecum	B	Mod
10.	MR	S305334	Control	Jun 95	M	74	Rectum	C	Poor
11.	DT	707357A	Yes (1)	Sept 95	F	75	Rectum	C	Mod
12.	MH	306326	Control	Sept 95	F	48	Ascend	C	Poor
13.	EN	S98383	Yes (2)	Sept 95	F	79	Sigmoid	B	Mod
14.	ER	S289263	Yes (3)	Sept 95	M	82	Caecum	C	Mod
15.	VH	640270	Control	Nov 95	M	64	Rectum	C	Mod
16.	JG	S927425	Yes (5)	Nov 95	M	64	Rectum	D	Mod
17.	-	-	Control	Nov 95	-	-	-	-	-
18.	MF	675748	Yes (7)	Dec 95	F	63	Rectum	B	Mod
19.	ES	75923A	Control	Dec 95	F	74	Ascend	B	Mod
20.	DH	S541069	Yes (9)	Dec 95	M	56	Rectum	B	Mod
21.	MB	750439	Yes (8)	Dec 95	M	75	Rectum	A	Mod
22.	IS	752730	Control	Dec 95	F	68	Sigmoid	C	Mod
23.	JA	50887A	Control	Dec 95	F	55	Rectum	B	Mod

Table 29. Trial and control patients undergoing tumour disaggregation.

No	Init.	Hosp no	105AD7 [1]	Date [2]	Sex	Age	Site [4]	Stage [5]	Diff [6]
24.	WL	992911	Control	Dec 95	M	68	Caecum	D	Mod
25.	VMc	214994	Yes (10)	Dec 95	M	63	Rectum	C	Mod
26.	N W	294189	Control	Jan 96	F	-	-	-	-
27.	DY	65473	Control	Jan 96	F	-	-	-	-
28.	JGa	367716	Yes (12)	Feb 96	M	58	Rectum	A	Mod
29.	JKirk	327877	Yes (11)	Feb 96	M	76	Sigmoid	A	Mod
30.	TB	S990904	Yes (13)	Mar 96	F	62	Rectum	C	Poor
31.	RF	-	Control	Mar 96	M	73	Anus	-	-
32.	JW	97622A	Control	Apr 96	M	82	Rectum	A	Mod
33.	F Cl	S521022	Yes (15)	Apr 96	F	67	Rectum	A	Mod
34.	G Cl	920255	Control	Apr 96	M	82	Caecum	B	Mod
35.	JD	419952	Control	Apr 96	M	78	Caecum	B	Mod
36.	JHa	S330368	Yes (16)	May 96	M	69	Rectum	A	Mod
37.	MD	92836A	Control	May 96	M	74	Caecum	C	Poor
38.	KK	48175A	Control	May 96	F	75	Caecum	B	Mod
39.	FP	51963A	Control	May 96	M	57	Rectum	B	Mod
40.	WC	972566	Control	May 96	M	64	Caecum	C	Mod
41.	FHa	S808637	Yes (17)	May 96	M	73	Rectum	C	Mod
42.	TS	98283A	Control	Jun 96	F	82	Spl flex	A	Poor
43.	PT	S823729	Yes (19)	Jun 96	M	73	Rectum	C	Mod
44.	JB	08536B	Control	Jul 96	F	56	Caecum	B	Mod
45.	TD	985934	Control	Jul 96	M	51	Rectum	C	Mod
46.	IM	959247	Control	Aug 96	F	72	Colon	C	Mod

Table 29. Trial and control patients undergoing tumour disaggregation.

No	Init.	Hosp no	105AD7 [1]	Date [2]	Sex	Age	Site [4]	Stage [5]	Diff [6]
47.	AP	58589A	Control	Sept 96	M	56	Rectum	C	Poor
48.	JB	475592	Control	Sept 96	F	65	Rectum	C	Mod
49.	W A	19669B	Control	Sept 96	M	75	Rectum	C	Mod
50.	EB	-	Control	Oct 96	M	61	Sigmoid	A	Well
51.	DW	-	Control	Nov 96	F	70	ReSig [7]	B	Mod
52.	**GF**	**S442424**	**Yes (20)**	**Nov 96**	**M**	**75**	**Rectum**	**B**	**Mod**
53.	**DP**	**S433883**	**Yes (21)**	**Nov 96**	**M**	**69**	**Rectum**	**C**	**Mod**
54.	DR	-	Control	Nov 96	F	70	Rectum	B	Mod

1 Patients immunised with 105AD7 by the author, followed by their trial number in parentheses (see Table 14). These patients are shown in bold. Tumours were also disaggregated prospectively from control patients who did not receive 105AD7 prior to surgery.

2 Month and year that tumour separation occurred.

3 Patients recruited by the previous CRC Fellow. These patients received 10μg of 105AD7 i.d. followed by 100μg i.m. 48 hours later.

4 Site of primary tumour

5 Modified Dukes stage of tumour

6 Differentiation of primary tumour, as determined by routine histopathological examination (Well, Moderate or Poor).

7 Rectosigmoid tumour

Tumour Disaggregation.

1cm^3 of tumour was finely chopped in a Petri dish using a scalpel, and added to 20mls of 5% Collagenase A (Sigma, Fancy Rd, Poole, Dorset). After 10 minutes incubation at 37°C, the solution was filtered through a coarse filter, and the resultant mixture centrifuged at 190g (1100rpm) for 10 minutes. Residual unfiltered tumour was once again mixed with 20 mls of RPMI, and the procedure repeated. After centrifugation, the cells were treated with 200μl of DNAase (Sigma), washed by centrifugation, and resuspended in RPMI.

Separation of lymphocytes from epithelial cells and red cells was achieved using a discontinuous Percoll gradient (Pharmacia, Sweden). The gradient was made using SIP. The SIP consisted of Percoll diluted in HEPES (Sigma) buffered Hanks balanced salt solution. The osmolarity of the SIP was made up to 285mOsm/l, and its density calculated. Varying densities (1.044,1.055 and 1.077) of gradient were made by adding RPMI. Five ml of each density was layered in a Universal container in order to create the discontinuous gradient.

Ten millilitres of disaggregated tumour suspension in RPMI was added to the top of the gradient, and centrifuged for 30 minutes at 4°C and 800g (2000rpm). The resultant band of lymphocytes was harvested from the interface between 1055 and 1077 densities, and washed in RPMI, prior to resuspension. Five millilitres of unlabelled cell suspension was aliquoted into a FACS tube, and run through the FACScan (Becton Dickinson), to check for the presence of lymphocytes

Flow cytometry of tumour infiltrating lymphocytes.

Two hundred microlitre samples of cell suspension were stained with a panel of MAbs. These MAbs included CD45/CD14, an isotype matched control (IgG_1 and IgG_{2a}), CD3/CD19 (B cells), CD4/CD8, CD3/CD16+56 (NK cells), CD4/HLA-Dr, CD4/CD25, and CD3/CD69. Monoclonal antibodies were used undiluted, and obtained from the same sources, as outlined in Table 24. In addition CD4/CD45RA/CD45RO and CD8/CD45RA/CD45RO MAb (Dako) were used. Samples were incubated in the dark, on ice, for 30 minutes. Following this, they were centrifuged at 1200 rpm for 5 minutes, and washed twice with RPMI/1% Fetal Calf Serum (FCS). Supernatant was removed, and 300μl of FACS Fix added. Samples were then analysed on the FACScan cytometer (Becton Dickinson).

Results.

A total of 54 fresh tumours were disaggregated, and analysed, as described. Of these, 35 were from unimmunised patients, while the remaining 19 were from patients who had received varying doses of 105AD7. TIL obtained from disaggregation and separation on the Percoll gradient were only labelled and analysed by flow cytometry, if at least 1000 lymphocytes were obtained. Results were therefore available on 16/19 patients who had received 105AD7, and 26/35 of the controls. Three of the 42 available results were lost because patients details were inadequately recorded, and one patient was excluded, as the pathology from his resection specimen confirmed squamous cell carcinoma of the anus.

Thirty-eight sets of results were therefore available from patients, for statistical analysis (16 immunised, and 22 unimmunised). There were 11 men, and 5 women in the immunised group, with a mean age of 69.6 years. Twelve tumours were rectal, 2 caecal, and 2 sigmoid. All but 1 were moderately differentiated adenocarcinomas, and 3 were stage A, 5 stage B, 7 stage C, and 1 stage D.

Twenty-two patients had not received 105AD7 prior to surgery, and acted as the control group. This group consisted of 10 men, and 12 women, with a mean age of 67.7 years. Seven tumours were rectal, 8 caecal, 3 sigmoid, 1 colonic, 1 rectosigmoid, 1 ascending colon, and 1 at the splenic flexure. The majority of tumours were moderately differentiated, on routine histopathological examination. Two cancers were Dukes stage A, 10 stage B, and 10 stage C. There were no stage D tumours in the control group.

Individual results for trial and control patients are shown in Appendix 2, Table 2-14. Figures in parentheses represent percentages as recorded from the FACScan, while those without brackets are corrected for

contamination, and represent the percentage of the total number of lymphocytes expressing the surface antigen assayed. It is the latter figures that are used in the statistical analysis.

Contamination of TIL by epithelial cells and erythrocytes was similar in both trial and control groups. Median figures clearly approach 70%, and in some cases a 95% pure population of lymphocytes was obtained.

A comparison was initially made between all trial and control patients irrespective of any confounding variables. Median percentages, and interquartile ranges, are shown in Table 31. Immunised patients had a higher percentage of their lymphocytes expressing CD3, CD4, and CD19 surface antigens at the tumour site, and an increased presence of activation markers such as CD4+/- HLA Dr, CD4+/- 25, CD3+69. None of these differences were however statistically significant. Percentage expression of various activation markers was also combined. CD3-/69 and CD4+/25 was higher in trial patients. The percentage of lymphocytes expressing CD25 (CD4+ and CD4-) was significantly higher in patients who had received 105AD7 prior to surgery (Figure 20). Percentage expression of CD8+ cells was however slightly lower in trial patients.

A further analysis was performed whereby the two groups were matched by site, stage and differentiation of the tumour, and patient sex (Table 30). The ages of trial and control groups were comparable, being 66.8 and 62.7 years, in the two groups respectively. Trial patients had greater numbers of TIL expressing CD3, CD4, CD4+/-25, and CD3/69. A number of phenotypes were lower in trial patients. These included CD8, CD16+56, CD4/HLADr, CD19 and CD3-/69. The combination of CD3-/69 and CD4/25 was also higher in control patients. The only difference to reach statistical significance was for CD4+/- 25, once again suggesting an activated population of TIL in patients receiving 105AD7 (Figure 21: $p<0.01$).

Figure 20. Expression of CD25 on CD4+ and CD4- lymphocytes in all trial and control patients.

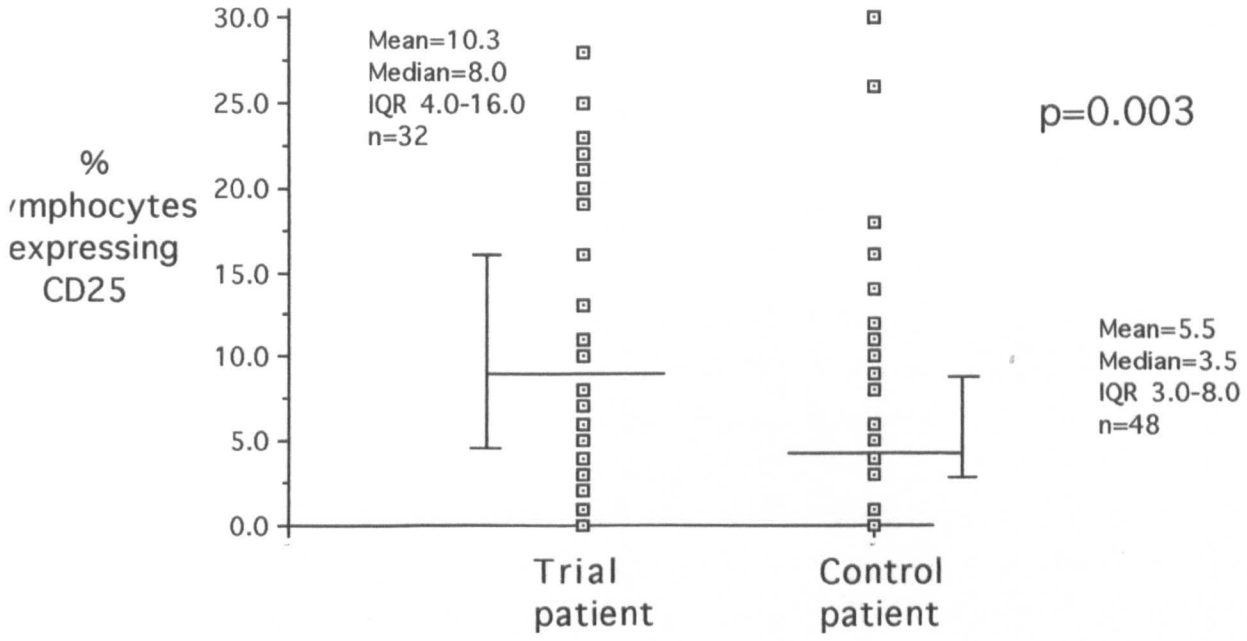


Figure 21. Expression of CD25 on CD4+ and - TIL, in trial and control patients matched according to Dukes stage, site and differentiation of tumour, sex and approximate age of patient.

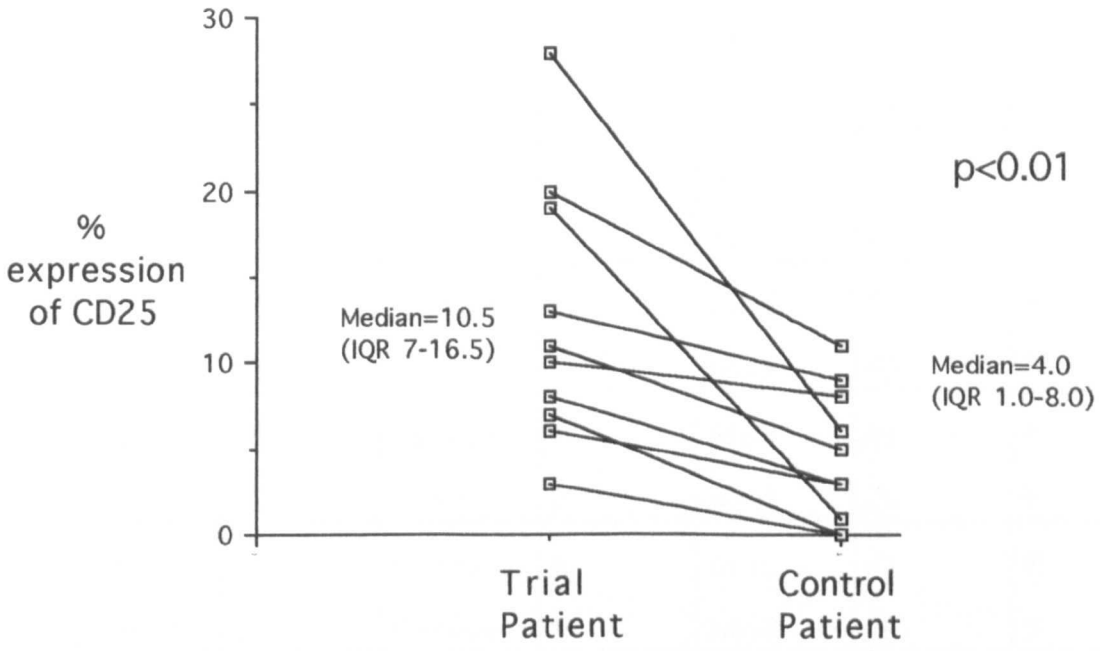


Table 30. Trial patients and controls matched, where possible, according to tumour site, stage,differentiation and sex and approximate age of patient.

Patient demography:

Pair		TIL no.	Site [3]	Stage [4]	Diff. [5]	Age	Sex
1.	Trial (9) [1]	20	Rectum	B	Mod	56	M
	Control	39	Rectum	B	Mod	57	M
2.	Trial (10)	25	Rectum	C	Mod	63	M
	Control	45	Rectum	C	Mod	51	M
3.	Trial (17)	41	Rectum	C	Mod	73	M
	Control	49	Rectum	C	Mod	75	M
4.	Trial (1)	11	Rectum	C	Mod	75	F
	Control	48	Rectum	C	Mod	65	F
5.	Trial (7)	18	Rectum	B	Mod	63	F
	Control	51	ReSig	B	Mod	70	F
6.	Trial [2]	5	Caecum	B	Mod	71	F
	Control	2	Rectum	B	Mod	58	F

1. Patients immunised with 105AD7 by the author, followed by their trial number in parentheses (see Table 14). Tumours were also disaggregated prospectively from control patients who did not receive 105AD7 prior to surgery.
2. Patients recruited by the previous CRC Fellow. These patients received 10μg of 105AD7 i.d. followed by 100μg i.m. 48 hours later.
3. Site of primary tumour.
4. Modified Dukes stage of tumour.
5. Differentiation of primary tumour, as determined at routine histopathological examination (Well, Moderate or Poor).

Table 31. Statistical analysis of TILS results

	Trial [1.]	Control [1.]	Trial [2.]	Control [2]
Lymph.	71.5 (63-84)	66.5 (65-76)	64.0 (48.5-84.8)	73 (61.0-84.5)
% CD3+	78 (59-89)	70 (69-80)	84.5 (63.0-88.5)	76.5 (64.0-89.0)
% CD4+	38 (29-52)	35 (27-48)	60.0 (43.0-82.0)	36.0 (29.0-48.0)
% CD8+	35.5 (23-42)	42.5 (36-46)	34.5 (22.0-39.0)	36.0 (22.0-51.8)
% CD19	7.5 (7-27)	14.5 (11-18)	9.5 (5.5-21.5)	11.5 (4.0-18.00
% CD16 + CD56	2.5 (1-10)	4.0 (2.5-5)	3.0 (2.0-5.0)	2.0 (1.0-5.0)
% CD4+ HLA Dr	16 (12.8-25.3)	22 (19.8-28.5)	26.0 (12.5-35.0)	23 (15.5-27.5)
% CD4- HLA Dr	51 (45.8-56)	61 (32.5-63.3)	54.0 (40.5-57.5)	48.0 (36.5-61.8)
% CD4 +/- HLA Dr	37 (14.5-49.5)	29 (22-61)	39 (26-56)	32.5 (23-52)

1. Analysis of 6 trial, and six control patients matched according to site, stage and differentiation of tumour , sex and approximate age of patient. Two-tailed Wilcoxon Signed Rank test used.
2 Statistical analysis of all trial and control patients, using a Mann-Whitney U test. Figures in parentheses represent interquartile ranges.

Table 31. Statistical analysis of TILS results.

	Trial [1.]	Control [1.]	Trial [2.]	Control [2]
% CD4+ 25 +	12 (10-20)	8 (4.5-9.5)	10.5 (8-18)	8.0 (4.8-11.3)
% CD4 - 25 +	6.5 (4-19)	1 (0-3.8)	4.5 (2.5-9.0)	3.0 (1.0-4.8)
% CD25 (CD 4 +/-)	10.5 (7-16.5)	4.0 **p<0.01** (1-8)	8.0 (4.0-16.0)	3.5 **p=0.003** (3.0-8.0)
% CD3+ CD69 +	70.5	61 (52.3-66.5)	66.0 (65-77)	58.0 (35-76)
% CD3 - CD69 +	6.0	14 (13.5-17.3)	10.0 (6.0-16.5)	14 (6.25-23.3)
% CD69 (CD3 +/-)	35.5 (6-71)	34.0 (14-61)	29.5 (10.0-66.0)	26.0 (14.0-59.5)
CD3-/69 CD4/25	10.5 (7-16.5)	11.5 (8-14)	10.0 (7.8-16.5)	10.5 (5.0-15.0)
CD4/ RO %	-	-	94.5 (92.5-96.5)	95.0 (88.3-97.8)
CD8/ RO %	-	-	88.0 (84.5-94.5)	95 (84.8-96.8)
CD4 & CD8 RO	91 (84-93)	94 (83-97)	93.0 (87.0-96.0)	95.0 (88.0-97.0)

1. 6 trial and control patients matched by site, differentiation and stage of tumour, sex and age. 2-tailed Wilcoxon Signed Rank test
2. Analysis of all trial and control patients, using a Mann-Whitney U test. Figures in parentheses represent interquartile ranges.

Discussion.

Analysis of all tumours disaggregated showed a significant increase in expression of the activation marker CD25 ($p=0.003$) in patients who had received 105AD7 pre-operatively. This is consistent with results obtained by immunohistochemical labelling of tumour sections. Other activation markers were raised in trial patients (CD4+HLA DR, CD4-HLA DR and CD3/69), though differences did not reach statistical significance. The percentage of lymphocytes expressing CD4 was also higher in immunised patients.

Matching trial and control patients for the same variables as used in the immunohistochemistry analysis, confirmed the findings above, for CD25 expression ($p<0.01$). The activation markers CD4+HLA DR, CD4-HLA DR and CD3/69 were also increased, though not significantly so.

It is reassuring to see results obtained immunohistochemically are confirmed by flow cytometry. The advantage of the former technique is that it allows multiple analyses to be performed, by separate observers in some cases, and comparisons to be made between different areas of the tumour. Flow cytometry is capable of quantifying the percentage of lymphocytes expressing various markers in a much larger amount of tumour tissue. Both techniques are subjective. Analysis of immunohistochemically labelled slides relies on the observer defining brown stain relative to the blue background. Determination of 'gates' on the FACScan is similarly subjective. The strength of the work lies in the close matching of trial and control patients, and analysing tumours blind.

A disadvantage of tumour disaggregation and flow cytometry, is that it is prospective. This means that a large number of tumours need to be 'processed', before adequate matching can occur. The separation is also techniqually difficult. If less than 1000 'events' (CD45+CD14- lymphocytes) were recorded from the tumour digest, then the computer analysis

software (Consort 30 and Lysys) would not run. As such only 42 out of 54 patients tumours were available. Flow cytometric analysis of tumour sections was performed blind by one observer (AG). Each dot plot is analysed separately and 'gates' inserted subjectively. This explains why results do not always add up to 100%. If percentage of lymphocytes expressing CD4 and CD8 were summed, this should broadly equal the percentage of lymphocytes expressing CD3. This is clearly not always the case.

The results obtained in trial and control patients are consistent with those in the literature. Work in colorectal cancer has also shown that there are more CD4+ cells in TIL than CD8+ cells [Balch CM 1990]. This is consistent with results presented here. A further study compared frozen sections from 14 normal colons with 14 colorectal adenocarcinomas. Sections were stained using antibodies to the T-cell associated antigens CD2, CD7, CD4, CD8, and the $\alpha\beta$ and $\gamma\delta$ subunits of the T-cell receptor [Banner BF 1993]. The distribution of cells was similar to that in small bowel, with CD8 expressing cells present in both the lamina propria and the epithelium. CD4 cells were concentrated in the luminal half of the mucosa. It was found that the major components of the immune response are Th cells, macrophages and HLA-DR+ cells [Banner BF 1993].

