AN EVALUATION OF EPITHELIAL MEMBRANE ANTIGEN AS A TARGET FOR MONOCLONAL ANTIBODIES IN THE INVESTIGATION OF PATIENTS WITH COLORECTAL CANCER

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Summary

The Epithelial Membrane Antigen(EMA) is known to be strongly expressed by the majority of adenocarcinomas including those of the breast, ovary and colon and also weakly by a variety of normal glandular tissues. This antigen has not, however, been investigated specifically in colorectal cancer. This thesis investigates the clinical applications in colorectal cancer of a monoclonal antibody(MAb), labelled ICR2 after the Institute of Cancer Research, Sutton, Surrey, which recognises EMA.

The expression of EMA in histological sections was explored by immunohistochemical techniques using human paraffin embedded tissues and the indirect immunoperoxidase staining method. The majority of primary colorectal cancers and metastases were found to strongly express the antigen whereas EMA was rarely expressed by normal colon and by adenomatous polyps only at sites of severe dysplasia. A further study compared the expression of EMA with Carcinoembryonic Antigen (CEA) in tissues from a consecutive series of patients undergoing resection for a colorectal cancer. EMA expression was found to be more restricted than CEA in that fewer primary and metastatic cancers expressed the antigen but more specific in that EMA, unlike CEA, was not expressed by normal colon nor benign adenomatous polyps. When the antigen expression of the cancers was graded and compared with standard pathological variables the expression of CEA was shown to be greater than EMA in most cancers and that CEA expression of cancers increased from proximal to distal in the colon.

Immunohistochemistry has been suggested as a means of detecting microscopic tumour deposits which may be overlooked

by routine staining methods. This was investigated in a series of lymph nodes from patients undergoing resection of a colorectal cancer using MAb's targeting both EMA and CEA and the indirect immunoperoxidase staining method. The results, however, did not suggest this to be a clinically useful technique.

Having demonstrated the presence of EMA in colorectal cancer sections a study was carried out to determine whether this antigen is present in the circulation of cancer patients, and if so whether it might be used as a tumour marker. A radioimmunoassay (RIA) using the MAb ICR2 was used to measure EMA in serum from healthy controls and patients with breast and colorectal cancer. All serum samples showed evidence of circulating antigen and the levels in patients with breast or colon cancer were not significantly different from controls.

To allow the MAb ICR2 to be used for the detection of colorectal cancer in patients the antibody was labelled with the gamma emitting radionuclide ^{111}Indium (^{111}In) and its in-vitro stability analysed. Previous studies have demonstrated that the major limitation to the detection of gastro-intestinal cancers using ^{111}In labelled MAb's is the uptake of radioactivity by the liver. This problem was investigated prior to clinical studies by the use of an in-vitro model consisting of freshly isolated hepatocytes and the EMA expressing tumour cell line MCF7. Various reagents were analysed for their ability to reduce the uptake of radioactivity by the hepatocytes without affecting tumour cell uptake when ^{111}In-ICR2 was applied to both cell groups. The synthetic metal chelating agent diethylene-triamine-pentacetic acid (DTPA) was found to significantly reduce the hepatocyte uptake without affecting the tumour uptake of radioactivity when incubated with the cells or added to the radiolabelled antibody either at the time of or prior to its application to the cells. The effect of incubating DTPA

with the radiolabelled antibody was dependent on the concentration of DTPA and the period of incubation with the radiolabelled antibody.

An imaging study was carried out in patients with known or suspected colorectal cancer using the ^{111}In labelled MAb ICR2. A control group was compared with a group whose injectate was treated with DTPA and a group whose injectate was treated with DTPA and who recieved unlabelled MAb prior to ^{111}In-ICR2 administration in an attempt to reduce the radiolabelled antibody forming circulating immune complex. The images were independently interpreted by a clinician with knowledge of the clinical details and investigations and completely blind by a nuclear medicine specialist provided with no clinical information. Imaging with ^{111}In-ICR2 was found to be a sensitive method of detecting primary cancers and detected metastatic lymph node deposits pre-operatively. False positive images, however, were produced with inflammatory lesions and dysplastic benign polyps and liver metastases could not be detected.

The imaging results were not significantly different in the three groups of patients outlined above. Analysis of the biodistribution of ^{111}In-ICR2 in these groups of colorectal cancer patients has also shown there to be no significant difference in the percentage of the injected dose taken up in the tumour tissue or in the ratio of activity in the tumour to that of normal colon. The uptake of activity in the tumour was, however, shown to be related to the antigen expression of the cancer and also the level of circulating antigen. Chelation of the ^{111}In-ICR2 with DTPA prior to its administration reduced the urinary excretion of radioactivity but did not reduce the liver uptake of radioactivity. Unlabelled MAb administration produced a minimal reduction in the level of circulating immune complex.