Analysis of sections from 58 large bowel adenocarcinomas, and 20 adenomas has recently been performed [Jackson PA 1996]. They found that the phenotype of the inflammatory infiltrate remained constant irrespective of intensity. The infiltrate was predominantly made up of CD4+ and CD3+ cells, with fewer CD8+ lymphocytes. HLA changes, notably ABC loss, A2 loss and DR gain, were commoner in poorly differentiated tumours. Further work has shown that the majority of TIL are CD4+, with the remainder being CD8+ [Keller H 1995][Moy P 1985]. Analysis of all tumours disaggregated, showed that 60% of all lymphocytes

were CD4+ in trial patients, as compared with 36% in controls. This is consistent with the results of our immunohistochemical analysis of tumour sections. Expression of CD8 however at the tumour site is essentially equal in the two groups. The proportion of NK cells in TIL is thought to be low, and our results confirm this, with <5% of CD45+ cells expressing CD16 and CD56.

Lymphocytes were labelled with a monoclonal antibody against the low molecular weight (180 kDa) isoform of the Leucocyte Common Antigen (CD45). The external domain of the molecule may be expressed in a number of different isoforms. Alternate mRNA splicing-out of exons 4,5 and 6, which encode products termed A,B and C could in theory produce 8 CD45R isoforms (ABC, AB, AC, BC, A, B, C and the null isoform O), ranging in molecular weight from 180 kDa to 220 kDa [Hargreaves M 1997]. CD4+ T cells may be functionally subdivided based upon expression of either high molecular weight CD45 RA+CD45RO- isoform or the low molecular weight CD45RO+CD45RA-. Expression of these two phenotypes is thought to reflect overall cell maturation, with the former representing the 'naive' or resting population, and the latter the 'memory' or activated lymphocyte population. Upon stimulation, T cells are thought to switch from synthesis of CD45RA mRNA to CD45RO mRNA within 24 hours of stimulation, and expression of the RO glycoprotein within 24-48 hours. CD45 RA glycoprotein has been shown to remain on the cell surface for 48-72 hours, and then disappear [Deans JP 1989]. The difference phenotypically between RA an RO relates to cytokine production. CD4+ T cells expressing CD45RO+ are capable of producing cytokines such as IL-1, IL-2, IL-5, IL-6, IFN-γ, TNF-α and TNF-β, when stimulated. CD4+ lymphocytes with the RA+ phenotype however, are only able to produce IL-2 and TNF-β in any quantity [Adamthwaite 1994]. In assessing expression of CD45RO at the tumour site of immunised and

unimmunised patients, an attempt was being made to semi-quantify the number of stimulated/memory lymphocytes at the tumour site. Expression of CD45RO however at the tumour site of all patients tumours was approximately the same in immunised patients as controls, on both CD8+ and CD4+ lymphocytes. This labelling was performed only on tumours disaggregated in the second half of the study, and insufficient numbers were available to match according to stage, site, differentiation age and sex.

The activation markers CD69 and HLA DR were also assessed at the tumour site of trial and control patients. There was no significant difference in expression of the former in the two groups, consistent with results obtained by immunohistochemical labelling of tumour sections. The significance of this has been discussed in this section. MHC Class II expression, which has been shown to increase on T cells following activation, was also similar on CD4 + and - TIL in trial and control patients [Pichler WJ 1994].

In conclusion, this work shows that expression of CD25 is increased following immunisation with 105AD7. This is consistent with the results obtained immunohistochemically, further sugesting a population of activated lymphocytes at the tumour site of patients receiving the vaccine. There is however no significant difference in terms of expression of other activation markers, such as CD69, HLA DR and CD45RO. This technique is slightly flawed in that while it may be ideal for expressing the percentage of activated lymphoctes, it does not compare infiltration of CD4 or CD8 cells per se, as the numbers quoted are percentage of all TIL. In future it might be more appropriate to assess more definitively absolute cell numbers.

Part 4.

Survival Analysis.

Contents.

Aim.

Previous chapters have attempted to assess the immunological changes that occur at the tumour site following pre-operative immunisation with 105AD7. The aim of this work is to assess whether these have any effect on overall survival.

A total of 23 patients were recruited to the 105AD7 adjuvant study between June 1993 and April 1995, by the previous CRC fellow Mr T.J.D. Buckley. This analysis was performed in March/April 1997, when all of these patients were at least 2 years following operation. The main outcomes were time to recurrence, and death within 2 years. The aim was to compare these outcomes in trial patients, with a well matched group, randomly selected from the Trent Colorectal Audit.

Materials and Methods.

Patients and Clinical Protocol.

A total of 23 patients were recruited to the 105AD7 adjuvant study between June 1993 and April 1995 by the previous CRC Fellow, Mr. T.J.D. Buckley (Table 32). All patients had colorectal cancer, either diagnosed at endoscopy and biopsy, or on Double Contrast Barium enema. There were 18 men, and 5 women, with a mean age of 69.7 years (range 55 to 82 years). Fourteen were rectal tumours, while the remaining nine were colonic.

Patients attended the Department of Surgery, where they received 10μg of 105AD7 intradermally, followed 48 hours later by a 100μg intramuscularly. Patients then underwent resection of their primary tumours. At 6 and 12 weeks after operation patients were boosted with the same treatment course.

Follow up post-operatively was in the Department of Surgery Colorectal Clinic, by five Surgical Research Registrars, according to a set protocol. All patients were seen 3 monthly for the first year, and then 4 monthly thereafter. At each visit, History and Physical Examination were performed, and a venous blood sample assayed for Carcinoembryonic Antigen (CEA). Patients underwent flexible sigmoidoscopy at 6 months, and 3 years, if their anastomosis was within reach of the endoscope. A persistently raised CEA was investigated by CT, Chest X-ray and colonoscopy. Other investigations were performed if clinically indicated.

Control Group.

The control group consisted of patients randomly selected from the Trent Colorectal Cancer Audit. This data base was originally established on

the 1st August 1992, to document outcome in patients with colorectal cancer, seen in the ensuing 12 months, in hospitals in the Trent region. The audit was extended to cover patients treated in Wales between January 1993 and December 1993, and in total 52 hospitals were covered, and the notes of 3520 patients scrutinised. Funded by the Department of Health through the Royal College of Surgeons of England, it involved an independent notes review by six trained research assistants, and ongoing GP contact. Patients were identified using a number of approaches, including hospital record systems, histopathology records, audit clerks, operation notes, and consultant secretaries. The only patients not included in the various centres were those who had had previous colorectal cancer.

Trial patients were matched to controls for the following eight variables :

Dukes stage of tumour.	ASA [1] grade.
Tumour site (colon v rectum).	Chemotherapy
Tumour differentiation.	Radiotherapy
Sex.	Approximate Age.

[1] American Society of Anaesthesiologists.

Table 32. Patient Demographics. Survival analysis.

No.	Sex	Age. [1]	No Con[2]	Age Con[3]	Dukes Stage.	Diff.[4]	Site [5]	ASA	Chemo [6].	RTH [6].
1.	M	64	5.	55.6	C	Mod.	Rectum.	1.	No	No
2.	M	55	2.	59.0	D	Poor.	Rectum.	NK.	5-FU+FA	No
3.	M	66	5.	59.4	C	Mod.	Rectum.	2.	No	No
4.	F	82	5.	68.0	C	Mod.	Rectum.	3.	No	No
5.	F	67	5.	75.8	B	Poor.	Colon.	3.	No	No
6.	M	64	1.	50.0	C	Mod.	Rectum.	2.	5-FU+FA	No
7.	F	60	5.	64.8	C	Mod.	Colon.	NK.	No	No
8.	F	75	5.	71.8	C	Mod.	Colon.	3.	No	No
9.	M	74	2.	74.0	D	Mod.	Rectum.	3.	No	No
10.	F	64	5.	69.6	B	Mod.	Colon.	NK.	No	No
11.	M	81	5.	63.2	B	Mod.	Rectum.	NK.	No	No
12.	M	71	5.	74.2	B	Mod.	Rectum.	NK.	No	No
13.	M	71	2.	63.0	C	Poor.	Rectum.	2.	No	No
14.	M	76	5.	69.8	B	Mod.	Rectum.	2.	No	No
15.	M	71	5.	73.2	B	Mod.	Rectum.	1.	No	No
16.	M	77	5.	73.6	A	Mod.	Rectum.	2.	No	No
17.	M	79	5.	63.2	A	Mod.	Rectum.	NK.	No	No
18.	M	70	5.	66.4	A	Mod.	Rectum.	1.	No	No
19.	M	65	5.	68.0	C	Mod.	Colon.	NK.	Gastr [7].	No
20.	M	70	2.	74.5	B	Poor.	Colon.	3.	No	No
21.	M	72	2.	54.5	C	Mod.	Colon.	2.	5FU+FA	No
22.	M	67	5.	72.2	A	Mod.	Colon.	NK.	No	No
23.	M	61	5.	67.2	C	Mod	Colon.	1.	No	No

Legend for Table 32.

1 Age of patient (years) at time of resection of primary tumour.

2 Number of control patients selected from Trent Audit for trial patient.

3 Mean age of control patients at time of operation.

4 Degree of differentiation of primary tumour (well, moderate or poor).

5 Site of primary tumour (colon v rectum).

6 A number of patients received adjuvant chemotherapy or radiotherapy, following resection of their primary tumours.

7 Patient received Gastrimmune™ for advanced disease, as part of a Phase I study.

Results.

From the database it was possible to obtain between 1 and 70 potential controls for each of the patients who had received 105AD7. It was decided, following consultation with a statistician, to use a maximum of 5 randomly selected controls for each, where possible. Having more than 5 controls per patient would have little effect on the power of the analysis. Ultimately, 96 controls were used for the 23 trial patients.

Twenty-three patients were recruited by the previous CRC Fellow, over a 23 month period, ending April 1995. The group consisted of 18 men, and 5 women, with a mean age of 69.7 years. Fourteen of the primary tumours were rectal, and 9 colonic. Four (17%) tumours were Dukes stage A, 7 stage B (31%), 10 stage C (43%), and the remaining 2 (9%), stage D. All were described as moderately differentiated on routine histological assessment, except for 4 tumours, which were classified as poorly differentiated. Patient demographics are shown in Table 32.

The survival, and recurrence data for these patients is shown in Table 33. Sixteen of the 23 (69.6%) patients who had received 105AD7 were alive and disease free at 2 year follow up. Five patients had died (21.7%), and a further 2 (8.7%) had developed liver metastases. Of the patients who died, 2 had Dukes D primary tumours. The remaining 3 died at between 60 and 209 days following operation. All had Dukes C tumours originally. A diagnosis of carcinomatosis was made on each. The mean time to death was 180 days. The two patients who had recurrences at 2 year follow up had a Dukes B and C at original presentation. It was not clear on reviewing these patients notes whether any treatment in terms of chemotherapy or radiotherapy was given for these recurrences.

Ninety-six control patients were matched to patients who had received 105AD7, according to the aforementioned 8 variables. Between 1

and 5 control patients were available for each trial patient (mean 4.2). The group as a whole consisted of 71 men, and 25 women, with a mean age of 67.4 years. Fifty-seven tumours were rectal, and 39 colonic. Eighty-five and 11 tumours were moderately, and poorly differentiated, respectively. Twenty tumours (21%) were Dukes stage A, 32 (33%) stage B, 40 (42%) stage C, and 4 (4%) stage D.

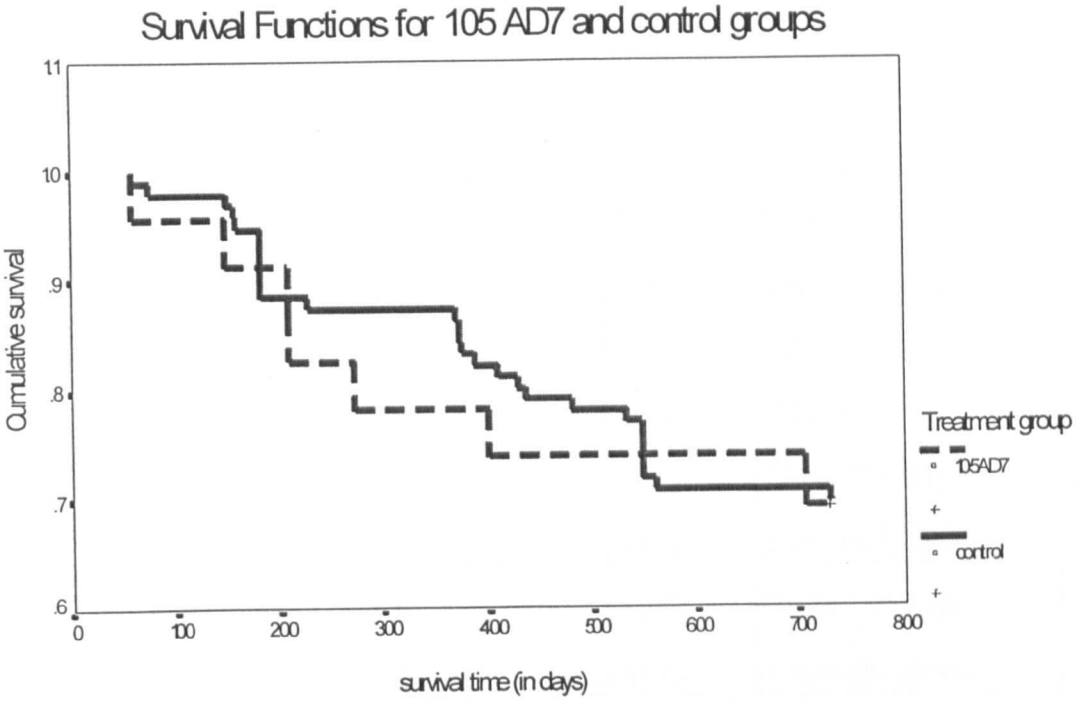
Survival and recurrence data for patients in the control group is shown in Table 33. There were 96 patients in the control group, of whom 67 (69.8%) were alive and disease free at 2-year follow up. A total of 15 (15.6%) patients had died, while 14 (14.6%) had evidence of local recurrence, or distant metastases. The mean time to death in this group was 403 days. Of the fifteen patients who died, 1 had a Dukes A primary tumour, 5 Dukes B, 6 Dukes C and 3 Dukes D tumours. Fourteen patients had evidence of local recurrence, or distant metastases at 2 year follow up of whom 2 were originally Dukes A tumours, 3 Dukes B, 8 Dukes C, and 1 Dukes D.

A log rank test was performed to assess if there was any difference between trial and control patients in terms of death, or recurrence. No significant difference existed between the two groups at the 5% level.

A Cox proportional hazards model was used to adjust for Dukes stage of disease. The risk ratio in the 105AD7 arm was 1.080 (CI 0.473-2.466), suggesting that overall survival in the 105AD7 arm is worse than controls. The 95% confidence intervals do however span 1, suggesting that no firm conclusions can be drawn. Analysis of Dukes A and B tumours relative to those graded C and D showed a risk ratio of 0.376 (CI 0.188-0.751), as one would expect, indicating that patients with the former tumours have a better survival than the latter. When the multivariate analysis was performed for Dukes stage A/B as one group, and C/D as another, a relative risk ratio of 0.387 (CI 0.050 to 2.998) was seen in the 105AD7 arm.

The corresponding figure for stages C and D was 1.532 (CI 0.607-3.866). These are shown in Table 34. Once again the 95% confidence intervals span 1, suggesting that no firm conclusions can be drawn.

Kaplan-Meier curves for trial and control patients is shown overleaf. The upper graph shows the time to a negative outcome (death or recurrence) for all patients, while the lower graph considers Dukes stages A and B versus C and D, for the two groups.


Survival Functions for 105 AD7 and control groups
Cumulative survival
1.1
1.0
.9
.8
.7
.6
0
100
200
300
400
500
600
700
800
survival time (in days)
Treatment group
105AD7
control

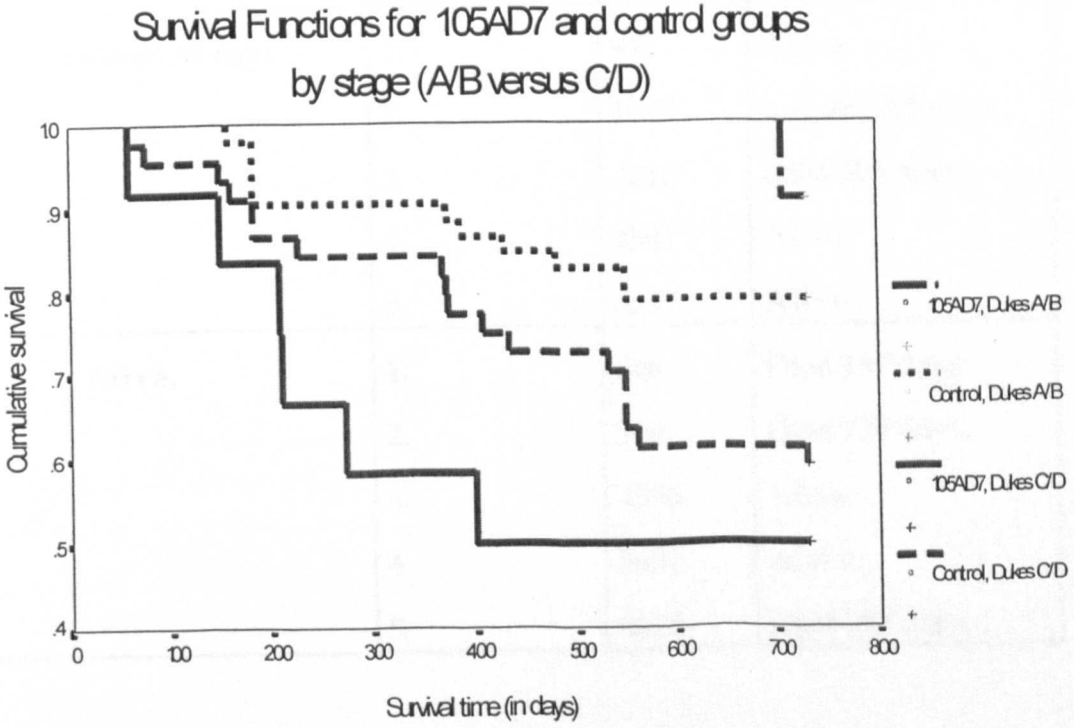

Survival Functions for 105AD7 and control groups
by stage (A/B versus C/D)
Cumulative survival
1.0
.9
.8
.7
.6
.5
.4
0
100
200
300
400
500
600
700
800
Survival time (in days)
105AD7, Dukes A/B
Control, Dukes A/B
105AD7, Dukes C/D
Control, Dukes C/D

Table 33. 2 Year Follow up data for 23 patients immunised by the previous CRC fellow between June 1993 and April 1995, and their matched controls from the Trent Audit.

No [1].	Status [2].	Control [3].	Ref no.[4]	Status of control [5]
1.	Alive.	1.	3136	Died 409 days.
		2.	4328	Alive
		3.	4400	Alive
		4.	4653	Died 375 days.
		5.	4661	Loc rec [6] 730 days.
2.	Died 273 days.	1.	4173	Died 160 days.
		2.	4499	Died 561 days.
3.	Alive.	1.	1681	Alive.
		2.	719	Alive.
		3.	4104	Loc rec 730 days.
		4.	1879	Alive.
		5.	2537	Alive.
4.	Died 209 days.	1.	947	Alive.
		2.	1647	Loc. rec 365 days.
		3.	4218	Died 365 days.
		4.	4581	Alive.
		5.	6230	Alive.
5.	Alive.	1.	246	Died 157 days.
		2.	508	Died 730 days.
		3.	4556	Alive.
		4.	5651	Alive.
		5.	8625	Died 387 days.

Table 33. 2 Year Follow up data for 23 patients immunised by the previous CRC fellow between June 1993 and April 1995, and their matched controls from the Trent Audit.

No [1].	Status [2].	Control [3].	Ref no [4].	Status of Control [5].
6.	Alive.	1.	4104	Loc rec 730 days.
7.	Alive.	1.	1081	Loc rec 76 days.
		2.	1301	Alive.
		3.	4624	Alive.
		4.	6178	Alive.
		5.	6221	Alive.
8.	Died 149 days.	1.	2808	Alive.
		2.	4502	Alive.
		3.	4236	Alive.
		4.	2842	Recurr 435 days.
		5.	4290	Alive.
9.	Died 210 days.	1.	5282	Died 373 days.
		2.	8825	Recurr 60 days.
10.	Alive.	1.	1321	Alive.
		2.	2174	Alive.
		3.	2113	Alive.
		4.	3585	Alive.
		5.	1890	Alive.

Table 33. 2 Year Follow up data for 23 patients immunised by the previous CRC fellow between June 1993 and April 1995, and their matched controls from the Trent Audit.

No [1]	Status [2]	Con [3]	Ref no [4]	Status of Control [5]
11.	**Alive**	1.	4406	Died 428 days.
		2.	8111	Alive.
		3.	4455	Alive.
		4.	4151	Alive.
		5.	6130	Alive.
12.	**Alive**	1.	765	Alive.
		2.	8550	Loc rec 365 days.
		3.	838	Alive.
		4.	885	Alive.
		5.	5300	Alive.
13.	**Died 60 days.**	1.	2236	Died 339 days.
		2.	4715	Loc rec 730 days.
14.	**Alive.**	1.	1278	Loc rec 730 days.
		2.	2365	Loc rec 365 days.
		3.	230	Alive.
		4.	1086	Alive.
		5.	4260	Alive.
15.	**Alive**	1.	6160	Alive.
		2.	6012	Alive.
		3.	4216	Alive.
		4.	4049	Alive.
		5.	3183	Alive.

Table 33. 2 Year Follow up data for 23 patients immunised by the previous CRC fellow between June 1993 and April 1995, and their matched controls from the Trent Audit.

No [1].	Status [2].	Control [3]	Ref no [4]	Status of Con [5].
16.	Alive.	1.	1150	Alive.
		2.	764	Alive.
		3.	2599	Alive.
		4.	2324	Alive.
		5.	744	Alive.
17.	Alive.	1.	5742	Alive.
		2.	775	Alive.
		3.	4109	Alive.
		4.	1185	Alive.
		5.	6359	Alive.
18.	Alive.	1.	2659	Loc rec 365 days.
		2.	3252	Alive.
		3.	4240	Alive.
		4.	1783	Alive.
		5.	8585	Alive.
19.	Recurrs 400 days.	1.	2773	Alive.
		2.	5690	Died 533 days.
		3.	6379	Alive.
		4.	4516	Alive.
		5.	2822	Died 369 days.

Table 33. 2 Year Follow up data for 23 patients immunised by the previous CRC fellow between June 1993 and April 1995, and their matched controls from the Trent Audit.

No [1]	Status [2]	Control [3].	Ref no [4].	Status of Control [5].
20.	Recurrs 707 days	1.	5254	Died 373 days.
		2.	49	Alive.
21.	Alive.	1.	4412	Alive.
		2.	1448	Alive.
22.	Alive.	1.	1058	Alive.
		2.	6156	Died 480 days.
		3.	361	Alive.
		4.	4746	Alive.
		5.	8815	Recurrs 365 days.
23.	Alive.	1.	4514	Alive.
		2.	8631	Alive.
		3.	2773	Alive.
		4.	3313	Alive.
		5.	2433	Recurrs 228 days.

Key to Table 33.

1 Patients recruited to study by the previous CRC fellow. The patient number corresponds to that in Table 32.

2 Status of the patient at 2-year follow up. Patients were either alive and presumed disease free, dead (all causes), or were alive, but had evidence of recurrent disease.

3 Number of controls matched to each trial patient from the Trent Colorectal Audit.

4 The reference number corresponds to the number allocated to the patient in the Trent Colorectal Audit. This should enable comparison of immunised patients with controls at 3 and 5 years.

5 Status of control patient at 2-year follow up. Patients may be alive, and presumed disease free, dead, or alive, but with evidence of recurrent disease.

6 Local recurrence.

Table 34 Multivariate analysis adjusting for Dukes stage.

Covariate	*Risk ratio*	*95% Confidence Interval*
105AD7	1.080	0.473-2.466
Control	1	
Dukes stage		
105AD7	0.376	0.188-0.751
Control	1	

Table 34. Effect of 105AD7 by Dukes Stage.

Dukes Stage	*Treatment*	*Risk ratio*	*95% CI*
A/B	105AD7	0.387	0.050 to 2.998
	Control	1	
C/D	105AD7	1.532	0.607 to 3.866
	Control	1	

Discussion.

This work has attempted to assess whether any of the immunological changes described, confer a survival advantage on patients receiving 105AD7 as adjuvant therapy. A comparison is made with a historical control group, that is well matched to trial patients according to 8 different variables. It differs from the Phase II study in that it is not prospective, randomised or blind, and these are the major criticisms of the analysis. In addition, trial and control patients were not operated on at exactly the same period in time - 1993/1995 in the former, and 1992/1993 in the latter group.

This analysis also takes no account of the treatments patients may have received for their recurrent disease. This data was not available from the Trent Audit, and was not reliably obtainable from Nottingham patients immunised with 105AD7. The post-operative follow up of patients receiving 105AD7 is likely to have been better than that of patients in the control group. Patients in the former group were seen every three months for the first year, and every 4 months in the second. Physical examination and CEA were performed on each occasion, with flexible sigmoidoscopy at 6 months, in cases where the anastomosis was visible. The follow up regimes used by the various consultants whose patients were included in the Trent Audit, and thus constituted the control group, has recently been documented [Mella J 1997]. There was wide variation in the regimes employed. Approximately 15% of all patients undergoing operation were followed up for only 1 year. As well as potentially closer follow up, more specific data relating to time taken to death or recurrence was available in the 105AD7 group. In some control patients exact dates of recurrence were known, though in others it was only known from the data base that they had recurred at 2 years, when in fact it may have been

earlier. In these cases the time to a negative outcome had to be assumed to be 2 years.

At the time of performing this survival analysis, only 2-year follow up data was available from the Trent Audit database, and this time point was therefore chosen as the censuring point. There is evidence however to suggest that 80% of all recurrences will occur within the first 2 years of surgery. Seven out of 23 patients (30%) receiving 105AD7 had either died or recurred, as compared with 29/96 (30%) in the control group. Ideally an analysis should be performed at a follow up of 5 years. If 50% of all patients with colorectal cancer ultimately die of their disease, then it is possible that more patients will die or recurr, in the 3 years after this analysis was performed. This may be the case. The caveat however is that the original 23 patients immunised with 105AD7 have earlier stage disease than would be expected. This observation is reflected in the control group which is stage-matched. Approximately 20% of tumours were Dukes stage A, and 4% stage D in the two groups, and the literature suggests that these figures should be 5% and 30% respectively [Gill P 1978]. It is likely that patients receiving 105AD7 had their tumours diagnosed as part of the faecal occult blood screening study that was running at the time in Nottingham. Tumours detected by screening have been shown to be detected at an earlier stage [Hardcastle JD 1996].

The ultimate role of a colorectal cancer vaccine is in the treatment of patients with primary disease. These patients have low tumour burdens, and are more likely to mount an effective immune response than patients with advanced disease. 40-45% of patients will have either Dukes A or B tumours and are unlikely to be referred for chemotherapy. These patients may benefit from a non-toxic adjuvant therapy, that has a small, but not insignificant effect on 5-year survival. It is interesting to note, that of the 11 immunised patients with Dukes A or B tumours, only 1 had recurred at

2 year follow up (9.1%). This compared with 11 of the 52 controls (21.2%), and was reflected in the relative risk ratio of 0.387. The difference was not however statistically significant, and the 95% confidence intervals spanned 1.0 (0.050 - 2.998), suggesting that 105AD7 could not be assumed to be conferring a survival advantage.

This analysis does nothing towards answering the question of whether 105AD7 prolongs survival when used as adjuvant therapy. Too few patients were immunised 2-3 years ago, and thus with wide confidence intervals, firm conclusions cannot be drawn. A prospective, randomised, placebo-controlled study recruiting over 200 patients is the only satisfactory way to answer this question.

Chapter 4.

Conclusion.

Conclusion.

There were two aims of this work. The first was to test the survival results of the Phase I study in a randomised, placebo-controlled, double-blind, Phase II study, in patients with advanced colorectal cancer. The second aim was to use 105AD7 as adjuvant therapy in patients with primary malignancy, and assess the immunological changes that may be occurring in the peripheral blood, and at the tumour site. A comparison was also made in terms of survival, and local recurrence between 23 patients who had received 105AD7 from the previous CRC Fellow, and a matched control group.

No significant survival difference was seen between patients receiving 105AD7 and placebo, in the Phase II study. It is likely that any immunological responses generated in these patients were insufficient to have any major effect on tumour growth. This was felt to be due to a number of factors, including the number of immunisations given, the adjuvant used, the site of disease, and whether sufficient time was left after completion of chemotherapy. The patient population was different from the Phase I study, in so much as patients with local recurrences, and multiple disease sites were included. The argument that tumour burden may equate to immunosuppression is persuasive, and it is likely that recruited patients failed to develop sufficiently effective immune responses to cause slowing of tumour growth, and prolongation in survival.

A number of encouraging results were however shown in the Phase II study. The effect of chemotherapy and radiotherapy in prolonging survival was seen in the multivariate analysis, consistent with the results of a number of other published studies. Survival of patients who received chemotherapy and 105AD7 was appreciably worse than other groups,

contradicting the hypothesis that immunotherapy and chemotherapy may act synergistically. The toxicity profile of 105AD7 was encouraging, with only one serious adverse event felt potentially due to immunisation. In view of this, and the fact that patients with advanced disease are not an ideal group for immunotherapy, work has concentrated on immunising patients with primary colorectal cancer, as part of an adjuvant study.

In contrast to the Phase II study, the adjuvant study has shown a number of interesting, and encouraging results. The toxicity profile was once again confirmed, with none of the 24 patients recruited by the author showing any side effects. Immunohistochemical analysis of tumour sections showed statistically significant infiltration of CD4, CD8 and CD56 expressing lymphocytes in patients who had received 105AD7 pre-operatively, as compared with a control group matched according to site, stage and differentiation of tumour, sex and age of patient. Evidence has been presented suggesting that such lymphocytic infiltration may confer a survival advantage on patients with colorectal cancer. Results for labelling with MAb against CD4 and CD8 lymphocyte subsets were also independently significantly higher in trial patients. This work also showed higher levels of the activation marker CD25 on lymphocytes at the tumour site of immunised patients. This latter observation was confirmed by disaggregating fresh tumour from 16 trial and 22 control patients, labelling the lymphocytes obtained with CD25 and analysing by flow cytometry.

These results suggested an enhanced population of activated lymphocytes at the tumour site of patients who received 105AD7 prior to surgery. Having established this, the aim was to assess whether these lymphocytes were functional, and capable of killing tumour cells by apoptosis. Tumour sections from trial and control patients were therefore labelled immunohistochemically with APO2.7, an MAb against a

mitochondrial antigen (7A6) expressed only on apoptotic cells. Results showed higher levels of apoptosis at the tumour site of trial patients. It was not however clear whether this reflected enhanced kiling of tumour cells by invading lymphocytes, or the converse; ie Fas mediated destruction of lymphocytes by tumour cells. Work is currently ongoing to address this issue.

Peripheral blood samples were taken from patients, prior to immunisation with 105AD7, and at various time points up until operation. Analysis by flow cytometry failed to show any significant increase in any of the lymphocyte subsets considered. This may reflect the fact that stimulated lymphocytes are accumulating at the tumour site, or that the technique is not sensitive enough to detect small changes in lymphocyte numbers.

An attempt was made to assess if any of the immunological changes described after immunisation, correlated with a survival advantage. Twenty-three patients were immunised with 105AD7 by the previous CRC Fellow, between June 1993 and April 1995. The survival and recurrence data at 2 year follow up was compared with 97 controls from the Trent Colorectal Audit, matched according to stage, site and differentiation of tumour, age, sex and ASA of patient, and whether they had chemotherapy or radiotherapy. No significant difference was seen between the two groups, almost certainly reflecting the small numbers involved in the analysis.

These results suggest that 105AD7 is capable of inducing immune responses in patients with primary colorectal cancer, with minimal if any associated toxicity.

Future work using the anti-idiotypic monoclonal antibody 105AD7 needs to concentrate on several different areas. The premise that the vaccine is capable of mimicking the antigen needs further substantiation.

With current advances in molecular biology it should be possible to clone and sequence the CDR3 region of 105AD7. In addition gp72 could be affinity purified, and protein sequenced. Ultimately, nieve lymphocytes could be stimulated with gp72, and then further re-stimulated with 105AD7 to confirm that the primed lymphocytes are specific for the antigen/anti-idiotypic antibody. This work is currently ongoing.

Clinical studies need to concentrate further on immunising patients with primary tumours, who are likely to get better immune responses than those with advanced disease. Work using APO2.7 MAb to immunohistochemically label tumour specimens was encouraging, though debate still exists as to the nature of the apoptotic cells concerned. In order to address this important issue, one or both of two techniques could be employed. These include either 2-colour immunohistochemical staining of tumour sections, or flow cytometric analysis of distinct apoptotic populations of disaggregated TIL/ tumour epithelium. No significant increase post-immunisation could be seen when peripheral blood was phenotyped, as discussed. If future work is going to look at this area, then it should concentrate on assaying cytokines such as IL-2, IL-4, TNFα and β.

The ideal method for assessing whether any of the immune responses seen in this thesis have any effect on patient survival would be a randomised, placebo-controlled survival study, whereby patients are immunised pre-operatively, and for up to two years after resection of primary tumours. An alternative approach would be to perform a further survival analysis of all patients recruited to the adjuvant study by the author, and the previous CRC Fellow, and compare it with controls from the Trent Audit. In addition future work should concentrate on the efficacy of combined adjuvant chemotherapy and 105AD7, and optimising the dose of the vaccine.

APPENDICES.

Appendix 1. Part 2. The Phase II Study.

APPENDICES.

Appendix 1 **Part 2. The Phase II Study.**

APPENDICES.

Appendix 2 **Part 3. Use of 105AD7 as adjuvant therapy.**

Chapter 1.

Table 2-1 Immunohistochemistry results - CD25

Table 2-2 Immunohistochemistry results - CD4

Table 2-3 Immunohistochemistry results - CD8

Table 2-4 Immunohistochemistry results - CD56

Table 2-5 Immunohistochemistry results - ζ chain expression.

Table 2-6 Immunohistochemistry results - CD68

Table 2-7 Immunohistochemistry results - CD69

Table 2-8 Immunohistochemistry results - IgG_1

Table 2-9 Immunohistochemistry control results. Tris buffered saline (TBS).

Table 2-10 Immunohistochemistry results for APO2.7. Control IgG_1

Table 2-11 Immunohistochemistry results - APO2.7

Table 2-12 Assessment of intraobserver variation.

Table 2-13 Assessment of interobserver variation. Analysis of a control patient by 2 independent observers.

Chapter 2.

Table 2-14 Results of all tumours disaggregated.

Table 1-1 Patient Demography

		Treatment 105AD7 n=85	Placebo n=77
Age	Mean (sd)	63.3 (11.9)	62.2 (11.2)
	Median	64	62
	Min	27	33
	Max	85	85
	n	85	77
Sex	Male	51 (60%)	40 (52%)
	Female	34 (40%)	37 (48%)
Time from diagnosis of advanced disease to entry into the study (in days)	Mean (sd)	277.1 (303.4)	278.6 (271.1)
	Median	172	179
	Minimum	0	5
	Maximum	1629	1131
	n	83	72
	Missing	2	5
Grade	Well	3 (5%)	4 (7%)
	Moderate/well	3 (5%)	2 (3%)
	Moderate	43 (78%)	42 (71%)
	Mod / Poor	1 (2%)	3 (5%)
	Poor	5 (9%)	8 (14%)
	Missing	30	18
Dukes stage	A	2 (3%)	2 (3%)
	B	14 (18%)	13 (18%)
	C	35 (45%)	33 (45%)
	D	26 (34%)	26 (35%)
	Missing	8	3
Primary site of tumour	Colon	48 (58%)	46 (60%)
	Rectum	36 (42%)	31 (40%)
	Unknown	1	0

Table 1-2 Initial surgery for resection of the primary tumour

Surgery for resection of primary tumour		Treatment 105AD7	Placebo
n		77	74
Time from surgery to entry into the study (in days)	Mean (sd)	640.8 (579.4)	803.7 (720.1)
	Median	465.0	666.5
	Min	20	41
	Max	3475	3615
	n	77	74
Operation	Colectomy	17 (22%)	9 (12%)
	Colectomy+debulk	0	1 (1%)
	Resection	5 (6%)	4 (5%)
	Hartmann's	4 (5%)	6 (8%)
	Right hemicolectomy	19 (25%)	15 (20%)
	Left hemicolectomy	4 (5%)	5 (7%)
	Subtotal colectomy	0	1 (1%)
	Anterior resection	21 (27%)	22 (30%)
	Abdomino-perineal resection	7 (9%)	11 (15%)
Site	Colon	44 (57%)	37 (50%)
	Recto-sigmoid	2 (3%)	0
	Rectum	26 (34%)	34 (46%)
	Rectum+small bowel.	0	1 (1%)
	Not documented	5 (6%)	2 (3%)
Residual disease at site	None	41 (53%)	40 (54%)
	Microscopic	4 (5%)	7 (9%)
	Macroscopic	21 (27%)	19 (26%)
	Unknown	11 (14%)	8 (11%)

Table 1-3 Any subsequent operations

Patient number	105AD7/ Placebo	Time[1]	Operation[2]	Site	Residual disease
11	105AD7	34	rev ileo	-	micro
13 *	105AD7	164	EUA+biopsy	rectum	macro
19	105AD7	194	laparotomy	-	macro
24 *	105AD7	161	ileo bypass	colon	macro
29	105AD7	579	excision	umbil lesion	none
29	105AD7	33	def ileost	-	macro
30	105AD7	277	cryotherapy	liver mets	macro
37 *	105AD7	790	endo+biopsy	rectum	macro
42	105AD7	308	exc loc rec	anastomosis	macro
42	105AD7	201	laparotomy	-	macro
44	105AD7	590	lobectomy	right lung met	none
46	105AD7	83	colectomy	sigmoid colon	macro
51	105AD7	218	exc loc rec	anastomosis	micro
63 *	105AD7	603	EUA+biopsy	rectum	macro
63 *	105AD7	181	def end col	-	macro
66 *	105AD7	161	def end col	colon	none
73	105AD7	485	resection	left liver lobe	unknown
79	105AD7	1325	resection	right liver	none
84	105AD7	916	resection	liver	macro
85 *	105AD7	35	ileo-sig byp	colon	macro
87	105AD7	936	def col	colon	none
87	105AD7	756	rev Hart	-	macro
98 *	105AD7	95	il trans byp	-	
130	105AD7	206	cryotherapy	liver	unknown
149	105AD7	362	resection	right liver	none
149	105AD7	256	resection	-	none
152 *	105AD7	195	def col	colon	macro
152 *	105AD7	41	laparotomy	unknown	macro
162	105AD7	644	resection	anastomosis	none
7	Placebo	455	excision	skin nodules	none
7	Placebo	358	laparotomy	-	macro
9	Placebo	868	resection	liver mets	none
9	Placebo	716	resection	liver mets	none
9	Placebo	412	resection	small bowel	macro
9	Placebo	351	aspiration	abdom abscess	macro
9	Placebo	198	aspiration	abdom abscess	macro
14	Placebo	304	EUA+biopsy	rectum	macro

* Primary tumour not removed.

Table 1-3 Any subsequent operations (continued)

Patient number	105AD7/	Time[1]	Operation[2]	Site [2]	Residual disease
18	Placebo	222	Laparotomy	-	macro
20	Placebo	254	bypass	ileocaecum	macro
21*	Placebo	725	ileostomy	-	macro
23	Placebo	322	excision	skin nodule	unknown
28	Placebo	470	excision	perineal nod	micro
41	Placebo	635	exc loc rec	anastomosis	unknown
45	Placebo	809	colostomy	rectum	unknown
45	Placebo	450	excision	perineal rec ca	macro
49	Placebo	601	exc loc rec	anastomosis	macro
49	Placebo	74	resection	small bowel	macro
53	Placebo	279	resection	liver	none
54	Placebo	1354	sub tot col	colon	none
56	Placebo	61	transv col	colon	macro
57	Placebo	278	rev col	-	macro
59	Placebo	69	Hartmann's	colon	none
60	Placebo	692	exc loc res	anastomosis	unknown
62	Placebo	402	resection	pelvic mass	macroscopi
69	Placebo	1100	resection	liver	none
69	Placebo	890	debulking op	rect+uterus+ omentum+liv	unknown
78	Placebo	1202	resection	right liver	none
88	Placebo	638	colectomy	colon	unknown
93	Placebo	69	laparotomy	unknown	macro
94	Placebo	126	laparotomy	smal bowel res	macro
95	Placebo	351	tran col.	-	none
97	Placebo	45	bypass	unknown	micro
100	Placebo	347	rev col	-	unknown
106	Placebo	1246	excision	wound rec	none
106	Placebo	1127	excision	rec nod groin	none
107*	Placebo	104	il trans byp	colon	macro
115	Placebo	49	laparotomy	-	macro
135	Placebo	171	colostomy	colon	macro
141*	Placebo	84	colostomy	colon	macro
160	Placebo	491	resection	right liver	none

1 Time from surgery to entry into the study.

2
exc loc rec	excision of local recurrence
sub tot col	subtotal colectomy
transv col	transverse colostomy
rev col	reversal of colostomy
il transv byp	ileotransverse bypass
rec ca	recurrent carcinoma
nod	nodule

Table 1-4 Radiotherapy prior to diagnosis of advanced disease

Radiotherapy prior to diagnosis of advanced disease		Treatment 105AD7	Placebo
n		10	10
Time since start of radiotherapy to entry into the study (in days)	Mean (sd) Median Min Max	353.7 (330.3) 178.5 125 1107	823.8 (495.5) 678 307 1946
Time since end of radiotherapy to entry into the study (in days)	Mean (sd) Median Min Max n	315.6 (330.9) 144.5 89 1076 10	784.1 (500.2) 641.0 264 1916 10
Length of radiotherapy (in days)	Mean (sd) Median Min Max n	38.1 (17.6) 34 10 72 10	39.7 (10.4) 37 30 61 10
Site of Radiotherapy	Left iliac fossa Pelvis	0 10 (100%)	1 (10%) 9 (90%)
Total Dose (cGy)	2 40 45 133 300 4500 5000 missing	1 (10%) 1 (10%) 0 1 (10%) 1 (10%) 1 (10%) 1 (10%) 4 (40%)	0 0 1 (10%) 0 0 1 (10%) 0 8 (80%)
Type of Radiotherapy	Radical Palliative Unknown	3 (30%) 5 (50%) 2 (20%)	4 (40%) 2 (20%) 4 (40%)
Response	CR PR NC PD NE NK	0 0 1 (10%) 3 (30%) 3 (30%) 3 (30%)	1 (10%) 1 (10%) 0 2 (20%) 2 (20%) 4 (40%)

Table 1-5 Any subsequent courses of radiotherapy prior to diagnosis of advanced disease

Pt no.	105AD7/ Placebo	Time[1]	Time[2]	Length of RTH	Site	Response
45	Placebo	140	110	30	Perineum	Not evaluable

[1] Time since start of radiotherapy (RTH) to entry into the study

[2] Time since end of radiotherapy to entry into the study

Table 1-6 Radiotherapy where date of diagnosis of advanced disease is not known

Pt no.	105AD7/ Placebo	Time[1]	Time[2]	Length of RTH	Site	Response
18	Placebo	194	163	31	Pelvis	Not Evaluable
28	Placebo	259	238	21	Perineum	Not Evaluable
36	Placebo	Not known[3]	Not known[3]	Not known[3]	Pelvis	Not Evaluable
158	105AD7	Not known[4]	73	Not known[4]	Pelvis	Not Known

[1] Time since start of radiotherapy to entry into the study

[2] Time since end of radiotherapy to entry into the study

[3] Day and month of start and end of radiotherapy not known

[4] Start of radiotherapy not known

Table 1-7 Radiotherapy where date of start of radiotherapy is not known

Patient No.	105AD7/ Placebo	Time[1]	Time[2,3]	Length of RTH	Site	Response
5	Placebo	Not known	Not known	Not known	Not known	Not Known
116	105AD7	Not known	Not known	Not known	Pelvis	Not Known
153	Placebo	Not known	Not known	Not known	Pelvis	Not Known

[1] Time since start of radiotherapy to entry into the study

[2] Time since end of radiotherapy to entry into the study

[3] Date of end of radiotherapy not known for all patients

Table 1-8 Radiotherapy after diagnosis of advanced disease

Radiotherapy after diagnosis of advanced disease		Treatment 105AD7	Placebo
n		13	13
Time since start of radiotherapy to entry into the study (in days)	Mean (sd) Median Min Max n	226.3 (160.1) 188 63 609 13	370.1 (268.4) 478 48 748 13
Time since end of radiotherapy to entry into the study (in days)	Mean (sd) Median Min Max n	215.7 (158.5) 163.5 77 581 12	326.7 (275.7) 168.0 27 716 11
Length of radiotherapy (in days)	Mean (sd) Median Min Max n	24.2 (16.4) 28 0 61 12	52.9 (108.3) 30 0 377 11
Site of Radiotherapy	Pelvis Lung Right femur	11 (85%) 1 (8%) 1 (8%)	12 (92%) 1 (8%) 0
Total Dose (cGy)	8 45 450 1770 2550 3000 3250 3500 4000 4500 5350 missing	1 (8%) 0 0 0 1 (8%) 1 (8%) 0 0 1 (8%) 2 (15%) 1 (8%) 6 (46%)	0 2 (15%) 1 (8%) 1 (8%) 0 1 (8%) 1 (8%) 1 (8%) 0 1 (8%) 0 5 (38%)
Type of Radiotherapy	Radical Palliative Adjuvant Unknown	3 (23%) 10 (77%) 0 0	2 (15%) 9 (69%) 1 (8%) 1 (8%)
Response	NA PR PD NE Not Known	1 (8%) 1 (8%) 2 (15%) 6 (46%) 3 (23%)	0 1 (8%) 2 (15%) 2 (15%) 8 (61%)

Table 1-9 Any subsequent courses of radiotherapy after diagnosis of advanced disease

Pt no.	105AD7/ Placebo	Time[1]	Time[2]	Length of RTH	Site	Response
162	105AD7	244	244	0	Abdo wall	NK
162	105AD7	184	184	0	Pelvis	NK
31	Placebo	118	104	14	Pelvis	NE

[1] Time since start of radiotherapy to entry into the study

[2] Time since end of radiotherapy to entry into the study

Table 1-10 Hormone/immuno/biological treatment prior to diagnosis of advanced disease

Hormone/immuno biological treatment prior to diagnosis of advanced disease		Treatment 105AD7 Placebo
n		none

Table 1-11 Hormone/immuno/biological treatment after diagnosis of advanced disease

Hormone/immuno /biological treatment after diagnosis of advanced disease		Treatment 105AD7	Placebo
n		2	4
Time since start of treatment to entry into the study (in days)	Mean (sd) Median Min Max n	588 (268.7) 588 398 778 2	694 (418.6) 742.5 198 1093 4
Time since end of treatment to entry into the study (in days)	Mean (sd) Median Min Max n	507 (250.3) 507 330 684 2	591 (350.8) 651 137 925 4
Length of treatment (in days)	Mean (sd) Median Min Max n	81 (18.4) 81 68 94 2	103 (87.6) 114.5 0 183 4
Treatment	Interferon Interleukin 2 Investigational	1 (50%) 0 1 (50%)	2 (50%) 1 (25%) 1 (25%)
Dose schedule	3 courses 3 inj. dose 6 million units Not Known	1 (50%) 0 0 1 (50%)	0 1 (25%) 1 (25%) 2 (50%)
Response	PR PD NK	0 1 (50%) 1 (50%)	1 (25%) 2 (50%) 1 (25%)

Table 1-12 Any subsequent hormone/immuno/biological treatment after diagnosis of advanced disease

Pt No.	Treatment group	Time[1]	Time[2]	Length of treatment	Treatment	Response
69	Placebo	1093	925	168	Interferon	PR

[1] Time since start of hormone/immuno/biological treatment to entry into the study

[2] Time since end of hormone/immuno/biological treatment to entry into the study

Table 1-13 Hormone/immuno/biological treatment where start date is not known

Pt no.	105AD7/ Placebo	Time[1]	Time[2,3]	Length of treatment	Treatment	Response
59	Placebo	not known	not known	not known	Not known	NK

[1.] Time since start of hormone/immuno/biological treatment to entry into the study.