In addition to carrying out external imaging of the colorectal cancer patients following ^{111}In-ICR2 administation the use of a radiation detecting probe for the localisation of metastases at the time of operation was also investigated. This technique, first described in 1984, should theoretically allow smaller lesions to be detected than by external imaging. The majority of patients with cancer had higher counts recorded over the tumour than over normal colon whereas those with benign lesions showed no preferential uptake. Counting over the lymph nodes in the large bowel mesentery could differentiate glands containing cancer deposits from those which did not by the uptake of radioactivity relative to normal colon.

The limitations of intra-operative probing following radiolabelled MAb administration have not previously been investigated. A model of the clinical situation was therefore established using hollow spheres filled with a radioactive solution to represent 'tumour' deposits placed within a tank containing a mildly radioactive solution to represent the background radioactivity of normal tissues. By altering the size of 'tumours' and the ratio of radioactivity in 'tumour' to that of background the ability was investigated of the two currently available forms of radiation detector to locate the 'tumour' deposit within the background radioactivity using a statistical criteria of detectability. This study demonstrated that for the reliable detection of small tumour deposits using an intra-operative probe a high tumour to background ratio is required, but that this is within the range of values which have been reported from patient studies.

Abbreviations used in this thesis

AFP	Alpha-foetal protein
ApoTf	Apotransferrin
BCG	Bacillus Calmette Guerin
BSA	Bovine serum albumin
Ca	Carcinoma
ccDTPA	Bicyclic anhydride of DTPA
CBH	Chester Beattie Hooded
CdTe	Cadmium Telluride
CEA	Carcinoembryonic antigen
Cs	Caesium
CT	Computerised axial tomography
DAB	Diamino benzidine
DFO	Desferrioxamine
DIPY	2,2' dipyridyl
DTPA	Diethylene Triamine Pentacetic Acid
EDHPA	Ethylendiamine-di (0-hydroxyphenylacetic acid)
EDTA	Ethylendiaminetetracetic acid
EGFR	Epidermal Growth Factor Receptor
EMA	Epithelial Membrane Antigen
F	False
F(ab)	Fraction (antibody binding)
Fc	Fraction (constant)
FCS	Foetal calf serum
Fe	Female
Flex	Flexure
(5)FU	5 Flourouracil
GDP	Gamma Detecting Probe
GICA	Gastro-Intestinal Cancer Antigen
HAMA	Human Anti-Mouse Antibody
H & E	Haematoxylin and Eosin
HCl	Hydrochloric acid
Hemi	Hemicolectomy
Hep	Hepatocyte
HMFGM	Human Milk Fat Globule Membrane
HPLC	High pressure liquid chromatograhy

Abbreviations (continued)

I	Iodine
I.C.	Immune Complex
I.D.	Injected dose
Ig	Immunoglobulin
In	Indium
I.O.	Intra-Operative
I.P.	Intra-Peritoneal
I.V.	Intra-Venous
Lt	Left
M	Male
MAb	Monoclonal Antibody
MDP	Methylene Di-Phosphonate
MFGM	Milk Fat Globule Membrane
MTX	Methotrexate
MWt	Molecular Weight
PBS	Phosphate Buffered Saline
PMT	Photo Multiplier Tube
PVC	Poly Vinyl Chloride
RES	Reticulo-Endothelial System
R/S	Recto-Sigmoid
RIA	Radio-Immuno Assay
RIL	Radio Immuno Localisation
S.D.	Standard Deviation
S.E.	Standard Error
SPECT	Single Photon Emission Computerised Tomography
TBS	Tris Buffered Saline
Tc	Technetium
Tf	Transferrin
Tl	Thallium
Trans	Transverse
T/NC	Tumour to Normal Colon ratio
U.K.	United Kingdom
U.S.A.	United States of America
U.V.	Ultra Violet

Abbreviation (continued)

Weights and measures

g	gram	Ci	Curie
mg	milligram	cpm	counts per minute
ug	microgram	cps	counts per second
ng	nanogram	MBq	Megabecquerel
l	litre	V	Volts
ml	millilitre	kD	kiloDaltons
ul	microlitre		
M	molar		
mM	millimolar		
uM	micromolar		
nM	nanomolar		

Statement of originality

The use of monoclonal antibodies to epithelial membrane antigen in the investigation of patients with colorectal cancer has not previously been investigated. The work reported in this thesis has been carried out entirely by myself in collaboration with and assisted by other researchers as outlined in the acknowledgements.