[2.] Time since end of hormone/immuno/biological treatment to entry into the study.

[3.] Date of end of hormone/immuno/biological treatment not known.

Table 1-14 Chemotherapy prior to diagnosis of advanced disease

Chemotherapy prior to diagnosis of advanced disease		Treatment 105AD7	Placebo
n		11	13
Time since start of chemotherapy to entry into the study (in days)	Mean (sd) Median Min Max n	463.6 (302.6) 376 125 1076 11	518.2 (309.6) 375 193 1080 13
Time since end of chemotherapy to entry into the study (in days)	Mean (sd) Median Min Max n	334.5 (233.9) 290 89 711 11	412.2 (341.4) 283 117 1045 13
Length of chemotherapy (in days)	Mean (sd) Median Min Max n	129.1 (100.2) 105 4 365 11	105.9 (57.9) 92 0 197 13
Chemotherapy[1]	5-FU 5-FU+FA 5-FU+Leuc 5-FU+Levam Invest	5 (45%) 4 (36%) 1 (9%) 1 (9%) 0	5 (38%) 6 (46%) 0 1 (8%) 1 (8%)
No of courses	1 2 3 4 5 6 52 missing	1 (9%) 0 1 (9%) 0 0 6 (55%) 1 (9%) 2 (18%)	1 (8%) 2 (15%) 1 (8%) 2 (15%) 1 (8%) 5 (38%) 0 1 (8%)
Response	CR NC PD NE NK	0 2 (18%) 5 (45%) 2 (18%) 2 (18%)	1 (8%) 0 5 (38%) 5 (38%) 2 (15%)

[1]Leuc=Leucovorin, Levam=Levamisole, Invest=Investigational drug

Table 1-15 Any subsequent courses of chemotherapy, prior to diagnosis of advanced disease

Pt no.	105AD7/ Placebo	Time[1]	Time[2]	Length of chemo.	Chemo agent.[3]	Response
11	105AD7	85	84	1	5-FU+FA	NE
130	105AD7	571	448	123	5-FU+FA	PD
45	Placebo	291	171	120	Mitomycin C	PD
78	Placebo	1045	912	133	5-FU+Levam	CR

Table 1-16 Chemotherapy where date of diagnosis of advanced disease is not known

Pt no.	105AD7/ Placebo	Time[1]	Time[2]	Length of chemo.	Chemo agent.[3]	Response
36	Placebo	268	118	150	5-FU+FA	NC
127	Placebo	1036	700	336	5-FU+Levam	NK
158	105AD7	NK	NK	NK	FA	PD
158	105AD7	NK	NK	NK	5-FU	PD
158	105AD7	NK	94	NK	Raltitrexed	NC

Table 1-17 Chemotherapy where date of start of chemotherapy is not known

Pt no.	Treatment	Time[1]	Time[2]	Length of chemo.	Chemotherapy	Response
59	Placebo	NK	NK	NK	Unspecified	NK
37	105AD7	NK	77	NK	Mitomycin C	NK
124	105AD7	NK	NK	NK	Fluorouracil	NK
124	105AD7	NK	NK	NK	Folinic acid	NK

1 Time since start of chemotherapy to entry into the study
2 Time since end of chemotherapy to entry into the study
3 Levam = Levamisole.

Table 1-18 Chemotherapy after diagnosis of advanced disease

Chemotherapy after diagnosis of advanced disease		Treatment 105AD7	Placebo
n		30	21
Time since start of chemotherapy to entry into the study (in days)	Mean (sd) Median Min Max n	368.7 (211.1) 291.5 108 888 30	443.8 (280.3) 357 157 1093 21
Time since end of chemotherapy to entry into the study (in days)	Mean (sd) Median Min Max n	247.4 (188.3) 161 84 698 29	309.8 (251.2) 174 91 925 21
Length of chemotherapy (in days)	Mean (sd) Median Min Max n	109.0 (82.4) 90 21 402 29	134.0 (128.3) 105 28 640 21
Chemotherapy[1]	Doxorubicin 5-FU 5-FU+FA FA+Cisp+5-FU Investigational Raltitrexed	1 (3%) 4 (13%) 21 (71%) 1 (3%) 1 (3%) 2 (7%)	0 5 (24%) 15 (71%) 0 1 (5%) 0
No of courses	1-5 6-10 12 13 14 18 22 missing	12 (39%) 10 (33%) 0 1 (3%) 0 1 (3%) 0 6 (20%)	6 (24%) 7 (34%) 1 (5%) 0 1 (5%) 0 1 (5%) 5 (24%)
Response	PR NC PD NE NK	1 (3%) 3 (10%) 18 (60%) 1 (3%) 7 (23%)	1 (5%) 4 (19%) 8 (38%) 2 (10%) 6 (29%)

1 Investigational=Investigational drug; Cis=Cisplatin.

Table 1-19 Any subsequent courses of chemotherapy, after diagnosis of advanced disease

Pt no.	105AD7/ Placebo	Time[1]	Time[2]	Length of chemo.	Chemo agent.[3]	Response
37	105AD7	520	422	98	Zilascorb	NK
37	105AD7	419	NK	NK	5-FU	NK
39	105AD7	552	475	77	Invest	NK
111	105AD7	369	369	0	Mitomycin C	PD
119	105AD7	190	146	44	Lometrexol	PD
159	105AD7	176	92	84	5-FU+FA	NK
31	Placebo	366	NK	NK	5-FU+ Epir+ BCNU+FA	PD
41	Placebo	270	150	120	5-FU	NK
62	Placebo	235	119	116	5-FU+FA	PD
69	Placebo	862	452	410	5-FU+Leuc	CR
160	Placebo	226	117	109	5-FU+FA	PD

1 Time since start of chemotherapy to entry into the study
2 Time since end of chemotherapy to entry into the study
3 Leuc= Leucovorin, Epir=Epirubicin, NK=Not known.

Table 1-20 Off study treatment for malignant disease

Pt no.	105AD7/ Placebo	Treatment	Information	Time[2]	Indication
14	Placebo	Radiotherapy	Pelvis	574	Malignant disease
60	Placebo	Radiotherapy	Palliative	235	Malignant disease
70	Placebo	Surgery	-	135	Small bowel obstruction
101	Placebo	Chemotherapy	5-FU Bolus[1]	133	Malignant disease
102	Placebo	Chemotherapy	5-FU+FA[1]	159	Malignant disease
115	Placebo	Radiotherapy	Pelvis	118	Pain
155	Placebo	Radiotherapy	Para-aortic	41	Malignant disease
51	105AD7	Surgery	-	121	Intestinal obstruction
68	105AD7	Surgery	Sympathectomy	NK	NK
98	105AD7	Chemotherapy	5-FU+FA[1]	153	Malignant disease

1 NK= Not known, FA= Folinic acid (leucovorin).

2 Time since start of study (in days)

Table 1-21 Results of protocol investigations at time of entry to study and week 12

		Entry to study		Week 12	
		105AD7	Placebo	105AD7	Placebo
Chest X-ray	Normal	38 (48%)	25 (33%)	9 (29%)	16 (48%)
	Abnormal	28 (35%)	32 (42%)	16 (52%)	11 (33%)
	Not Done	13 (16%)	19 (25%)	6 (19%)	6 (18%)
	missing	6	1	54	44
CT scan	Abnormal	26 (37%)	23 (34%)	2 (7%)	6 (21%)
	Not Done	44 (63%)	45 (66%)	27 (93%)	23 (79%)
	missing	15	9	56	48

Table 1-22 Patient status

			Treatment	
			105AD7	Placebo
Weight (kg)	Week 0	Mean (sd)	66.9 (12.5)	69.0 (16.0)
		Median	66.3	67.0
		Min	38	40.3
		Max	97.2	105.4
		n	77	73
	Week 6	Mean (sd)	68.8 (12.2)	69.1 (15.7)
		Median	67.4	67.9
		Min	40	38.1
		Max	97.1	106
		n	48	51
	Week 12	Mean (sd)	67.6 (13.0)	69.9 (16.1)
		Median	66.6	68.6
		Min	42	44.2
		Max	94.3	104
		n	39	34
Height (cm)	Week 0	Mean (sd)	168.1 (8.9)	167.3 (9.1)
		Median	167.4	167.5
		Min	147	151
		Max	190	191
		n	76	68
Surface Area (mg/m^2)	Week 0	Mean (sd)	1.74 (0.18)	1.74 (0.25)
		Median	1.74	1.70
		Min	1.35	1.22
		Max	2.10	2.19
		n	24	28

Table 6 Patient status (continued)

			Treatment	
			105AD7	Placebo
WHO Performance status	Week 0	0	28 (33%)	20 (26%)
		1	43 (51%)	47 (61%)
		2	14 (16%)	10 (13%)
	Week 6	0	23 (42%)	13 (25%)
		1	22 (40%)	30 (57%)
		2	8 (15%)	9 (17%)
		3	2 (4%)	1 (2%)
		4	0	0
		missing	30	24
	Week 12	0	19 (49%)	9 (23%)
		1	16 (41%)	24 (62%)
		2	4 (10%)	5 (13%)
		3	0	0
		4	0	1 (3%)
		missing	46	38

Table 1-23 Haematology

		Week 0	
		105AD7	Placebo
WBC ($x10^9/l$)	Mean (sd) Median n	9.20 (4.27) 7.6 85	9.24 (4.73) 8.6 74
Platelets ($x10^9/l$)	Mean (sd) Median n	332.4 (158.6) 286 85	333.3 (117.9) 314 74
Haemoglobin (xg/dl)	Mean (sd) Median n	11.89 (1.69) 11.8 84	11.81 (1.84) 11.8 74
Neutrophils ($x10^9/l$)	Mean (sd) Median n	6.77 (3.74) 5.59 83	7.02 (4.47) 6.11 74
Lymphocytes ($x10^9/l$)	Mean (sd) Median n	1.35 (0.57) 1.27 84	1.36 (0.63) 1.20 74

		Week 6	
		105AD7	Placebo
WBC ($x10^9/l$)	Mean (sd) Median n	8.42 (3.53) 7.45 62	9.37 (3.74) 8.6 57
Platelets ($x10^9/l$)	Mean (sd) Median n	292.7 (118.2) 271.5 62	339.3 (114.7) 319.0 57
Haemoglobin (xg/dl)	Mean (sd) Median n	11.92 (1.78) 12.3 62	11.58 (1.99) 11.9 57
Neutrophils ($x10^9/l$)	Mean (sd) Median n	6.18 (3.44) 5.21 62	7.11 (3.54) 5.88 57
Lymphocytes ($x10^9/l$)	Mean (sd) Median n	1.45 (0.69) 1.30 62	1.42 (0.65) 1.26 57

Table 1-23 Haematology Results

		Week 12	
		105AD7	Placebo
WBC ($x10^9/l$)	Mean (sd) Median n	8.49 (3.44) 7.3 47	8.88 (2.77) 8.4 44
Platelets ($x10^9/l$)	Mean (sd) Median n	285.7 (106.3) 265.0 47	335.6 (141.4) 316.5 44
Haemoglobin (xg/dl)	Mean (sd) Median n	12.26 (1.78) 12.8 47	11.88 (1.86) 12.05 44
Neutrophils ($x10^9/l$)	Mean (sd) Median n	6.26 (3.38) 5.00 47	6.35 (2.53) 5.88 44
Lymphocytes ($x10^9/l$)	Mean (sd) Median n	1.44 (0.62) 1.40 47	1.47 (0.64) 1.33 44

Table 1-24 Chemical Pathology

		Week 0	
		105AD7	Placebo
Na (mmol/l)	Mean (sd) Median n	137.9 (4.0) 138 83	138.3 (3.4) 139 75
K (mmol/l)	Mean (sd) Median n	4.44 (0.43) 4.4 82	4.27 (0.47) 4.2 75
Urea (mmol/l)	Mean (sd) Median n	5.57 (3.22) 4.8 83	6.01 (3.91) 5.1 76
Creatinine (mmol/l)	Mean (sd) Median n	85.1 (34.6) 79 83	91.4 (72.1) 79 75
Total Protein (g/l)	Mean (sd) Median n	69.1 (6.3) 69.5 12	70.6 (8.3) 70 12
Albumin (g/l)	Mean (sd) Median n	37.9 (4.6) 38 84	38.3 (5.7) 39 76
Bilirubin (mmol/l)	Mean (sd) Median n	20.4 (59.7) 10 84	14.9 (31.7) 10 76

Table 1-24 Chemical Pathology (continued).

		Week 0	
		105AD7	Placebo
Alk Phos (IU/l)	Mean (sd) Median n	303.2 (388.0) 149 83	260.0 (273.4) 158.5 76
ALT (IU/l)	Mean (sd) Median n	39.2 (39.8) 29.5 84	39.5 (30.4) 32 76
AST (IU/l)	Mean (sd) Median n	26.7 (12.4) 23.5 10	30.0 (22.2) 20.5 6
Urate (mmol/l)	Mean (sd) Median n	0.33 (0.06) 0.32 6	0.34 (0.12) 0.32 10
GGT (IU/l)	Mean (sd) Median n	240.5 (308.5) 104 75	233.1 (307.9) 105 71
CEA (mg/l)	Mean (sd) Median n	669.2 (1135.5) 134.5 80	475.4 (1174.8) 61 70

Table 1-24 Chemical Pathology (continued)

		Week 6	
		105AD7	Placebo
Na (mmol/l)	Mean (sd) Median n	138.4 (3.9) 139 60	138.2 (3.2) 139 56
K (mmol/l)	Mean (sd) Median n	4.36 (0.39) 4.4 60	4.34 (0.50) 4.2 55
Urea (mmol/l)	Mean (sd) Median n	5.41 (2.37) 4.75 60	5.62 (2.50) 5.1 56
Creatinine (mmol/l)	Mean (sd) Median n	90.8 (36.1) 79.5 60	85.4 (28.4) 79.5 56
Total Protein (g/l)	Mean (sd) Median n	68.8 (7.1) 67 9	69.7 (7.4) 71 10
Albumin (g/l)	Mean (sd) Median n	38.4 (4.2) 39 60	38.4 (5.4) 39 56
Bilirubin (mmol/l)	Mean (sd) Median n	13.6 (13.5) 10 60	12.9 (14.9) 9.5 56

Table 1-24 Chemical Pathology (continued)

		Week 6	
		105AD7	Placebo
Alk Phos (IU/l)	Mean (sd) Median n	270.6 (333.8) 145.5 60	238.6 (232.7) 151 56
ALT (IU/l)	Mean (sd) Median n	32.2 (21.1) 26.5 60	35.2 (23.4) 26.5 56
AST (IU/l)	Mean (sd) Median n	30.6 (14.3) 27 11	35.4 (30.5) 22.5 8
Urate (mmol/l)	Mean (sd) Median n	0.34 (0.07) 0.33 8	0.36 (0.15) 0.34 7
GGT (IU/l)	Mean (sd) Median n	218.9 (301.1) 79 55	243.5 (362.9) 93 52
CEA (mg/l)	Mean (sd) Median n	881.1 (1325.8) 181.5 56	341.6 (554.4) 91.5 52

Table 1-25 Adverse events- non-serious.

		Treatment	
		105AD7	Placebo
n		57	30
Highest CTC Grade	1 2 3 4 missing	26 (47%) 15 (27%) 9 (16%) 5 (9%) 2	11 (38%) 13 (45%) 5 (17%) 0 1
Relation to Study drug	Almost certainly Possibly Unlikely	1 (2%) 9 (16%) 47 (82%)	0 8 (27%) 22 (73%)
Outcome	Resolved Improved Unchanged Worse	20 (35%) 2 (4%) 29 (51%) 6 (11%)	11 (37%) 3 (10%) 15 (50%) 1 (3%)
Time from entry into the study & start of adverse event (days)	Mean (sd) Median Minimum Maximum n Missing	28.2 (29.9) 22 0 120 46 11	31.2 (28.5) 26 0 90 29 1
Duration of adverse event.	Mean (sd) Median Minimum Maximum n Missing	2.73 (2.05) 2 1 7 11 46	24.1 (29.8) 14 2 84 7 23

Table 1-25 Adverse events- Serious.

Patient number.	105AD7/ Placebo	Highest CTC grade.	Relation to study drug [1]	Outcome.	Time [2]	Time [3]
2	Placebo	3	Unlikely	Improved	26	NK
7	Placebo	3	Unlikely	Unchanged	NK	NK
22	105AD7	3	Unlikely	Unchanged	9	NK

1. Excludes all adverse events classified by investigators as "unrelated" to the study drug.

2. Time to onset (days).

3. Time at which adverse event ceased (if applicable).

Table 2-1. Immunohistochemistry Results CD25.

Patient No	Site [1]	Trial Abs [2]	Con Abs	Trial T/N [3]	Con T/N
1. DT	Centre	0.59	0.80	0.62	1.13
	Edge	0.52	0.30	0.55	0.42
2. EN	Centre	0.89	0.05	0.93	0.10
	Edge 1	0.74	0.07	0.77	0.13
	Edge 2	0.95	0.08	0.99	0.16
3.ER	Centre	0.218	0.93	0.68	0.34
	Edge	0.50	0.13	1.55	0.05
5. JGr	Centre	0.28	0.61	0.45	2.00
	Edge 1	0.24	0.59	0.38	1.93
	Edge 2	0.11	0.34	0.18	1.12
6. ET	Centre	1.33	0.64	0.86	1.07
	Edge	1.62	1.20	1.05	2.00
8.MB	Centre	0.40	1.53	0.62	1.09
	Edge 1	0.43	1.33	0.66	0.97
	Edge 2	0.71	0.21	1.08	0.15
9. DH	Centre	1.45	0.14	1.80	0.27
	Edge 1	1.00	0.26	1.25	0.44
	Edge 2	0.51	0.11	0.63	0.21
11. JK	Centre	0.44	0.79	3.01	0.42
	Edge 1	0.92	1.48	6.33	0.79
	Edge 2	1.69	0.78	11.59	0.41
12. JGa	Centre	0.33	1.50	1.59	0.99
	Edge 1	0.11	0.15	0.51	0.10
	Edge 2	0.79	0.11	3.74	0.21

13. TB	Centre	0.40	1.24	0.70	1.06
	Edge 1	0.77	1.29	1.34	1.10
15.FCl	Centre	2.14	2.36	1.40	15.67
	Edge 1	1.91	0.94	1.24	5.79
17.FHa	Centre	0.98	0.51	1.38	1.38
	Edge 1	1.20	0.14	1.70	0.39
18.FCh	Centre	1.46	0.79	1.56	0.41
	Edge 1	1.08	0.32	1.15	0.16
	Edge 2	1.96	1.32	2.09	0.68
A. [4]	Centre.	-	0.45	-	-
AC	Edge.	1.46	0.56	1.08	-
B. [4]	Centre.	1.87	0.08	3.22	0.16
HS	Edge 1.	0.76	0.20	1.31	0.40
	Edge 2.	0.47	0.14	0.80	0.28
C. [4]	Centre	0.14	0.18	0.32	-
AGe	Edge 1.	0.08	0.48	0.18	-
	Edge 2.	0.10	0.33	0.23	-

1 Site of tumour specimen was taken from.

2 Absolute infiltration of lymphocytes into tumours from trial patients who had received 105AD7, and their matched controls.

3 Ratios of tumour edge and centre lymphocyte infiltration, to that of infiltration into normal bowel.

4 Specimens from patients immunised by the previous CRC Fellow.

Table 2-2 Immunohistochemistry results - CD4

Patient No	Site [1]	Trial Abs [2]	Con Abs	Trial T/N [3]	Con T/N
1. DT	Centre	1.12	0.62	0.47	1.17
	Edge	2.48	1.00	1.04	1.88
2. EN	Centre	1.33	0.16	1.04	0.93
	Edge 1	0.56	0.20	0.44	1.13
	Edge 2	1.89	0.27	1.48	1.53
3.ER	Centre	1.20	1.17	1.38	0.52
	Edge	0.78	0.81	0.99	0.36
5. JGr	Centre	0.57	0..75	0.45	0.19
	Edge 1	0.37	0.83	0.29	0.22
	Edge 2	0.19	1.25	0.15	0.32
6. ET	Centre	0.92	0.19	0.59	0.34
	Edge	2.00	0.33	1.25	0.60
8.MB	Centre	1.20	1.44	2.13	1.25
	Edge 1	0.59	0.26	1.04	0.23
	Edge 2	0.59	1.30	1.05	1.12
9. DH	Centre	1.87	1.35	1.95	2.05
	Edge 1	0.78	0.68	0.82	1.02
	Edge 2	0.66	0.58	0.69	0.88
11. JK	Centre	0.99	2.43	0.91	1.04
	Edge 1	1..57	2.84	1.44	1.21
	Edge 2	0.75	1.83	0.69	0.78
12. JGa	Centre	1.13	0.56	1.11	1.04
	Edge 1	1.02	0.42	1.00	0.78
	Edge 2	1.22	0.58	1.19	1.08

13. TB	Centre	0.94	1.41	0.88	0.88
	Edge 1	1.20	2.42	1.14	1.52
15.FCl	Centre	2.26	2.25	1.52	5.41
	Edge 1	2.20	2.28	1,48	5.47
17.FHa	Centre	1.54	0.15	1.17	0.23
	Edge 1	1.79	0.08	1.35	0.12
18.FCh	Centre	2.16	1.04	2.34	0.72
	Edge 1	1.86	1.46	2.02	1.01
	Edge 2	3.30	1.42	3.58	0.98
A [4]	Centre.	-	0.96	-	0.73
AC	Edge.	1.49	1.20	1.01	0.91
B [4]	Centre.	2.18	0.44	2.13	0.80
HS	Edge 1.	0.95	0.27	0.93	0.49
	Edge 2.	1.98	0.09	1.93	0.18
C [4]	Centre.	0.77	0.79	0.69	-
AGe	Edge 1.	0.58	0.20	0.52	-
	Edge 2.	0.10	1.63	0.23	-

1 Site of tumour specimen was taken from.

2 Absolute infiltration of lymphocytes into tumours from trial patients who had received 105AD7, and their matched controls.