CHAPTER 1

HISTORICAL REVIEW AND BACKGROUND TO THE PRESENT STUDY

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1.1. DIAGNOSIS AND TREATMENT OF COLORECTAL CANCER

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ii) Immunohistochemical studies

iii) Radioimmunolocalisation of cancers

1.6. CONCLUSIONS AND PURPOSE OF THE PRESENT STUDY

1.1. DIAGNOSIS AND TREATMENT OF COLORECTAL CANCER

Colorectal cancer is the second most common cause of death from malignant disease, with over 18,000 deaths annually in the United Kingdom [1]. Despite improvements in the standard of patient care the mortality from this condition has not improved significantly over the last two decades[2-4], with a mean five year survival generally less than 50% [5,6]. The stage of the disease at presentation is the most important factor affecting survival, patients with cancers confined to the bowel mucosa having greater than 90% 5 year survival, those with invasion of the muscular coat 50-80% and approximately 30% when regional lymph nodes are involved[3,7,8].

1.1.i Screening and early detection

Unfortunately only about 1 in 20 people presenting with colorectal cancer will have mucosal lesions and hence a good prognosis[3]. The relationship between the presence of localised disease and a good prognosis has focused attention on the importance of the early detection of colorectal cancer and the management of pre-malignant lesions such as adenomatous polyps[9-11]. Sigmoidoscopy may be used as a method of screening for colorectal cancer and in 1978 Gilbertson reported the findings of proctosigmoidoscopy on asymptomatic patients over 45 years of age[12]. The initial procedure discovered 27 cancers in 21,150 patients and

on annual follow up (mean 5.4 examinations / patient) a further 13 cancers. By removing all adenomatous polyps the expected incidence of colorectal cancer in this defined population was decreased by 90% over the period 1948-1976. Although these results would suggest a beneficial effect of screening by sigmoidoscopy, this may not be an acceptable method of screening for many asymptomatic patients. With symptomatic patients, however, the use of endoscopy and improvements in radiological techniques appear to have increased the number of patients being diagnosed with early disease[13].

An alternative, non-invasive approach to the early detection of colorectal cancer is the use of population screening with faecal occult blood testing[14-16]. The major drawbacks to this form of screening study are that the patient compliance is generally poor, the detection rate and test sensitivity low and a long follow up period is required to prove a survival advantage. Despite these problems occult blood screening has been shown to increase the number of early stage cancers detected and should therefore improve the prognosis[17,18].

1.1.ii) Surgical treatment

Surgery remains the best treatment option for patients with colorectal cancer. Although the operative technique employed is often controversial little evidence exists that any particular method carries a

more favourable prognosis. In 1908 Moynihan suggested high ligation of the inferior mesenteric vessels to be an important factor in preventing the recurrence of rectosigmoid carcinoma[19]. This was not supported, however, by the study of Pezim and Nicholls[20] which analysed the 5 year survival of 1370 patients undergoing surgery for cancers of the rectum and rectosigmoid at St Marks Hospital. No difference was found between those having ligation above and those having ligation below the left colic vessels. The importance of a radical removal of regional lymphatics has also been questioned, with no benefit being shown in a group of patients undergoing extended abdominal-iliac lymphadenectomy as opposed to conventional resection[21].

A no-touch operative technique in which the supplying and draining blood vessels are divided prior to mobilising the tumour has been advocated by Turnbull[22] to limit the known dissemination of tumour cells at the time of operation[23] and reduce the risk of recurrence. However, in Turnbull's study the survival results were compared between one surgeon practising, and a group of surgeons who were not practising the no-touch technique, and the patients were not randomised. In a prospective controlled trial the no-touch isolation technique decreased the number and delayed the development of liver metastases but with no improvement in survival[24]. The importance of

operative technique to the results of surgery for rectal cancer was emphasised in the study by Heald reported in 1986 in which the local recurrence rate of 4% and long term survival of 80% was attributed to the careful dissection of the mesorectum [25]. This study was, however, uncontrolled and therefore open to misinterpretation.

1.1.iii) Adjuvant therapy

Because of the advanced nature of the disease at presentation surgery is curative in less than half of those treated [3]. In order to improve the results obtained adjuvant therapies have been investigated.