3 Ratios of tumour edge and centre lymphocyte infiltration, to that of infiltration into normal bowel.

4 Specimens from patients immunised by the previous CRC Fellow.

Table 2-3 Immunohistochemistry results - CD 8

Patient No	Site [1]	Trial Abs [2]	Con Abs	Trial T/N [3]	Con T/N
1. DT	Centre	1.17	0.18	0.92	0.60
	Edge	0.61	0.21	0.43	0.71
2. EN	Centre	0.91	0.12	-	0.45
	Edge 1	2.25	0.10	-	0.38
	Edge 2	1.10	0.29	-	1.11
3. ER	Centre	0.17	0.24	0.22	0.11
	Edge	0.58	0.07	0.74	0.03
5. JGr	Centre	0.24	0.47	0.38	1.79
	Edge 1	0.32	0.40	0.51	1.54
	Edge 2	0.17	0.04	0.28	0.15
6. ET	Centre	0.92	0.81	1.15	0.16
	Edge	1.57	0.58	1.96	0.11
8. MB	Centre	0.33	2.47	3.34	3.09
	Edge 1	0.45	1.13	4.46	1.42
	Edge 2	0.68	2.04	6.82	2.57
9. DH	Centre	0.69	0.69	0.88	0.50
	Edge 1	0.69	0.61	0.89	0.45
	Edge 2	0.81	0.24	1.03	0.18
11. JK	Centre	0.27	1.38	0.42	0.58
	Edge 1	0.74	1.54	1.12	0.65
	Edge 2	0.99	1.25	1.51	0.53
12. JGa	Centre	0.46	1.48	0.43	1.52
	Edge 1	0.76	1.06	0.70	1.09
	Edge 2	1.12	1.01	1.04	1.04

13. TB	Centre	1.39	1.02	1.00	0.63
	Edge 1	0.39	1.34	0.28	0.82
15. FCl	Centre	2.33	1.45	1.13	1.55
	Edge 1	1.86	0.98	0.91	1.04
17. FHa	Centre	0.91	0.26	0.63	0.49
	Edge 1	0.85	0.26	0.59	0.48
18. FCh	Centre	0.81	1.39	0.47	1.30
	Edge 1	1.41	1.96	0.81	1.83
	Edge 2	1.15	2.03	0.66	1.90
A [4]	Centre.	-	-	-	-
AC	Edge.	1.46	0.94	10.81	1.47
B [4]	Centre.	1.85	0.03	4.14	0.04
HS	Edge 1.	1.53	0.23	3.41	0.30
	Edge 2.	0.84	0.03	1.88	0.04
C [4]	Centre.	0.10	1.82	0.40	-
AGe	Edge 1.	0.52	0.44	2.06	-
	Edge 2.	0..60	1.27	2.38	

1 Site of tumour specimen was taken from.

2 Absolute infiltration of lymphocytes into tumours from trial patients who had received 105AD7, and their matched controls.

3 Ratios of tumour edge and centre lymphocyte infiltration, to that of infiltration into normal bowel.

4 Specimens from patients immunised by the previous CRC Fellow.

Table 2-4 Immunohistochemistry results - CD 56

Patient No	Site [1]	Trial Abs [2]	Con Abs	Trial T/N [3]	Con T/N
1. DT	Centre	0.79	0.76	0.28	0.53
	Edge	1.32	0.40	0.46	0.28
2. EN	Centre	1.23	0.04	1.71	0.07
	Edge 1	0.45	0.06	0.63	0.10
	Edge 2	-	0.48	-	0.80
3. ER	Centre	0.12	0.60	0.08	0.22
	Edge	0.68	0.84	0.46	0.31
5. JGr	Centre	0.19	0.26	0.33	0.09
	Edge 1	0.29	0.92	0.51	0.33
	Edge 2	0.10	0.81	0.17	0.29
6. ET	Centre	0.22	0.09	0.09	0.07
	Edge	2.93	0.41	1.16	0.32
8. MB	Centre	0.92	1.73	6.45	1.01
	Edge 1	0.92	0.17	6.48	0.10
	Edge 2	0.73	0.43	5.11	0.25
9. DH	Centre	0.84	0.04	1.09	0.12
	Edge 1	0.61	0.14	0.79	0.41
	Edge 2	1.17	0.13	1.51	0.40
11. JK	Centre	0.44	2.56	0.22	1.03
	Edge 1	0.77	1.76	0.38	0.71
	Edge 2	1.23	1.02	0.60	0.41
12. JGa	Centre	0.99	0.44	0.46	4.42
	Edge 1	0.65	0.22	0.31	2.22
	Edge 2	1.27	0.33	0.59	3.32

13. TB	Centre	0.55	1.65	0.43	1.37
	Edge 1	2.18	1.69	1.69	1.41
15. FCl	Centre	4.28	1.34	1.64	1.28
	Edge 1	2.43	1.99	0.94	1.89
17. FHa	Centre	1.41	0.18	0.88	0.36
	Edge 1	2.23	0.15	1.39	0.31
18. FCh	Centre	1.78	1.24	2.44	1.19
	Edge 1	0.96	1.43	1.32	1.36
	Edge 2	0.51	1.45	0.70	1.38
A [4]	Centre.	-	2.12	-	1.57
AC	Edge.	-	0.62	-	0.46
B [4]	Centre.	-	0.15	-	0.08
HS	Edge 1.	-	0.16	-	0.09
	Edge 2.	-	0.23	-	0.13
C [4]	Centre.	0.03	0.25	0.03	-
AGe	Edge 1.	0.63	0.54	0.56	-
	Edge 2.	-	0.42	-	-

1 Site of tumour specimen was taken from.

2 Absolute infiltration of lymphocytes into tumours from trial patients who had received 105AD7, and their matched controls.

3 Ratios of tumour edge and centre lymphocyte infiltration, to that of infiltration into normal bowel.

4 Specimens from patients immunised by the previous CRC Fellow.

Immunohistochemistry results - ζ chain expression.

Patient No	Site [1]	Trial Abs [2]	Con Abs	Trial T/N [3]	Con T/N
2. EN	Centre	0.40	0.49	0.45	0.56
	Edge 1	0.44	0.89	0.50	1.02
	Edge 2	1.63	0.44	1.85	0.51
3. ER	Centre	0.50	0.89	0.24	0.89
	Edge	0.96	0.81	0.47	0.81
5. JGr	Centre	2.36	1.91	7.47	0.70
	Edge 1	2.12	0.81	6.70	0.30
	Edge 2	0.34	-	1.08	-
6. ET	Centre	0.76	0.42	0.70	0.48
	Edge	0.59	0.09	0.54	0.10
8. MB	Centre	0.36	0.55	0.63	0.49
	Edge 1	0.45	0.26	0.78	0.24
	Edge 2	0.18	0.22	0.32	0.19
9. DH	Centre	0.12	-	0.14	-
	Edge 1	0.45	0.94	0.51	0.98
	Edge 2	0.30	0.59	0.34	0.61
11. JK	Centre	0.22	0.61	0.11	0.38
	Edge 1	0.55	0.37	0.28	0.22
	Edge 2	-	1.30	-	0.80
13. TB	Centre	0.35	1.37	0.41	1.07
	Edge 1	0.59	1.09	0.68	0.85
15. FCl	Centre	0.58	0.23	0.57	0.16
	Edge 1	0.42	0.33	0.41	0.24

17. FHa	Centre	0.31	0.97	0.30	0.50
	Edge 1	0.56	1.01	0.55	0.52
18. FCh	Centre	1.19	0.35	0.59	0.25
	Edge 1	0.86	0.80	0.43	0.57
	Edge 2	2.39	0.62	1.18	0.44

1 Site of tumour specimen was taken from.

2 Absolute infiltration of lymphocytes into tumours from trial patients who had received 105AD7, and their matched controls.

3 Ratios of tumour edge and centre lymphocyte infiltration, to that of infiltration into normal bowel.

Table 2-6 Immunohistochemistry results - CD 68

Patient No	Site [1]	Trial Abs [2]	Con Abs	Trial T/N [3]	Con T/N
2. EN	Centre	0.99	1.24	0.45	0.80
	Edge 1	1.26	1.17	0.57	0.76
	Edge 2	1.16	0.71	0.53	0.45
3. ER	Centre	1.06	1.70	0.72	-
	Edge	1.44	0.91	0.98	-
5. JGr	Centre	2.24	0.88	1.82	0.55
	Edge 1	1.23	2.09	0.64	1.31
	Edge 2	1.32	-	1.08	-
6. ET	Centre	0.83	0.89	1.02	0.84
	Edge	0.87	1.19	1.07	1.12
8. MB	Centre	0.69	1.02	0.89	2.12
	Edge 1	0.41	0.43	0.53	0.90
	Edge 2	0.57	0.48	0.74	1.00
9. DH	Centre	1.10	-	0.93	-
	Edge 1	0.27	0.94	0.23	0.57
	Edge 2	0.99	0.64	0.83	0.39
11. JK	Centre	1.22	2.08	0.89	0.96
	Edge 1	1.06	2.22	0.78	1.03
	Edge 2	-	1.36	-	0.63
13. TB	Centre	2.45	0.61	1.18	0.40
	Edge 1	0.57	1.05	0.27	0.69
15. FCl	Centre	1.12	1.65	0.90	2.64
	Edge 1	1.13	1.01	0.91	1.62

17. FHa	Centre	0.49	1.53	0.36	0.75
	Edge 1	0.91	1.46	0.67	0.72
18. FCh	Centre	2.45	0.68	1.45	0.62
	Edge 1	1.86	1.69	1.10	1.54
	Edge 2	1.41	0.89	0.83	0.81

1 Site of tumour specimen was taken from.

2 Absolute infiltration of lymphocytes into tumours from trial patients who had received 105AD7, and their matched controls.

3 Ratios of tumour edge and centre lymphocyte infiltration, to that of infiltration into normal bowel.

Table 2-7 Immunohistochemistry results - CD 69

Patient No	Site [1]	Trial Abs [2]	Con Abs	Trial T/N [3]	Con T/N
2. EN	Centre	0.74	0.45	1.00	0.24
	Edge 1	0.92	0.53	1.25	0.29
	Edge 2	0.90	1.21	1.21	0.66
3. ER	Centre	0.45	1.62	0.51	-
	Edge	1.40	0.84	1.59	-
5. JGr	Centre	0.33	0.74	0.26	0.45
	Edge 1	0.55	1.77	0.45	1.08
	Edge 2	0.47	-	0.38	-
6. ET	Centre	0.52	0.98	0.84	1.81
	Edge	0.31	0.52	0.50	0.96
8. MB	Centre	0.15	0.29	0.32	0.58
	Edge 1	0.14	0.64	0.29	1.29
	Edge 2	0.39	0.28	0.80	0.57
9. DH	Centre	0.49	-	0.38	-
	Edge 1	2.90	0.57	2.22	0.47
	Edge 2	0.59	0.98	0.45	0.82
11. JK	Centre	1.02	1.51	1.40	0.90
	Edge 1	0.44	1.47	0.61	0.87
	Edge 2	-	1.68	-	1.00
13. TB	Centre	1.33	1.18	0.97	1.51
	Edge 1	0.66	1.10	0.48	1.41
15. FCl	Centre	0.41	0.45	0.66	0.73
	Edge 1	0.48	0.83	0.78	1.33

17. FHa	Centre	0.64	0.65	0.66	1.44
	Edge 1	0.43	1.92	0.45	4.26
18. FCh	Centre	0.73	0.79	0.34	0.69
	Edge 1	0.47	0.40	0.28	0.35
	Edge 2	1.21	0.63	0.56	0.55

1 Site of tumour specimen was taken from.

2 Absolute infiltration of lymphocytes into tumours from trial patients who had received 105AD7, and their matched controls.

3 Ratios of tumour edge and centre lymphocyte infiltration, to that of infiltration into normal bowel.

Table 2-8 Immunohistochemistry control results. Ig G_1.

Patient No	Site [1]	Trial Abs [2]	Con Abs	Trial T/N [3]	Con T/N
2. EN	Centre	0.05	0.16	0.13	0.15
	Edge 1	0.20	0.17	0.46	0.16
	Edge 2	0.55	0.25	1.28	0.24
3. ER	Centre	0.24	0.55	0.19	0.76
	Edge	0.40	0.24	0.31	0.33
5. JGr	Centre	0.13	0.24	0.62	0.29
	Edge 1	0.36	0.81	1.71	0.98
	Edge 2	0.86	-	4.11	-
6. ET	Centre	0.11	0.06	0.46	0.26
	Edge	0.20	1.19	0.84	1.12
8. MB	Centre	0.13	0.21	0.60	0.51
	Edge 1	0.05	0.08	0.22	0.18
	Edge 2	0.07	0.02	0.32	0.05
9. DH	Centre	0.52	-	2.87	-
	Edge 1	0.19	0.15	1.05	1.06
	Edge 2	0.24	0.12	1.32	0.82
11. JK	Centre	0.08	0.48	0.59	1.23
	Edge 1	0.30	0.14	2.30	0.36
	Edge 2		0.16	-	0.41
13. TB	Centre	0.33	0.34	0.68	0.81
	Edge 1	0.28	0.21	0.57	0.49
15. FCl	Centre	0.17	0.03	0.62	0.74
	Edge 1	0.16	0.06	0.57	1.53

17. FHa	Centre	0.07	0.13	0.17	0.25
	Edge 1	0.15	0.30	0.40	0.59
18. FCh	Centre	0.81	0.06	6.80	0.09
	Edge 1	0.47	0.24	4.02	0.32
	Edge 2	0.40	0.89	3.41	1.18
A [4]	Centre	-	-	-	-
AC	Edge	-	0.01	-	0.02
B [4]	Centre	-	0.12	-	0.63
HS	Edge 1	-	0.07	-	0.37
	Edge2	-	0.09	-	0.47
C [4]	Centre	-	0.11	-	-
AGe	Edge 1	-	0.05	-	-
	Edge 2	-	0.17	-	-

1 Site of tumour specimen was taken from.

2 Absolute infiltration of lymphocytes into tumours from trial patients who had received 105AD7, and their matched controls.

3 Ratios of tumour edge and centre lymphocyte infiltration, to that of infiltration into normal bowel.

4 Patients immunised by previous CRC Fellow.

Table2-9 Immunohistochemistry control results. Tris buffered saline (TBS)

Patient No	Site[1]	Trial Abs[2]	Con Abs	Trial T/N[3]	Con T/N
2. EN	Centre	0.03	0.15	0.15	0.22
	Edge 1	0.09	0.12	0.38	0.71
	Edge 2	0.16	0.04	0.67	0.22
3. ER	Centre	0.03	0.47	10.29	0.23
	Edge	0.17	0.10	0.47	0.05
5. JGr	Centre	0.05	0.25	0.29	1.84
	Edge 1	0.63	0.20	0.92	1.48
	Edge 2	0.92	0.25	0.63	1.87
6. ET	Centre	2.07	0.04	1.85	0.77
	Edge	0.58	0.48	0.52	9.31
8. MB	Centre	0.15	0.14	1.29	0.44
	Edge 1	0.39	0.05	3.31	0.05
	Edge 2	0.13	3.00	1.10	3.00
9. DH	Centre	0.73	0.03	1.39	0.05
	Edge 1	0.68	0.02	1.30	0.03
	Edge 2	0.50	0.06	0.96	0.10
11. JK	Centre	0.03	0.03	2.29	0.26
	Edge 1	0.11	0.11	8.00	0.48
	Edge 2	0.23	0.24	17.00	0.19
13. TB	Centre	0.53	0.51	1.71	0.96
	Edge 1	0.11	0.28	0.34	1.16
15. FCl	Centre	2.22	0.88	1.63	14.7
	Edge 1	0.92	0.51	0.68	8.5

17. FHa	Centre	0.36	0.06	6.74	0.18
	Edge 1	0.64	0.15	11.89	0.44
18. FCh	Centre	1.09	0.52	2.30	0.67
	Edge 1	0.13	0.23	0.27	0.30
	Edge 2	0.43	1.07	0.90	1.39
A [4]	Centre				
AC	Edge 1	0.53	-	0.49	-
	Edge 2				
B [4]	Centre	0.18	-	0.23	-
HS	Edge 1	0.35	-	0.44	-
	Edge2	0.17	-	0.22	-
C [4]	Centre	0.03	-	0.26	-
AGe	Edge 1	0.03	-	0.26	-
	Edge 2	0.04	-	0.38	-

1 Site of tumour specimen was taken from.

2 Absolute infiltration of lymphocytes into tumours from trial patients who had received 105AD7, and their matched controls.

3 Ratios of tumour edge and centre lymphocyte infiltration, to that of infiltration into normal bowel.

4 Patients immunised by previous CRC Fellow.

Table 2-10 Immunohistochemistry APO 2.7 results. Control Ig G_1.

Patient No	Site [1]	Trial Abs [2]	Con Abs	Trial T/N [3]	Con T/N
2. EN	Centre	0.72	0.73	1.25	1.41
	Edge 1	0.98	0.89	1.72	1.73
3. ER	Centre	0.32	0.31	-	-
	Edge	1.35	1.22	-	-
6. ET	Edge	1.99	1.39	1.14	0.95
8. MB	Centre	0.37	0.37	0.74	0.82
9. DH	Centre	0.01	0.01	0.01	0.01
11. JK	Centre	0.02	0.24	0.53	0.38
	Edge 1	0.02	0.01	0.53	7.50
13. TB	Edge 1	0.02	0.02	-	-
18. FCh	Centre	0.73	0.70	0.65	0.83

1 Site of tumour specimen was taken from.

2 Absolute infiltration of lymphocytes into tumours from trial patients who had received 105AD7, and their matched controls.

3 Ratios of tumour edge and centre lymphocyte infiltration, to that of infiltration into normal bowel.

Table 2-11 Immunohistochemistry results. APO 2.7

No.	Site [1]	Trial abs infil [2]			Control abs inf			Trial T/N [3]			Control T/N		
2. EN	Centre	3.95	2.49	3.68	2.17	1.73	2.59	1.20	0.55	0.76	0.57	0.43	0.73
	Edge 1	3.51	4.12	5.65	1.67	1.88	1.77	1.06	0.91	1.17	0.44	0.46	0.50
3. ER	Centre	1.61	1.92	2.65	2.62	1.55	1.70	-	-	-	-	-	-
	Edge	3.49	3.42	3.24	1.82	2.10	1.63	-	-	-	-	-	-
6. ET	Edge	1.75	2.41	2.86	1.52	0.96	1.56	0.27	0.38	0.43	0.42	0.35	0.46
8. MB	Centre	7.66	6.93	5.66	2.14	2.62	2.22	2.16	2.24	2.00	0.59	0.97	0.72
9. DH	Centre	0.64	0.87	0.60	0.53	0.62	0.81	0.50	0.26	0.29	2.24	0.53	0.80
11. JK	Centre	2.06	1.91	1.82	2.99	2.21	3.00	0.57	0.51	0.58	0.82	0.56	0.66
	Edge 1	0.90	1.96	1.82	3.63	3.56	2.04	0.25	0.52	0.59	0.99	0.90	0.45
13. TB	Edge 1	6.32	5.23	4.51	1.65	1.77	1.76	-	-	-	-	-	-
18.FCl	Centre	2.92	2.89	2.22	0.87	0.94	1.04	0.66	0.47	0.51	0.28	0.23	0.28

1 Site of tumour specimen was taken from.

2 Absolute infiltration of lymphocytes into tumours from trial patients who had received 105AD7, and their matched controls.

3 Ratios of tumour edge and centre lymphocyte infiltration, to that of infiltration into normal bowel.

Table 2-12. Assessment of intraobserver variation.

JG. Trial pt number 12

Site	CD Ag	Abs inf [1]	Abs inf [2]	T/N [1].	T/N [2].
Edge 1.	CD56	0.65	0.78	0.31	0.71
	CD4	1.02	1.20	1.00	0.59
	CD8	0.76	1.97	0.70	0.98
	CD25	0.11	0.66	0.51	2.06
Edge 2.	CD56	1.27	1.0	0.59	0.91
	CD4	1.22	0.91	1.19	0.45
	CD8	1.12	1.17	1.04	0.58
	CD25	0.79	0.59	3.74	1.84
Centre	CD56	0.99	0.68	0.44	0.62
	CD4	1.13	1.65	1.11	0.82
	CD8	0.46	1.23	0.43	0.61
	CD25	0.33	1.24	1.59	3.88

1 Image analysis of immunohistochemically stained sections.

2 Second image analysis performed by the same observer (CMA), approximately 9 months following the first analysis.

Table 2-12. Assessment of intraobserver variation.

JG. Trial pt number 12

Site	CD Ag	Abs inf [1]	Abs inf [2]	T/N [1].	T/N [2].
Edge 1.	CD56	0.65	0.78	0.31	0.71
	CD4	1.02	1.20	1.00	0.59
	CD8	0.76	1.97	0.70	0.98
	CD25	0.11	0.66	0.51	2.06
Edge 2.	CD56	1.27	1.0	0.59	0.91
	CD4	1.22	0.91	1.19	0.45
	CD8	1.12	1.17	1.04	0.58
	CD25	0.79	0.59	3.74	1.84
Centre	CD56	0.99	0.68	0.44	0.62
	CD4	1.13	1.65	1.11	0.82
	CD8	0.46	1.23	0.43	0.61
	CD25	0.33	1.24	1.59	3.88

1 Image analysis of immunohistochemically stained sections.

2 Second image analysis performed by the same observer (CMA), approximately 9 months following the first analysis.

Table 2-12. Assessment of intraobserver variation.

ET. Trial pt number 6

Site	CD Ag	Abs inf [1]	Abs inf [2]	T/N [1].	T/N [2].
Edge 1.	CD56	2.93	1.16	1.16	1.51
	CD4	2.00	0.55	1.25	1.38
	CD8	1.57	0.30	1.96	0.28
	CD25	1.62	0.22	1.05	0.39
Centre	CD56	0.22	0.06	0.09	0.08
	CD4	0.92	0.44	0.59	1.1
	CD8	0.92	0.32	1.15	0.30
	CD25	1.33	0.34	0.86	0.61

1 Image analysis of immunohistochemically stained sections.

2 Second image analysis performed by the same observer (CMA), approximately 9 months following the first analysis.

Table 2-12. Assessment of intraobserver variation.

Control patient for EN.

Site	CD Ag	Abs inf [1]	Abs inf [2]	T/N [1].	T/N [2].
Edge 1.	CD56	0.06	0.08	0.10	0.24
	CD4	0.20	0.11	1.13	0.41
	CD8	0.10	0.06	0.38	0.15
	CD25	0.07	0.03	0.13	0.10
Edge 2.	CD56	0.48	0.53	0.80	1.56
	CD4	0.27	0.41	1.53	1.52
	CD8	0.29	-	1.11	-
	CD25	0.08	0.33	0.16	1.10
Centre	CD56	0.04	0.22	0.07	0.65
	CD4	0.16	0.06	0.93	0.22
	CD8	0.12	0.16	0.45	0.41
	CD25	0.05	0.17	0.10	0.57

1 Image analysis of immunohistochemically stained sections.

2 Second image analysis performed by the same observer (CMA), approximately 9 months following the first analysis.

Table 97. Assessment of intraobserver variation.

Control patient for DT.

Site	CD Ag	Abs inf 1	Abs inf 2	T/N 1.	T/N 2.
Edge.	CD56	0.40	0.31	0.28	1.24
	CD4	1.00	0.24	1.88	0.37
	CD8	0.21	0.98	0.71	2.18
	CD25	0.30	0.37	0.42	0.37
Centre	CD56	0.76	0.53	0.53	2.12
	CD4	0.62	0.54	1.17	0.83
	CD8	0.18	0.58	0.60	1.29
	CD25	0.80	0.50	1.13	0.50

1 Image analysis of immunohistochemically stained sections.

2 Second image analysis performed by the same observer (CMA), approximately 9 months following the first analysis.

Table 2-13. Assessment of interobserver variation. Analysis of a control patient by two independent observers.

MAb	Tumour specimen	Observer 1	Observer 2	Observer 1 T/N ratios.	Observer 2 T/N ratios.
CD25	Centre	0.14	0.52	0.27	0.91
	Edge 1	0.26	0.26	0.44	0.46
	Edge 2	0.11	0.41	0.21	0.72
CD8	Centre	0.69	0.65	0.50	0.87
	Edge 1	0.61	0.46	0.45	0.61
	Edge 2	0.24	0.80	0.18	1.07
CD4	Centre	1.35	1.56	2.05	1.22
	Edge 1	0.68	1.41	1.02	1.10
	Edge 2	0.58	1.71	0.88	1.34
CD56	Centre	0.04	0.56	0.12	0.32
	Edge 1	0.14	0.98	0.41	0.56
	Edge 2	0.13	0.85	0.40	0.49
TBS	Centre	0.03	0.22	0.05	0.71
	Edge 1	0.02	0.11	0.03	0.35
	Edge 2	0.06	0.19	0.10	0.61

Observer 1 (CMA) and Observer 2 (RM) performed both analysis using identical techniques, approximately 2 months apart.

Table 2-14. Results of all tumour specimens disaggregated.

No.	% Lym	%T cell	% CD4	% CD8	%CD19	CD16+56	CD4+ Dr	CD4- Dr
1.	-	-	-	-	-	-	-	-
2.	62	(43) 69	(17) 27	(28) 45	(10) 16	(1) 2	-	-
3.	-	-	-	-	-	-	-	-
4.	59	(34) 58	(26) 44	(12) 20	(15) 25	(2) 3	(14) 24	(22) 37
5.	91	(81) 89	(53) 58	(21) 23	(6) 7	(1)	1	(32) 35
6.	50	(40)	(23)	(16)	(4)	(1)	(3)	(11)
7.	55	(49) 89	(16) 29	(34) 62	(2) 4	(0) 0	(9) 16	(28) 51
8.	94	(77) 82	(49) 52	(28) 30	(11) 12	(1) 1	(32) 34	(38) 40
9.	91	(81) 89	(60) 66	(22) 24	(3) 3	(1) 1	(45) 49	(23) 25
10.	73	(60) 82	(42) 58	(16) 22	(6) 8	(1) 1	-	-
11.	63	(37) 59	(25) 40	(13) 21	(17) 27	(0) 0	(8) 13	(30) 48
12.	-	-	-	-	-	-	-	-
13.	42	(26) 62	(18) 43	(7) 17	(6) 14	(3) 7	(11) 26	(29) 69
14.	21	(19) 90	(14) 67	(7) 33	(3) 14	(1) 5	(10) 48	(21)
15.	-	-	-	-	-	-	-	-
16.	90	(83) 92	(49) 54	(31) 34	(2) 2	(4) 4	(38) 42	(35) 39
17.	73	(62) 85	(23) 32	(41) 56	(6) 8	(1) 1	(15) 21	(48) 66
18.	79	(41) 52	(19) 24	(28) 35	(21) 27	-	(13) 16	(44) 56
19.	36	(29) 81	(21) 58	(7) 19	(3) 8	-	(9) 25	(11) 31
20.	84	(65) 77	(30) 36	(35) 42	(6) 7	(14) 17	(16) 19	(43) 51
21.	87	(76) 87	(15) 17	(60) 69	(4) 5	(6) 7	(10) 11	(65) 75
22.	73	(59) 81	(30) 41	(31) 42	(4) 5	(4) 5	(19) 26	(34) 47
23.	-	-	-	-	-	-	-	-

Table 3-1 Results of all tumour specimens disaggregated.

No.	% Lym	% Tcell	% CD4	% CD8	B cell	NK cell	4+ Dr	4- Dr
24.	-	-	-	-	-	-	-	-
25.	52	(41) 79	(15) 29	(31) 60	(4) 8	(1) 2	(6) 12	(29) 56
26.	52	(37) 71	(10) 19	(28) 54	(7) 13	(4) 8	(6) 12	(38) 73
27.	67	(62) 93	(22) 33	(36) 54	(1) 1	(1) 1	(16) 24	(40) 60
28.	85	(75) 88	(52) 61	(20) 24	(14) 16	(1) 1	(22) 26	(32) 38
29.	56	(47) 84	(28) 50	(20) 36	(6) 11	(2) 4	(15) 27	(30) 54
30.	31	(27) 87	(21) 68	(5) 16	(1) 3	(1) 3	(19) 61	(14) 45
31.	60	(38) 63	(26) 43	(13) 22	(12) 20	(1) 2	(5) 8	(20) 33
32.	48	-	-	-	-	-	-	-
33.	48	(50)	(40)	(12)	(9)	(3)	(19)	(19)
34.	-	-	-	-	-	-	-	-
35.	62	(43)	(30)	(17)	(4)	(12)	(19)	(21)
36.	43	(29)	(16)	(15)	(14)	(1)	(5)	(28)
37.	23	(14) 61	(10) 43	(5) 22	(4) 17	(1) 4	(4) 17	(11) 48
38.	77	(48) 62	(22) 29	(25) 32	(26) 34	(3) 4	(11) 14	(49) 64
39.	76	(60) 80	(53) 70	(10) 13	(10) 13	(4) 5	(21) 28	(19) 25
40.	97	(94) 97	(18) 19	(76) 78	(3) 3	(1) 1	(15) 15	(79) 81
41.	64	(56) 89	(33) 52	(23) 36	(4) 6	(2) 3	(28) 44	(25) 39
42.	95	(89) 94	(62) 65	(26) 27	(4) 4	(2) 2	(54) 57	(33) 35
43.	67	(57) 85	(21) 31	(38) 57	(1) 1	(2) 3	(19) 28	(44) 66
44.	95	(34) 36	(30) 32	(8) 8	(60) 63	(1) 1	(12) 13	(58) 61
45.	77	(53) 69	(22) 29	(28) 36	(24) 31	(1) 2	(16) 21	(54) 70
46.	92	(59) 64	(33) 36	(26) 28	(13) 14	(6) 7	(29) 32	(34) 37

Table 3-1 Results of all tumour specimens disaggregated.

No.	% Lym	% Tcell	% CD4	% CD8	B cells	NK cell	4+ Dr	4- Dr
47.	88	(48) 54	(30) 34	(17) 19	(36) 41	(1) 1	(10) 11	(42) 48
48.	66	(47) 71	(27) 41	(26) 40	(7) 11	(7) 3	(20) 30	(40) 61
49.	65	(60) 92	(31) 48	(30) 46	(1) 2	(3) 5	(14) 22	(23) 35
50.	81	(59) 73	(29) 36	(31) 38	(6) 7	(4) 5	(22) 27	(43) 53
51.	67	(44) 66	(14) 21	(34) 51	(12) 18	(9) 13	(11) 16	(41) 61
52.	**89**	**(57) 64**	**(32) 36**	**(31) 35**	**(30) 34**	**(1) 1**	**(23) 26**	**(52) 58**
53.	**82**	**(31) 38**	**(18) 22**	**(8) 10**	**(51) 62**	**(2) 2**	**(7) 9**	**(46) 56**
54.	79	(77) 97	(20) 25	(61) 77	(2) 2	(3) 4	(20) 25	(66) 83

1 Figures in parentheses are the percentage of lymphocytes, as analysed from FACScan.

2 Lymphocyte numbers corrected for the lymphocyte percentage.

Table 3-1. Results of all tumours disaggregated.

No.	+4/25	-4/25	+3/69	-3/69	CD4RO	CD4RA	CD8RO	CD8RA
1.	-	-	-	-	-	-	-	-
2.	-	-	-	-	-	-	-	-
3.	-	-	-	-	-	-	-	-
4.	(8) 14	(3) 5	-	-	-	-	-	-
5.	(23) 25	(4) 4	-	-	-	-	-	-
6.	-	-	-	-	-	-	-	-
7.	(6) 11	(2) 4	-	-	-	-	-	-
8.	(15) 16	(3) 3	-	-	-	-	-	-
9.	(16) 18	(3) 3	-	-	-	-	-	-
10.	-	-	-	-	-	-	-	-
11.	(5) 8	(4) 6	-	-	-	-	-	-
12.	-	-	-	-	-	-	-	-
13.	(2) 5	(1) 2	-	-	-	-	-	-
14.	(2) 10	(1) 5	-	-	-	-	-	-
15.	-	-	-	-	-	-	-	-
16.	(20) 22	(4) 4	-	-	94%	6%	85%	15%
17.	(7) 10	(2) 3	-	-	100%	0%	98%	2%
18.	(10) 13	(22) 28	-	-	89%	11%	69%	31%
19.	(3) 8	(3) 8	(27) 75	(9) 25	-	-	-	-
20.	(17) 20	(6) 7	-	-	-	-	-	-
21.	(9) 10	(20) 23	(72) 83	(9) 10	-	-	-	-
22.	(12) 16	(8) 11	(8) 11	(1) 1	98%	2%	79%	21%
23.	-	-	-	-	-	-	-	-

Table 3-1 Results of all tumour specimens disaggregated.

No.	+4/25	-4/25	+3/69	-3/69	CD4RO	CD4RA	CD8RO	CD8RA
24.					-	-	-	-
25.	(5) 10	(10) 19	(33) 65	(3) 6	93%	7%	84%	16%
26.	(4) 8	(3) 6	(30) 58	(8) 15	83%	17%	95%	5%
27.	(7) 10	(2) 3	(51) 76	(1) 1	-	-	-	-
28.	(18) 21	(4) 5	(68) 80	(12) 14	92%	8%	96%	4%
29.	(9) 16	(6) 11	(37) 66	(9) 16	96%	4%	85%	15%
30.	(5) 16	(1) 3	(20) 65	(2) 6	-	-	-	-
31.	(2) 3	(0) 0	(20) 33	(8) 13	91%	9%	80%	20%
32.	-	-	-	-	-	-	-	-
33.	(4)	(0)	(38)	(6)	-	-	-	-
34.	-	-	-	-	-	-	-	-
35.	(3)	(1)	(33)	(12)	-	-	-	-
36.	(5)	(1)	-	-	-	-	-	-
37.	(6) 26	(7) 30	-	-	-	-	-	-
38.	(3) 4	(1) 1	(36) 47	(14) 18	96%	4%	96%	4%
39.	(8) 11	0	(31) 41	(11) 14	80%	20%	83%	17%
40.	(3) 3	(1) 1	(78) 80	(3) 3	95%	5%	96%	4%
41.	(7) 11	(2) 3	(50) 76	(4) 6	97%	3%	93%	7%
42.	(11) 12	(1) 1	(73) 77	(6) 6	98%	2%	96%	4%
43.	(5) 7	(0) 0	(51) 76	(6) 9	100%	0%	96%	4%
44.	(3) 3	(1) 1	(25) 26	(42) 44	89%	11%	100%	0%
45.	(6) 8	(1) 1	(47) 61	(11) 14	92%	8%	97%	3%
46.	(7) 8	(1) 1	(49) 53	(24) 26	98%	2%	90%	10%

Table 3-1 Results of all tumour specimens disaggregated.

No.	+4/25	-4/25	+3/69	-3/69	CD4RO	CD4RA	CD8RO	CD8RA
47.	(3) 3	(1) 1	(19) 22	(23) 26	70%	30%	71%	29%
48.	(2) 3	(2) 3	(37) 56	(9) 14	-	-	-	-
49.	(3) 5	0	(50) 77	(8) 12	-	-	-	-
50.	(4) 5	0	(51) 63	(11) 14	97%	3%	90%	10%
51.	(6) 9	(4) 6	(42) 63	(18) 27	96%	4%	98%	2%
52.	(7) 8	(1) 1	(58) 65	(16) 18	95%	5%	91%	9%
53.	(3) 4	(1) 1	(21) 26	(27) 33	-	-	-	-
54.	(5) 6	-	(74) 94	(3) 4	88%	12%	93%	7%

1. Figures in parentheses are the percentage of lymphocytes, as analysed from FACScan.

2. Lymphocyte numbers corrected for the lymphocyte percentage.

Glossary.

Adjuvant	Any substance that enhances the immune response to an antigen with which it is mixed.
Allograft	A graft of tissue from an allogeneic or non-self donor of the same species; such grafts are rejected unless the recipient is immunosuppressed.
Anergy.	Is a state of non-responsiveness to antigen.
ADCC	Antibody-dependent cell-mediated cytotoxicity is the killing of antibody coated cells by cells with Fc receptors which recognise the Fc region of the bound antibody (usually NK cells)
Apoptosis	Cell death in which the cell activates an internal death programme. It is characterised by nuclear DNA degradation, nuclear degeneration and condensation, and the phagocytosis of cell residua.
Cytokines	Proteins made by cells, that affect the behaviour of other cells.
Cytotoxic T cells	T cells that can kill other cells. Usually MHC class I restricted.
Delayed-type hypersensitivity	A form of cell-mediated immunity elicited by antigen in the skin, and mediated by inflammatory CD4 T cells.
Epitope	A site on an antigen recognised by an antibody. A T-cell epitope is a short peptide derived from a protein antigen, that binds to an MHC molecule, and is recognised by a particular T cell.
Hapten	Molecules that can bind antibody, but cannot themselves elicit an adaptive immune response.

Helper CD4 T cell (T_H2)	CD4+ T cells that help B cells make antibody in response to antigenic challenge. Also make cytokines such as IL-4 and IL-5.
Idiotopes	Antigenic epitopes on the variable regions of specific antibody molecules. a collection of idiotopes on an antibody is an idiotype.
Inflammatory CD4 T cell (T_H1)	Armed effector T cells that make the cytokines interferon γ and TNF upon recognition of antigen. Their major function is in macrophage activation, though some have cytotoxic activity.
MHC	Major Histocompatibility Complex. Antigen recognition by T cells is MHC restricted. This means that a given T cell will recognise antigen, only when its peptide fragments are bound to a particular MHC molecule
Oncogene	Genes involved in regulating cell growth. When they are defective in structure or expression, they can cause cells to grow continuously to form a tumour

References.

Adams DH, Yanelli JR, Newman W, Lawley T, Ades E, Rosenberg SA and Shaw S.

Adhesion of tumour-infiltrating lymphocytes to endothelium: a phenotypic and functional analysis. *Br J Cancer* 75: 1421-1431, 1997.

Adamthwaite D and Cooley MA.

CD8+ T cell subsets defined by expression of CD45 isoforms, differ in their capacity to produce IL-2, IFN γ and TNF β. *Immunology* 81: 253-260,1994.

Allen C and Hogg N.

Elevation of infiltrating mononuclear phagocytes in human colorectal tumors. *J. Nat Cancer Inst* 78: 465-470, 1987.

Alving CR, Wassef NM, and Richards RL.

Use of adjuvants for enhancement of antibody responses. In Handbook of Experimental Immunology. eds Weir D, Blackwell C and Herzenberg L 1995.

Apasov S, Redegeld F and Sitkovsky M

Cell-mediated cytotoxicty: contact and secreted factors. *Curr Opin. Immunol* 5: 404-410, 1993.

Armitage NC, Perkins AC, Pimm MV, Farrands PA, Baldwin RW and Hardcastle JD.

The localisation of an anti-tumour monoclonal antibody (791T/36) in gastrointestinal tumours. *Br J Surg* 71: 407-412, 1984.

Arnand JP, Buyse M, Nordhinga B et al.
Results of an EORTC double-blind randomised clinical trial. *Br J Surg* **76**: 284-289, 1959.

Austin EB, Robins RA, Durrant LG , Price MR & Baldwin RW.
Human monoclonal anti-idiotypic antibody to the tumour-associated antibody 791T/36. *Immunology* **67**: 525-530, 1989.

Austin EB, Robins RA, Baldwin RW and Durrant LG.
Induction of delayed hypersensitivity to human tumour cells with a human monoclonal anti-idiotypic antibody. *J Nat Cancer Inst* **83**: 1245-1248, 1991.

Balch CM, Riley LB, Yoon Joo Bae, et al.
Patterns of human tumour-infiltrating lymphocytes in 120 human cancers. *Arch Surg* **125**: 200-205, 1990.

Banner BF, Sarus L, Baher S and Wode BA.
Characterization of the inflammatory cell populations in normal colon and colonic carcinoma. *Virchows Archiv B Cell Pathol* **64**: 213-220, 1993.

Barnd DL, Lan M, Metzgar R, and Finn OJ.
Specific, MHC unrestricted recognition of tumour associated mucins by human cytotoxic T cells. *Proc Nat Acad Sci* **86**: 7159-7163, 1989.

Begent RHJ, Verhaar MJ, Chester KA, Casey JL et al.
Clinical evidence of efficient tumour targeting based on single-chain Fv antibody selected from a combinatorial library. *Nature Medicine* **2**: 979-984, 1996.

Berd D, Maguire HC Jr, Mastrangelo MJ.
Induction of cell-mediated immunity to autologous melanoma cells and regression of metastases after treatment with a melanoma cell vaccine, preceded by cyclophosphamide. *Cancer Res* **46**: 2572-2577, 1986.

Berd D, Maguire HC Jr, Mastrangelo MJ and Murphy G.
Activation markers on T cells infiltrating melanoma metastases after therapy with dinitrophenyl-conjugated vaccine. *Cancer Immunol Immunother* **39**: 141-147, 1994.

Besredka T

Du role de la peau dans la sarcomatose de la souris. *Ann Inst Pasteur* **55**: 402-416, 1935.

Bevan MJ.

Class discrimination in the world of immunology. *Nature* **325**: 192-194, 1987.

Bhattacharya Chatterjee M, Foon KA and Kohler H.

Idiotypic antibody immunotherapy of cancer. 75-82, 1994.

Bischof Delaloye A and Delaloye B

Radiolabelled monoclonal antibodies in tumor imaging and therapy: Out of fashion? *Eur J Nucl Med* **22**, 571-580, 1995.

Blay JY, Favrot MC, Negrier S, et al.

Correlation between clinical response to interleukin-2 therapy and sustained production of tumour necrosis factor. *Cancer Res* **50**: 2371-2381, 1990.

Bloem AC, Jurgens R, Eichmann K, and Emmrich F.

Human immune response to group A streptococcal carbohydrate (A-CHO). III. Comparison of the efficiencies of anti-idiotypic antibody and of nominal antigen in the induction of IgM anti-A-CHO-producing B cells. *Eur J Immunol* **18**:1959-1964, 1988.

Blomgren SE, Wolberg WH, Kishen WA.

Effect of fluoropyrimidines on delayed cutaneous hypersensitivity. *Cancer Res* **25**: 977, 1965.

Bowen Yacyshyn MB, Poppema S, and Berg A et al.
CD69+ and HLA-DR+ activation antigens on peripheral blood lymphocyte populations in metastatic breast and ovarian cancer patients: correlations with survival following active specific immunotherapy. *Int J Cancer* **61**: 470-474, 1995.

Brivio F, Lissini P and Alden G et al.
Preoperative IL-2 subcutaneous immunotherapy may prolong survival time in advanced colorectal cancer patients. *Oncology* **53**: 263, 1996.

Brooks AG, Posch PE, Scorzelli CJ, Borrego F and Coligan JE.
NKG2A complexed with CD94 defines a novel inhibitory Natural Killer cell receptor. *J. Exp Med* **185**: 795-800, 1997.

Buckley TJD, Robins RA and Durrant LG.
Clinical evidence that the human monoclonal anti-idiotypic antibody 105AD7 delays tumor growth by stimulating anti-tumor T-cell responses. *Hum. Antibod. Hybridomas* **6**: 68-72, 1995.

Burnett FM.
The concept of immunological surveillance. *Progress in Expt Tumor Research.* **13**: 1-27, 1970.

Buzzi S, Baccini C, Rubboli D, Morelli A, Monti G, Buzzi G and Buzzi AM.
Immunological effects of a boiled diphtheria toxoid on high risk cancer patients. *Proc Am. Assoc for Cancer Research* **38**: 397, 1997 (abstract).

Byers VS, Pimm MV, Scannon PJ, Pawluczyk IZA, and Baldwin RW. Inhibition of growth of human tumour xenografts in athymic mice treated with ricin toxin A chain-monoclonal antibody 791T/36 conjugates. *Cancer Res* **47**: 5042-5046, 1987.

Byers VS, Rodvein R, Grant K, Durrant LG, Hudson KH, Baldwin RW, and Scannon PJ.
Phase I study of monoclonal antibody-Ricin A chain immunotoxin Xomazyme-791 in patients with metastatic colon cancer.*Cancer Res* **49**: 6153-6160,1989.

Cameron DJ and Stromberg BV.
The ability of macrophages from head and neck cancer patients to kill tumour cells. Effect of prostaglandin inhibitors in cytotoxicity. *Cancer* **54**: 2403-2408, 1984.

Campbell MJ, Esserman L and Levy R.
Immunotherapy of established murine B cell lymphoma. Combination of idiotype immunisation and cyclophosphamide. *J Immunology* **141**: 3227-3233, 1988.

Castedo M, Macho A, Zamzami N, Hirsch T, Marchetti P, Uriel J and Kroemer G.
Mitochondrial perturbations define lymphocytes undergoing apoptotic depletion in vivo. *Eur J Immunol* **25**: 3277, 1995.

Chapman PB, Livingston PO, Steffens TA , Oettgen HF and Houghton AN.
Pilot trial of anti-idiotypic monoclonal antibody BEC2 in patients with metastatic melanoma. *Proc Am Assoc Cancer Research* **33**: 208-211, 1992.

Chattopadhyay P, Starkey J, Morrow WJW, and Raychaudhuri S.
Murine monoclonal anti-idiotype antibody breaks unresponsiveness and induces a specific antibody response to melanoma associated proteoglycan antigen in cynomolgus monkeys. *Proc Nat Acad Sci* **89**: 2684-2688, 1992.

Chen JJ, Saeki Y, Shi L and Kohler H.
Tumour idiotype vaccines. Synergistic anti-tumor effects with combined "Internal Image" anti-idiotypes and chemotherapy. *J of Immunology* **143**: 1053-1057, 1989.

Ciborowski P, Welch SN and Finn OJ.
Analysis of native and recombinant epithelial cell mucin (MUC-1) a tool for cancer immunotherapy. *Proc. of Am. Assoc for Cancer Res* **37**: 464-465, 1996.

Ciernik IF, Berzofsky JA, and Carbone DP.
Induction of Cytotoxic T-Lymphocytes and Anti-tumour immunity with DNA vaccines expressing single T-cell epitopes. *J. Immunol* **156**: 2369-2375, 1996.

Coles M, Rose M, and Yacoub M.
Appearance of cells bearing the interleukin-2 receptor in the peripheral blood of cardiac transplant patients., and their correlation with rejection episodes. *Transplantation Proc* **19**: 2546-2547, 1987.

Coley H
A report of recent cases of inoperable sarcoma successfully treated with mixed toxins of erisipelas and Bacillus prodigiosus. *Surg Gynecol Obstet* **256**: 495, 1911.

Conry RM, LoBuglio AF, Kantor J et al.
Immune Response to a Carcinoembryonic Antigen Polynucleotide Vaccine. *Cancer Res* **54**: 1164-1168, 1994.

Conry RM, Saleh MN, Schlom J, and LoBuglio AF.
Human Immune Response to carcinoembryonic antigen tumour vaccines. *J Immunother.* **18**: 137-141, 1995.

Courtney-Luk LS, Epenetos AA, Moore R, Larche M, Pectasides D, Dhokia B, and Ritter MA.

Development of primary and secondary immune responses to mouse monoclonal antibodies used in the diagnosis and therapy of malignant neoplasms. *Cancer Res* **46**: 6489-6493, 1986.

Dalgleish A.
Personal communication. 1996.

Deans JP, Boyd AW and Pilarski LM.
Transitions from high molecular weight isoforms of CD45 (T200) involve rapid activation of alternate mRNA splicing and slow turnover of surface CD45R. *J Immunol* **143**: 1233-1238, 1989.

Denton GWL, Durrant LG, Jack D Hardcastle, Eric B Austin, Herb F Sewell and R Adrian Robins.
Clinical Outcome of Colorectal Cancer Patients treated with Human Monoclonal Anti-Idiotypic Antibody.
Int J Cancer **57**: 10-14, 1994.

Durrant LG, Buckley TJD, Denton GWL, Hardcastle JD, Sewell HF, Robins RA.
Enhanced cell-mediated killing in patients immunized with a human monoclonal anti-idiotypic antibody 105AD7. *Cancer Res* **54**: 4837-4840, 1994.

Ebert EC, Brolin RE and Roberts AI.
Characterization of activated lymphocytes in colon cancer. *Clin Immunol Immunopathol* **50**: 72-81, 1989.

Edelman O.
Adjuvants. *Intern Rev Immunol.* **7**: 51, 1990.

Ehrlich P.
On immunity with special reference to cell life. *Proc Royal Soc* **66**: 424-448, 1900.

Eichmann K.
Expression and function of idiotypes on lymphocytes. *Adv Immunol* **26**: 195-254, 1978.

Eilber FR, Nizze JA and Morton DL.
Sequential evaluation of general immune competence in cancer patients: correlation with clinical course. *Cancer* **35**: 660-666, 1975.

Embleton MJ, Gunn B, Byers VS and Baldwin RW.
Antitumour reactions of a monoclonal antibody against a human osteogenic sarcoma cell line. *Br J Cancer* ,**43**: 582-587, 1981.

Embleton MJ, Byers VS, Lee HM, Scannon P, Blackhall NW and Baldwin RW.
Sensitivity and selectivity of ricin toxin A chain-monoclonal antibody 791T/36 conjugates against human tumour cell lines.*Cancer Res* **46**: 5524-5528, 1986.

Evans H.

Host cells in transplanted murine tumors and their possible relevence to tumour growth. *J Reticuloendothelial Soc* 26: 427-437, 1979.

Fagerberg J, Hjelm A-L, Ragnhammer P et al.
Tumour Regression in Monoclonal Antibody-treated patients correlates with the presence of Anti-idiotypic-reactive T lymphocytes.*Cancer Res* **55**: 1824-1827, 1995 a.

Fagerberg J, Steinitz M, Wigzell H et al.
Human anti-idiotypic antibodies induced a humoral and cellular immune response against a colorectal cancer associated antigen in patients. *Proc Nat Acad Sci USA* **92**: 4773-4777, 1995 b.

Fairlamb DJ.
Spontaneous regression of metastases of renal cancer. *Cancer* **47**: 2102-2106, 1981.

Falk RE, Mac Gregor AB, Landi S, Ambus U and Langer B.
Immunostimulation with intraperitoneally administered Bacille Calmette Guerin for advanced malignant tumors of the gastrointestinal tract. *Surg Gynecol and Obstet* **142**: 363-368, 1976.

Farrands PA, Perkins AC, Pimm MV, Hardy JD, Embleton MJ, Baldwin RW and Hardcastle JD.
Radioimmune detection of human colorectal cancers by an anti-tumour monoclonal antibody. *Lancet* ii 397-400, 1982.

Finn OJ, McKolanis JR, Nalesnik MA et al.
T cell defects in advanced breast, pancreatic, and colon cancer, and improvements after vaccination with a mucin peptide. *Proc Am Assoc for Cancer Res* **37**: 490, 1996.

Fontenot JD, Tjandra N, Bu D, Ho C, Montelaro RC and Finn OJ.
Biophysical characterization of one-, two-, and three-tandem repeats of human mucin (muc-1) protein core. *Cancer Res* **53**: 5386-5394, 1993.

Foon KA, Chakraborty M, John WJ, Sherratt A, Kohler H, and Bhattacharya-Chatterjee M.
Immune response to the carcinoembryonic antigen in patients treated with an anti-idiotype antibody vaccine. *J Clin Invest* **96**: 334-342, 1995.

Forstrum JW, Nelson KA, Nepom GT, Hellstrom L, and Hellstrom KE.
Immunization to a syngeneic sarcoma by a monoclonal auto-anti-idiotypic antibody. *Nature* **303**: 627-629, 1983.

Fulton AM, Loveless SE, and Heppner GH.
Mutagenic activity of tumour-associated macrophages in Salmonella typhimurium strains TAG8 and TA100. *Cancer Res* **44**: 4308-4311, 1984.

Gabrilovich DI, Nadaf S, Corak J, Berzofsky JA, Carbone DP.
Dendritic cells in antitumor immune-responses 2. dendritic cells grown from bone-marrow precursors, but not mature DC from tumor-bearing mice, are effective antigen carriers in the therapy of established tumors. *Cellular Imm.* **170**: 111-119, 1996.

Giacommini P, Fraioli R, Calabro AM, Difilipo F, Natali PG.
Class I Major Histocompatability Complex enhancement by recombinant leucocyte interferon in the peripheral blood mononuclear cells and plasma of melanoma patients. *Cancer Res* **51**: 652-656, 1991.

Gill P and Morris PJ.
The survival of patients treated with colorectal cancer treated in a regional hospital. *Br J. Surg* **65**: 17-20, 1978.

Gimmi C, Morrison BW, Mainprice BA et al.
Breast cancer-associated antigen, DF3/MUC1, induces apoptosis of activated human T cells. *Nature Medicine* **2**: 1367-1370, 1996.

Gjertsen MK, Bakka A, Breivik J, Saeterdal I, Solheim BG, Soreide O, Thorsby E and Gaudernack G.
Vaccination with mutant ras peptides and induction of T-cell responsiveness in pancreatic carcinoma patients carrying the corresponding RAS mutations. *Lancet* **346**: 1399-1400, 1995.

Golub SH, O'Connel TX and Morton DL.
Correlation of *in vitro* assays of immunological competence in cancer patients. *Cancer Res* **34**: 1833-1840, 1974.

Gourevitch MM, von Mensdorff-Pouilly, Litinov SV et al.
Polymorphic epithelial mucin (muc-1) containing circulating complexes in carcinoma patients. *Br J Cancer* , **72**: 934-938, 1995.

Goydos JS, Elder E, Whiteside TL, Finn OJ and Lotze MT.
A Phase I Trial of Synthetic Mucin Peptide Vaccine. Induction of Specific Immune reactivity in Patients with Adenocarcinoma. *J of Surg Res* **63**: 298-304, 1996.

Griffith KD, Read EJ, Carrasquillo JA, Carter CS,Yang JC, Fisher B, Aebersold P, Packard BS, Yu MY, and Rosenberg SA.
In vivo distribution of adoptively transferred indium-111 labelled tumor-infiltrating lymphocytes and peripheral blood lymphocytes in patients with metastatic melanoma. *J. Natl Cancer Inst* **81**: 1709-1717, 1989.

Grimm EA, Robb RJ, Roth JA.
Lymphokine-activated killer cell phenomenon II. Evidence that Interleukin-2 is sufficient for direct activation of peripheral blood lymphocytes into lymphokine activated killer cells. *J Exp Med* **158**: 1356-1361, 1983.

Gross M.
Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. *Cancer Res* **3**: 326-333, 1943.

Guilloux Y, Viret C, Gervois N et al.
Defective lymphokine production by most CD8+ and CD4+ tumour-specific T cell clones derived from human melanoma-infiltrating lymphocytes in response to autologous tumour cells in vitro. *Eur J. Immunol* **24**: 1966-1973, 1994.

Gupta RK, Relyveld EH, Lindblad EB, Bizzini B, Ben-Effraim S, and Gupta CK.
Adjuvants- a balance between toxicity and adjuvanticity. *Vaccine* **11**: 293-306, 1993.

Hahne M, Rimoldi D, Schroter M et al
Melanoma cell expression of Fas (APO-1/CD95) Ligand: Implications for Tumour immune escape. *Science* **274**: 1363-1366, 1996.

Hakansson L, Adell G, Boeryd B, Sjogren F and Sjodahl R
Infiltration of mononuclear inflammatory cells into primary colorectal carcinomas: an immunohistological analysis. *Br J Cancer* **75**: 374-380, 1997.

Hamilton JM, Chen AP, Nguyen B, Gram J, Abrams S, Chung Y, Kantor J, Phares JC, Bastian A, Brooks C, Morrison G, Allegra CJ, Schlom J.
Phase I study of recombinant vaccinia virus (rV) that expresses human carcinoembryonic antigen (CEA) in adult patients with adenocarcinomas. *Proc of the Am. Assoc Clin Oncol Annual Meeting* 961, 1994 (abstract).

Hardcastle JD, Chamberlain JO, Robinson MHE, Moss SM, Amar SS, Balfour TW, James PD and Mangham CM.
Randomised controlled trial of faecal-occult blood screening for colorectal cancer. *Lancet* **348**: 1472-1477, 1996.

Hardegree A.
Influence of antigens on release of free fatty acids from arlacel A (mannide moneolate) *Proc Soc Exp Biol Med* **123**: 179, 1966.

Harel-Bellan A, Bertoglio J, Quillet A et al
Interleukin-2 up-regulates its own receptor on a subset of human unprimed peripheral blood lymphocytes and triggers their proliferation. *J. Immunol* **136**: 2463-2469, 1986.

Hargreaves M and Bell EB.
Identical expression of CD45R isoforms by CD45RC+ 'revertant' memory and CD45RC+ naive CD4 T cells. *Immunology* **91**: 323-330, 1997.

Henney CS, Kuribayashi K, Kern DE and Gillis S.
Interleukin-2 augments Natural Killer cell activity. *Nature* **291**: 335-338, 1981.

Herlyn D, Lubeck M, Sears H and Koprowski H
Specific detection of anti-idiotypic immune responses in cancer patients treated with murine monoclonal antibody. *J Immunol Methods* **85**: 27-35, 1985.

Herlyn D, Ross AH, Koprowski H
Anti-idiotype antibodies bear the internal image of a human tumour antigen. *Science* **232**: 100-102, 1986.

Herlyn D, Wettendorf M, Schmoll E et al.
Anti-idiotype immunization of cancer patients : Modulation of the immune response. *Proc Nat Acad Sci USA,* **84**: 8055-8059, 1987.

Herlyn D, Benden A, Kane M, Somasundaram R, Zaloudik J et al.
Anti-idiotype cancer vaccines : preclinical and clinical studies. *In vivo* **5**: 615-624, 1991.

Herlyn D, Zaloudik J, Li W et al
Preclinical and clinical evaluation of baculovirus-derived gastrointestinal carcinoma-associated antigen CO17-1A/GA733. *Proc of the Am Assoc for Cancer Res* **38**: 399, 1997 (abst).

Herlyn M, Steplewski Z, Herlyn D and Koprowski H.
Colorectal carcinoma-specific antigen: Detection by means of monoclonal antibodies. *Proc Natl Acad Sci* **76**: 1438-1442, 1979.

Heys SD, Franks ER, Eremin O.
Interleukin-2 therapy: current role in surgical oncological practice. *Br J Surg* **80**: 155-162, 1993.

Heys SD, Segar A, SD Payne S, Bruce DM, Kernohan N, and Eremin O.
Dietary supplementation with L-Arginine: modulation of tumour-infiltrating lymphocytes in patients with colorectal cancer. *Br J Surg* **84**: 238-241, 1997.

Hilleman P.
Critical appraisal of emulsified oil adjuvants applied to viral vaccines. *Prog Med Virol* **8**: 131, 1966.

Hodge JW, Abrams S, Schlom J, Kantor JA.
Induction of anti-tumour immunity by recombinant vaccinia viruses expressing B7.1 or B7.2 co-stimulatory molecules. *Cancer Res* **54**: 5552-5555, 1994.

Hoover HC, Brandhorst JS, Peters LC et al.
Adjuvant Active Specific Immunotherapy for Human Colorectal Cancer: 6.5-Year Median Follow-up of a Phase III prospectively randomized trial. *J Clin Oncol* **11**: 390-399, 1993.

Hutchinson GH, Heinemann D, Symes MO and Williamson RCN.
Differential immune reactivity of tumor-intrinsic, and peripheral blood lymphocytes, against autoplastic carcinoma cells. *Br J Cancer* **44**: 396-402, 1981.

Hutter C.
The balance of macrophage subsets may be customised at mucosal surfaces. *FEMS Microbiol Immunol* **105**: 309, 1992.

Irvine KR and Schlom J.
Induction of delayed-type hypersensitivity responses by monoclonal anti-idiotypic antibodies to tumor cells expressing carcinoembryonic antigen and tumor-associated glycoprotein-72. *Cancer Immunol Immunother* **36**: 281-292,1993.

Irvine KR, Rao JB, Rosenberg SA and Restifo NP.
Cytokine enhancement of DNA Immunization Leads to effective Treatment of Established Pulmonary Metastases. *J of Immunol*, **156**: 238-245, 1996.

Irvine KR, Chamberlain RS, Shulman EP, Rosenberg SA and Restifo NP.
Route of immunization and the therapeutic impact of recombinant anticancer vaccines. *J. Nat Cancer Inst* **89** :390-392, 1997.

Irving F.
The cytoplasmic domain of the T cell receptor ζ is sufficient to couple to receptor-associated signal transduction pathways. *Cell* **64**: 891-901, 1991.

Itkowitz S, Kjeldsen T, Friera A, Hakamori S, Yang US, and Kim YS.
Expression of Tn, sialosyl Tn, and T antigens in human pancreas. *Gastroenterology* **100**: 1691-1700, 1991.

Itoh K, Tilden AB and Balch CM.
Interleukin-2 activation of cytotoxic T lymphocyte infiltration into malignant melanoma. *Cancer Res* **46**: 3011-3017, 1986.

Jackson PA, Green MA, Marks CG, King RJB, Hubbard R, and Cook MG.
Lymphocyte subset infiltration patterns and HLA antigen status in colorectal carcinomas and adenomas. *Gut* **38**: 85-89, 1996.

Jacob L, Somasundaram R, Smith W, Monos D, Basak S, Marincola F, Pereira S and Herlyn D.
Cytotoxic T-Cell clone against rectal carcinoma induced by stimulation of a patients peripheral blood mononuclear cells with autologous cultured tumour cells. *Int J Cancer* **71**: 325-332, 1997.

Janetzki S, Blachere NE, and Srivastava PK
Generation of tumour-specific CTL's and memory T-cells, *Science* 1994.

Jass JR.
Lymphocytic infiltration and survival in rectal cancer. *J Clin Pathol* **39**: 585-589, 1986 a.

Jass JR, Atkin WS, Cuzick J et al.
The grading of rectal cancer: historical perspectives, and a multivariate analysis of 447 cases. *Histopathology* **10**: 435-459, 1986 b.

Jass JR, Love and Northover J.
A new prognostic classification of rectal cancer. *Lancet* June 6th, 1303-1307, 1987.

Jass JR, Ajioka Y, Allen JP et al
Assessment of invasive growth pattern and lymphocytic infiltration in colorectal cancer. *Histopathology* **28**: 543-548, 1996.

Jerome KR, Barnd DL, Bendt KM, Boyer CM, Taylor-Papadimitriou J, McKenzie IFC, Bast RC Jr and Finn OJ.
Cytotoxic T lymphocytes derived from patients with breast adenocarcinoma recognize an epitope present on the protein core of a mucin molecule preferentially expressed by malignant cells. *Cancer Res* **51**: 2908-2916, 1991.

Jerome KR, Bu D, and Finn OJ.
Expression of tumour associated epitopes on EBV immortalized B cells and Burkitts lymphomas transfected with epithelial mucin cDNA. *Cancer Res* **52**: 5985-5990, 1992.

Jung S and Schleusner HJ
Human T lymphocytes recognize a peptide of single point-mutated, oncogenic ras proteins. *J. Exp Med* **173**: 273-276, 1991.

Kantor J, Irvine K, Abrams S, Snoy P, Olsen R, Greiner J, Kaufman H, Eggensperger D, and Schlom J.
Immunogenicity and safety of a recombinant vaccinia virus vaccine expressing the carcinoembryonic antigen gene in a non-human primate. *Cancer Res* 52:6917-6925, 1992.

Kantor J, Abrams S, Irvine K, Snoy P, Kaufman H Schlom J.
Specific immunotherapy using a recombinant vaccinia virus expressing human carcinoembryonic antigen. *Ann NY Acad Sci* , 690: 370-373, 1993.

Kaufman H, Schlom J, and Kantor J.
A recombinant vaccinia virus expressing human carcinoembryonic antigen (CEA) *Int J Cancer* 48: 900-907, 1991.

Kedar E and Weiss DW.
The *in vitro* generation of effector lymphocytes, and their employment in tumor immunotherapy. *Adv Cancer Res* 38: 171-287, 1983.

Keller H, Wimmenauer S, Rahner S et al.
Morphological and functional characteristics of TIL from human colorectal cancers after stimulation with rIL-2. *Europ Surg Res* 27: 258-268, 1995.

Kernohan NM, Sewell HF and Walker F.
Natural Killer cells in cutaneous malignant melanoma. *J Pathology* 161: 35-40, 1990.

Kerrebijn JD, Balm AJM, Knegt PP, Meeuwis CA, Drexhage HA.
Macrophage and dendritic cell infiltration in head and neck squamous-cell carcinoma; an immunohistochemical study. *Cancer Immunol Immunother* **38**: 31-37, 1994.

Khazaeli MB, Wheeler R, Rogers K, Teng N, et al.
Initial evaluation of a human monoclonal antibody (HA-1A) in man. *J Biol Resp Modif* **9**: 178-184, 1990.

Klein G, Sjogren HO, Klein E et al.
Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochronous host.*Cancer Res* **20**: 1561-1572,1960.

Klein JL, Leichner PK, Callahan KM, Kopher KA, and Order SE.
Effect of anti-antibodies on radiolabeled antibody therapy. *Antibody Immunoconj Rad. Pharm* **1**: 55-64, 1988.

Kohler P.
Continuous culture of fused cells secreting antibody of predefined specificity. *Nature* **256**: 495-497, 1975.

Koprowski H, Herlyn M, Steplewski Z et al.
Specific antigen in serum of patients with colon carcinoma.*Science* **212**: 53-55, 1981.

Kotera Y, Fontenot JD, Pecher G, Metzgar RS, Finn OJ.
Humoral Immunity against a Tandem Repeat Epitope of Human Mucin MUC-1 in sera from Breast, Paancreatic and Colon Cancer Patients. *Cancer Res* **54**: 2856-2860, 1994.

Kubota Y, Petras RE, Easley KA et al.
Ki-67-determined growth fraction vs standard staging and grading parameters in colorectal carcinoma . *Cancer* **70**: 2602-2609, 1992.

Kurt Jones EA, Hamberg S, Ohara J, Paul WE.
Heterogeneity of helper/inducer T lymphocytes. *J Exp Med* **166**: 1774-1789, 1987.

Lafreniere R and Rosenberg SA.
Successful immunotherapy of murine experimental hepatic metastases with lymphokine-activated killer cells and recombinant interleukin-2. *Cancer Res* **45**: 3735-3741, 1985.

Leclerc JC, Gomard E, Plata F et al
Cell-mediated immune reaction against tumours induced by oncornaviruses. *Int J Cancer* **11**: 426-432, 1973.

Lennox ES and Sikora K
Definition of human tumour antigens. in Monoclonal antibodies in clinical medicine. (Eds McMichael and Fabre). London Academic Press. 1982.

Leonard PC
Immunotherapy options for colorectal cancer 8-10, In Oncology in Practice, European School of Oncology, 1997.

Li Z, Srivastava PK.
Tumour rejection antigen gp96/grp94 is an ATPase: implications for protein folding and antigen presentation. *EMBO J* **12**: 3143-3151,1993.

Lindenman J and Jerne NK.
Speculations on Ids and homobodies. *Ann Immunol* **124**: 171-184, 1974.

Lissoni P, Tancini G, Rovelli F et al.
Serum interleukin-2 levels in relation to the neuroendocrine status in cancer patients. *Br J Cancer* **62**: 838-839, 1990.

Livingston PO, De Leo AB, Jones M and Oettgen HF.
Comparison of approaches for augmenting the serological response to the individually specific methylcholanthrene-induced sarcoma-meth A: pre-treatment with cyclophosphamide is most effective. *J Immunol* **131**: 2601-2605,1983.

LoBuglio AF, Khazaeli MB, Meredith R and Saleh MN.
Chimeric monoclonal antibodies in cancer therapy. *Ann Oncol* .**3**: 196, 1992 (abst).

Lotze MT, Matory YL, Ettinghausen SE et al.
In vivo administration of purified human interleukin 2 II Half-life, immunological effects and expansion of peripheral lymphoid cells *in vivo* with recombinant IL-2. *J Immunol* **135**: 2865-2875, 1995.

MacCarthy WC.
Principles of prognosis in cancer. *JAMA* **96**: 30-33, 1931.

Markey AC and MacDonald DM.
Identification of CD16/NKH1+ NK cells, and their relevence to cutaneous tumour immunity. *Br J Dermatol* **121**: 563-570,1989.

Martin SJ, Reutlingersperger CPM, McGahon AJ et al.
Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus. Inhibition by overexpression Bcl-2 and Abl *J Exp Med* **182**: 1545-1557, 1995.

Matsuda M, Petersson M, Lenkei R et al
Alterations in the signal-transducing molecules of T cells and NK cells in colorectal tumour-infiltrating gut mucosal and peripheral lymphocytes: correlation with the stage of the disease. *Int J Cancer* **61**: 765-772,1995.

Matthews JNS, Altman DG, Campbell MJ and Royston P.
Analysis of serial measurements in medical research. *BMJ* **300**: 230-235, 1990.

Mavligit GM, Burgess MA, Burton Seibert G et al.
Prolongation of postoperative disease free interval and survival in human colorectal cancer by BCG, or BCG plus 5-Fluorouracil. *Lancet* **i**: 871-876, 1976.

McGovern OP.
Spontaneous regression of melanoma. *Pathology* **7**: 91-99, 1975.

McKolanis JR, Pecher G, Lotze MJ, Finn OJ.
Mucin reactive CTL induced by *in vivo* immunization. *Proc of the Am Assoc for Cancer Res* **37**: 466, 1996 (abst).

McMillan DN, Kernohan NM, Flett ME et al
Interleukin-2 receptor expression and interleukin-2 localisation in human solid tumours in situ and in vitro : evidence for a direct role in the regulation of tumour cell proliferation. *Int J Cancer* **60**: 766-772, 1995.

Mella J, Datta SN, Biffin A, Radcliffe AG, Steele RJC and Stamatakis JD.
Surgeons follow up practice after resection of colorectal cancer. *Ann R Coll Surg Eng* **79**: 206-209, 1997.

Michich E.
Antileukaemic action of new aromatic bisguanylhydrazone derivatives. *Cancer* **20**: 880, 1967.

Mittelman A, Chen ZJ, Yang H , Wong GY and Ferrone S.
Human high molecular weight melanoma-associated antigen (HMW-MAA) mimicry by mouse anti-idiotypic monoclonal antibody MK2-23 : induction of humoral anti-HMW-MAA immunity and prolongation of survival of patients with stage IV melanoma. *Proc Nat Acad Sci USA* **89**: 466-470, 1992.

Mizoguchi H, O'Shea JJ, Longo DL, et al.
Alterations in signal transduction molecules in T lymphocytes from tumour bearing mice. *Science* **258**: 1795-1798, 1992.

Mokyr MB, Hengst JCD and Dray S

Role of anti-tumor immunity in cyclophosphamide-induced rejection of subcutaneous non-palpable MOPC 315 tumours. *Cancer Res* **42**: 974-979, 1982.

Morikawa K, Hosokawa M, Hamada M.

Host-mediated therapeutic effects produced by appropriately timed administration of bleomycin on a rat fibrosarcoma. *Cancer Res* **45**: 1502-1506, 1985.

Moy P, Holmes C and Golub SH.

Depression of natural killer cytotoxic activity in lymphocytes infiltrating pulmonary tumours. *Cancer Res* **45**: 57-60, 1985.

Nair SK, Boczkowski D, Snyder D and Gilboa E
Antigen-presenting cells pulsed with unfractionated tumor-derived peptides are potent tumor vaccines.*Eur J Immunol* **27**: 589-597, 1997.

Nakagomi H, Petersson M, Magnusson I et al
Decreased expression of the signal-transducing zeta chains in Tumour-Infiltrating T-cells, and NK cells of patients with Colorectal Carcinoma. *Cancer Res* **53**: 5610-5612, 1993.

Nisonoff A and Lamoyi E.
Implications of the presence of an internal image of the antigen anti-idiotypic antibodies : possible application to vaccine production. *Clin Immunol Immunopathol.* **21**: 397-406,1981.

Numata A, Minagawa T, Asano M et al
Functional evaluation of tumour-infiltrating mononuclear cells. *Cancer* **68**: 1937-1943, 1991.

O'Connell J, O'Sullivan GC, Collins JK, and Shanahan F.
The Fas counterattack: Fas mediated T-Cell killing by colon cancer cells expressing Fas Ligand. *J Exp Med* **184**: 1075-1082, 1996.

O'Hara RJ, Drew PJ, Lee PWR, Duthrie GS, Greenman J, and Monson JRT.
Jass score, immune suppression and interleukin-12 production: a functional link. *Br J Surg* **84**: 713, 1997 (abstract).

Old LJ, Boyse EA, Clarke DA et al.
Antigenic properties of chemically induced tumours. *Ann NY Acad Sci* **101**: 80-106, 1962.

Ortaldo JR, Mason TA, Gerard JP et al.
Effects of natural and recombinant Interleukin-2 on regulation of γ Interferon production and Natural Killer cell activity: lack of involvement of the TAC antigen for these immunorehulatory effects. *J Immunol* **133**: 779-783, 1984.

OPCS
Mortality statistics by cause : England and Wales 1992. Series DH2, no 20.London : HM Stationery Office. 1995.

Ostenstad B, Sioud M, Lea T, et al.
Limited heterogeneity in the T cell receptor V gene usage in lymphocytes infiltrating human colorectal tumours. *Br J Cancer* **69**: 1078-1082, 1994.

Palladino MA, Srivastava PK, Oettgen HF et al.
Expression of a shared tumour-specific antigen by two chemically induced BALB/c sarcomas. *Cancer Res* **47**: 5074-5079, 1987.

Paterson R, Tod M, Russel M
Tumours of various sites. In Livingstone E and Livingstone S eds. The results of Radium and X-ray therapy in malignant disease, compiled by Paterson, Tod and Russell, 1950.

Pecher T
Induction of cellular immunity in chimpanzees to human tumor-associated antigen mucin by vaccination with MUC-1 cDNA transfected Epstein-Barr virus-immortalized autologous B cells. *Proc Natl Acad Sci USA* **93**: 1699-1704, 1996.

Pervin S, Chakraborty M, Bhattacharya-Chatterjee M, Zeytin H, Foon KA and Chatterjee SK.
Induction of antitumor immunity by an anti-idiotype antibody mimicking carcinoembryonic antigen. *Cancer Res* **57**: 728-734, 1997.

Pichler WJ and Wyss-Coray T.
T cells as antigen presenting cells. *Immunol Today* **15**: 312-315, 1994.

Pimm MV, Perkins AC, Armitage NC, and Baldwin RW.
The characteristics of blood-borne radiollabeled antibodies and the effect of anti-mouse IgG antibodies on localization of radiolabelled monoclonal antibody in cancer patients. *J Nucl Med* **26**: 1011-1023, 1985.

Pockaj BA, Sherry R, Wei J, Yannelli JR, Carter CS, Leitman SF, Carrasquillo JR, White DE, Steinberg SM, Rosenberg SA and Yang JC.
Localisation of Indium-labelled tumor-infiltrating lymphocytes to tumor in patients receiving adoptive immunotherapy: augmentation with cyclophosphamide and association with response. *Cancer* **73**: 1731-1737, 1994.

Poulter LW, Janossy G, Power C, Sreenan S and Burke C
Immunological/physiological relationships in asthma: potential regulation by lung macrophages. *Immunol Today* **15**: 258, 1994.

Prehn R, Main JM.
Immunity to methylcholanthrene-induced sarcoma. *J Nat Cancer Inst* **18**: 769-778, 1957.

Press OW, Eary JF, Appelbaum FR, Martin PJ, Badger CC, Nelp WB, Glenn S, Butchko G, Fisher D, Porter B, Matthews DC, et al Radiolabelled-antibody therapy of B-cell lymphoma with autologous bone marrow support. *N Eng J Med* **329**: 1219-1224, 1993.

Pressman D and Korngald L
The *in vitro* localisation of anti-wagner osteogenic sarcoma antibodies. *Cancer* **6**: 619-625, 1953.

Proietti E, Tritarelli E, Gabriele L et al.
Combined interleukin 1b/interleukin-2 treatment in mice: synegistic myelostimulatory activity and protection against cyclophosphamide-induced myelosuppression. *Cancer Res* **53**: 569-576, 1993.

Pullyblank AM and Monson JRT.

Monoclonal antibody treatment of colorectal cancer. *Br J Surg* **84**: 1511-1517, 1997.

Rabinowich H, Cohen R, Bruderman I.
Functional analysis of mononuclear cells infiltrating into tumours. Lysis of autologous human tumour cells by cultured infiltrating lymphocytes. *Cancer Res* **47**: 173-177, 1987.

Ragnhammar P, Fagerberg J, Frodin J-E et al.
Effect of monoclonal antibody 17-1A and GM-CSF in patients with advanced colorectal carcinoma- long-lasting, complete remissions can be induced. *Int J Cancer* **53**: 751-758, 1993.

Raychaudhuri S
Anti-idiotype antibodies : An alternative approach to tumour immunotherapy. *Indian J Exp Biol* **27**: 671-680,1989.

Reddish MA, MacClean GD, Poppema S, Berg A, Longenecker BM.
Pre-immunotherapy serum CA27.29 (MUC-1) mucin level and CD69+ lymphocytes correlate with effects of Theratope sialyl-Tn-KLH cancer vaccine in active specific immunotherapy. *Cancer Immunol Immunother.* **42**:303-309, 1996.

Reithmuller G, Schneider- Gadicke E, Schlimok G et al.
Randomised trial of monoclonal antibody for adjuvant therapy of resected Dukes C colorectal carcinoma. *Lancet* **343**: 1177-1183, 1994.

Reithmuller G, Holz E, Schlimok G et al
Monoclonal antibody (MAb) adjuvant therapy of Dukes C colorectal carcinoma: 7-year update of a prospective randomized trial. *Proc of the Am Soc of Clin Oncol* **15**: 1385. 1996 (abstract).

Reyband JF.
Memoire sur un tumeur cancereuse affectant le colon. *Bull Acad Med Paris* 9:1031, 1844.

Robb H.
Interleukin-2: the molecule and its function. *Immunology Today* 5: 203-207, 1984.

Robins RA
T cell responses at the host:tumour interface. *Biochem Biophys Acta* 856: 289-305, 1986.

Robins RA, Denton GWL, Hardcastle JD , Austin EB , Baldwin RW and Durrant LG.
Antitumor Immune Response and Interleukin 2 Production Induced in Colorectal Cancer patients by Immunisation with Human Monoclonal Anti-Idiotypic Antibody *Cancer Res* 51: 5425-5429, 1991.

Roitt IM, Cooke A, Male DK, Hay FC, Guarnotta G, Lydyard PM et al.
Idiotypic networks and their possible exploitation for manipulation of the immune response. *Lancet* I: 1041-1045, 1981.

Rosenberg SA, Speiss P and Lafreniere R.
A new approach to the adoptive immunotherapy of cancer with Tumour-infiltrating Lymphocytes. *Science* 233: 1318-1321, 1986.

Rosenberg SA, Lotze MT, Muul LM et al.
A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2,or high dose interleukin-2 alone *N Engl J Med* **316**: 889-897, 1987.

Rosenberg SA.
The development of new immunotherapies for the treatment of cancer using Interleukin-2. *Annals of Surgery* **208**: 121-135, 1988.

Rosenberg SA, Lotze MT, Yang JC et al.
Experience with the use of high-dose interleukin-2 in the treatment of 652 cancer patients. *Ann Surg* **210**: 474-485, 1989.

Ross AH, Lubeck M, Steplewski Z and Koprowski H.
Identification and Characterisation of the CO 17-1A carcinoma associated antigen. *Hybridoma* **5** (suppl 1) S21-28, 1986.

Rothlein R, Czaikowski M, O'Neill MM, Martin SD, Mainolfi E and Merrluzzi VJ.
Induction of intercellular adhesion molecule-1 on primary and continuous cell lines by pro-inflammatory cytokines. *J Immunol* **141**: 1665-1669, 1988.

Rouse BT, Rollinghoff C, Warner NL.
Anti-θ serum induced suppression of the cellular transfer of tumour-specific immunity to syngeneic plasma cell tumour. *Nature Biol* **238**: 116-117, 1972.

Samonigg H, Wilders-Truschnig M, Loibner H, Plot R, Rot A, Kuss I, Werner G et al.
Immune response to tumour antigens in a patient with colorectal cancer after immunization with anti-idiotype antibody. *Clin Immunol Immunopathol* 65: 271-277, 1992.

Sasaki Y, Ohtsu A, Shimada Y et al
Simultaneous administration of CPT-11 and fluorouracil alteration of the pharmacokinetics of CPT-11 and SN-30 in patients with advanced colorectal cancer. *J Nat Cancer Inst* 86: 1096-1098, 1994.

Schroff RW, Foon KA, Beatty SM, Oldham RK,and Morgan AC.
Human antimurine immunoglobulin responses in patients receiving monoclonal antibody therapy. *Cancer Res* 45: 879-855, 1985.

Secco GB, Fardelli R, Lapertosa G et al.
Prognostic value of the Jass histopathological classification in left colon and rectal cancer: a multivariate analysis.*Digestion* 47:71-80, 1990.

Shih W, Baumhefner R, Tourtellote W, Haskett C, Korn E and Fakey J.
Difference in effect of single immunosuppressive agents (cyclophosphamide, CCNU and 5-FU) on peripheral blood immune cell parameters and central nervous system immunoglobulin synthesis rate in patients with multiple sclerosis. *Clin Exp Immunol.* 53: 122-132, 1983.

Shiraki K, Tsuji N, Shioda T, Isselbacher KJ, and Takahashi H.
Expression of Fas ligand in liver metastases of human colonic adenocarcinomas. *Proc Nat Acad Sci USA.* 94: 6420-6425, 1997.

Smith KA.

Interleukin-2: inception, impact and implications. *Science* **240** : 1169-1176, 1988.

Smith KA.

The Interleukin-2 receptor. *Ann Rev Cell Biol* **5**: 397-425, 1989.

Somasundaram R, Zaloudik J, Jacob L, Benden A, Sperlagh M, Hart E, Marks G, Kane M, Mastrangelo M and Herlyn D.

Induction of antigen-specific T and B cell immunity in colon carcinoma patients by anti-idiotypic antibody. *J Immunol* **155**: 3253-3261, 1995.

Sparks FC, Wile AG, Ramming KP, Silver HK, Wolk RW and Morton DC. Immunology and chemo-immunotherapy of breast cancer. *Arch Surg* **111**: 1057, 1976.

Srivastava PK and Das MR

The serologically unique cell surface antigen of Zajdela ascitis hepatoma is also its tumour associated transplantation antigen. *Int J Cancer* **33**: 417-422, 1984.

Srivastava PK, De Leo AB, Old LJ.

Tumour rejection antigens of chemically induced tumours of inbred mice. *Proc Natl Acad Sci.* **83**: 3407-3411, 1986.

Srivastava PK.

Personal communication. 1996.

Stein KE and Soderstrom T.
Neonatal administration of idiotype or anti-idiotype primes for protection against Escherichia coli K13 infection in mice.*JExpMed* **160**: 1001-1011,1984.

Steinman RM, Witmer-Pack M and Inaba K.
Dendritic cells : antigen presentation, accessory function and clinical relevence. *Adv Exp Med Biol* **329**: 1-9, 1993.

Strouss GJ and Decker J.
Mucin-type glycoproteins. *Crit Rev Biochem Mol Biol* **27**: 57-92, 1992.

Svennevig JL, Lunde OC, Holter J & Bjorgsvik D.
Lymphoid infiltration and prognosis in colorectal carcinoma. *Br J Cancer* **49**: 375-377, 1984.

Sy M-S, Brown AR, Benacerraf B, and Greene MI.
Antigen and receptor-driven regulatory mechanisms III. Induction of Delayed-Type Hypersensitivity to Azobenzenearsonate with Anti-cross-reactive Idiotypic Antibodies. *J Exp Med* **151**: 896-908, 1980.

Syrengelas AD, Chen TT and Levy R.
DNA immunization induces protective immunity against B-cell lymphoma. *Nature Medicine* **2**: 1038-1041, 1996.

Takii Y, Hashimoto S, Iiai T, Hatakeyama K and Abo T
Increase in the proportion of granulated CD56+ T cells in patients with malignancy. *Clin Exp Immunol* 97:522-527, 1994.

Teisa A, Sood U, Pietruk T et al.
In situ quantification of inflammatory mononuclear cells in ductal infiltrating breast cancer. *Am J Pathol* **128**: 52-56, 1987.

Thomas WR, Morahan G, Walker ID, and Miller JFAP.
Induction of delayed-type hypersensitivity to azobenzenearsonate by a monoclonal anti-idiotype antibody.*J Exp Med* **153**: 743-747, 1981.

Tjandra JJ, Pietersz GA, Teh JG, Cutherbertson AM, Sullivan JR, Penfold C and McKenzie IFC.
Phase I clinical trial of drug-monoclonal antibody conjugates in patients with advanced colorectal carcinoma : a preliminary report. *Surgery* **106**: 533-545, 1989.

Topalian SL, Solomon D and Rosenberg SA.
Tumour specific cytolysis by lymphoctytes infiltrating human melanomas. *J Immunol* **142**: 3714-3725, 1989.

Topalian SL, and Rosenberg SA.
Evidence for specific immune reactions against growing cancers in mice and men. in DeVita V, Hellman S, Rosenberg SA (eds). Important Advances in Oncology. Philadelphia, JB Lippencourt.19-41, 1990.

Tormey VJ, Faul J, Leonard C, Burke CM, Dilmec A and Poulter LW.
T-cell cytokines may control the balance of functionally distinct macrophage populations. *Immunology* 90: 463-469, 1997.

Trinchieri G, Matsumoto-Kobayashi M, Clark SC, Seehra J, London L, and Perussia B
Response of resting human peripheral blood natural killer cells to Interleukin. *J Exp Med* 160: 1147-1169, 1984.

Triozzi P, Kim JA, Martin EW, Young DC, Benzies T and Villasmil PM.
Phase I trial of escalating doses of Interleukin-1b in Combination with a fixed dose of Interleukin-2. *J Clin Oncol* 13: 482-489, 1995.

Tsang KY, Zaremba S, Nieroda CA, Zhu Mz, Hamilton JM, Schlom J.
Generation of Human Cytotoxic T-cells specific for Human Carcinoembryonic Antigen epitopes from patients immunized with Recombinant Vaccinia-CEA vaccine. *J Nat Cancer Inst* 87: 982-990, 1995.

Udono H and Srivastava PK.
Heat-shock protein 70 associated peptides elicit specific cancer immunity. *J Exp Med* **178**: 1391-1396, 1993.

Ullman KS, Northrop JP, Verweij CL and Crabtree GR.
Transmission of signals from the T lymphocyte antigen receptor to the genes responsible for cell proliferation and immune function: the missing link. *Annu Rev Immunol* **8**: 421, 1990.

Ullrich SJ, Robinson EA, Law LW et al
A mouse tumour-specific transplantation antigen is a heat shock related protein. *Proc Nat Acad Sci USA* **83**: 3121-3125, 1986.

Ulmer JB, Donnelly JJ, Parker SE et al
Heterologous protection against inluenza by injection of DNA encoding a viral protein. *Science* **259**: 1745-1749, 1993.

Urba WJ, Ewel C, Kopp W et al.
Anti-CD3 monoclonal antibody treatment of patients with CD3-negative tumours: a Phase IA/B study. *Cancer Res* **52**:2394-2401,1992.

Vaessen LMB, Baan CC, Ouwehand AJ et al.
Acute rejection in heart transplant patients is associated with the presence of committed donor-specific cytotoxic lymphocytes in the graft, but not in the blood. *Clin Exp Immunol* 88: 213-219, 1992.

Wadler S and Schwartz EL.
Anti-neoplastic activity activity of the combination of interferon and cytotoxic agents against experimental and human malignancies. *Cancer Res* **50**: 3673-3686, 1990 a.

Wadler S
The role of immunotherapy in colorectal cancer. *Seminars in Oncology* **18** suppl1: 27-38, 1991.

Waldmann TA, Goldman CK, Robb RJ et al.
Expression of Interleukin-2 receptors on on activated human B cells. *J Exp Med* **160**: 1450-1466, 1984.

Waldmann TA.
The Interleukin-2 receptor. *J Biol Chem.* **266**: 2681-2684, 1991.

Walsh GM, Williamson ML, Symon FA et al.
Ligation of CD69 induces apoptosis and cell death in Human eosinophils cultured with Granulocyte-macrophage Colony stimulating factor. *Blood* **87**: 2815-2821,1996.

Wang HM, Smith KA.
The Interleukin-2 receptor: functional consequences of its bimolecular structure. *J Exp Med* **166**: 1055-1067,1987.

Watt AG and House AK.
Colonic carcinoma. A quantitative assessment of lymphocyte infiltration at the periphery of colonic tumours related to prognosis. *Cancer* **41**: 279-282

Weidmann E, Sacchi M, Plaisance S, Heo DS et al.
Receptors for Interleukin-2 on human squamous cell carcinoma cell lines and tumor in situ. *Cancer Res* **52**: 5963-5970, 1992.

Weissman AM, Baniyash M, Hou D et al
Molecular cloning of the z chain of the T-cell antigen receptor. *Science* **239**: 1018-1021, 1988.

Werkmeister J, Phil E, Nind A, Flannery GR and Nain C.
Immunoreactivity by intrinsic lymphoid cells in colorectal carcinoma. *Br J Cancer* **40**: 839-847, 1979.

Winter G and Milstein C.
Man-made antibodies. *Nature* **349**: 293-299, 1991.

Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, and Felgner PL.
Direct gene transfer into mouse muscle *in vivo*. *Science* **247**: 1465-1468, 1990.

Working party on the clinical use of Antibodies.
Operation manual for control of production, pre-clinical toxicology, and Phase I trial of anti-tumour antibodies and drug antibody conjugates. *Br J Cancer* **54**: 557-568, 1986.

Yamaue H and Tanimura H.
Clinical application of IL-2 and IL-4 for cancer biotherapy. *Biotherapy* **10**: 1147, 1996.

Yasumura S, Lin W-C, Weidmann E, Hebda P and Whiteside TL
Expression of Interleukin-2 receptors on human carcinoma cell lines and tumor growth inhibition by interleukin-2. *Int J Cancer* **59**: 225-234, 1994.

Yoo Young Kul, Heo DS, Hata K.
Tumour infiltrating lymphocytes from human colon carcinomas. Function and phenotypic characteristics after long term culture in recombinant Interleukin-2. *Gastroenterology* **98**: 259-268, 1990.

Young JW and Inaba K
Dendritic cells as Adjuvants for Class I Major Histocompatibility Complex-restricted Antitumour Immunity. *J Exp Med* **183**: 7-11, 1996.

Zhang C, Zhaohui A, Seth A and Schlossman S.
A mitochondrial membrane protein defined by a novel monoclonal antibody is preferentially detected in apoptotic cells. *J Immunol* **157**: 3980-3987, 1996.

Zitvogel L, Mayordomo JI, Tjandrawan T, DeLeo AB, Clarke MR, Lotze MT, and Storkus WJ.
Therapy of Murine Tumours Peptide-pulsed Dendritic Cells : Depemdence on T Cells, B7 Costimulation, and T helper cell-associated Cytokines. *J Exp Med* **183**: 87-97, 1996.