In 1959 Stearns reported the results of the use of pre-operative radiotherapy for rectal carcinomas [26]. Giving 1000-2000 rads in 10 daily fractions resulted in both a better 5 and 10 year survival in treated over untreated patients although the study was not randomised. A similar dosage regime was used by Roswit in a prospective controlled randomised study reported in 1975[27] which suggested that radiotherapy reduced the incidence of Dukes stage C lesions. This finding has subsequently been supported by the MRC working party on adjuvant radiotherapy[28] who studied 824 patients with operable rectal cancer. This randomised multicentre study compared surgery alone, surgery and a single pre-operative 500 rad fraction of radiotherapy and surgery following 2000 rads in 10 daily fractions. Although the patients given the higher

radiotherapy dose had fewer Dukes C tumours and their tumours were significantly smaller the 5 year follow up has shown no difference in recurrence rate nor survival benefit.

Despite the many studies carried out on adjuvant chemotherapy few have shown any evidence of improvement in the prognosis for patients with colorectal cancer. A large randomised study carried out by the Gastrointestinal study group in the U.S.A. compared chemotherapy, immunotherapy and a combination of both with a control group [29]. The chemotherapy group receiving 5-fluorouracil(5FU) and semustine, the immunotherapy group receiving a methanol extract of the Bacillus Calmette Guerin(BCG), and the combination of both therapies showed no survival advantage over the control group. Systemic chemotherapy, however, delivers a relatively small drug concentration to the liver which is the main site of colorectal cancer metastases. For this reason considerable interest has surrounded the use of adjuvant regional therapy. In 1985 the results of a randomised controlled trial of adjuvant portal vein 5FU were reported [30]. Patients with no evidence of liver metastases were randomised to receive either 1g daily of 5FU into the portal vein over the first 7 post-op days or no adjuvant treatment.

Fewer liver metastases developed in the treatment group and the Dukes B patients showed an improved survival. Encouraging results have also been obtained in the use of adjuvant systemic 5FU in combination with

the immune stimulant Levamisole which has been shown to reduce the period of post-operative immunosuppression [31]. Comparing groups of patients receiving 5FU alone, 5FU with Levamisole and a control group showed death from tumour recurrence on 5 year follow up to be 44%, 32% and 52% respectively. This represented a significant survival advantage to those patients receiving 5 FU and levamisole [32].

Also of significant interest in the adjuvant treatment of colorectal cancer was the study on the anti-mitotic drug Razoxane reported in 1986 [33]. A 5 year follow up of 272 randomised patients showed a prolonged time to recurrence in the Dukes C patients receiving Razoxane. Unfortunately the side effect of the development of acute leukaemia in three of these patients would seem to prohibit further evaluation of this drug.

1.1.iv) Staging and the detection of recurrence

Adjuvant radiotherapy and chemotherapy seem to have benefitted only a small percentage of patients undergoing surgery for colorectal cancer.

One of the reasons for the poor results of treatment may be incorrect staging and hence inappropriate or inadequate management of the primary cancers. Routine staging of patients with colorectal cancer will be based on a clinical examination, sigmoidoscopy or colonoscopy, a barium series, chest X-ray, liver function test and either a radioisotope or

ultrasound scan of the liver. Newer technology may improve the results of pre-operative staging. Computerised axial tomography(CT) has been shown to be better than either ultrasound or radionuclide imaging in the detection of liver metastases[34] and can detect occult metastases in up to 24% of patients [35]. The liver may also be examined intra-operatively using an ultrasound probe which is capable of detecting metastases less than 0.5cm in diameter [36].

With rectal cancers the involvement of lymph nodes in the mesorectum as well as direct lateral spread have been shown to be important factors in prognosis and hence are important aspects of pre-operative staging [25,37]. Although some would claim that accurate staging of rectal cancers may be achieved clinically [38] other alternatives include CT scanning and endoluminal ultrasound. In a study comparing these techniques with clinical examination the closest approximation to operative and pathological findings was achieved by endoluminal ultrasound. All these techniques provided useful information on direct disease spread but none reliably discriminated involved from uninvolved lymph nodes [39].

Approximately 50% of patients undergoing resection for a colorectal cancer will die of disease recurrence [5,6]. By the time diagnosis of recurrence is made the majority have disseminated disease and will not benefit from any operative intervention. Local disease was found in 27% of patients with recurrence at

post mortem but diagnosed in 55% of patients in a prospective clinical study, the difference being attributed to the clinical under-staging of disease [40]. Methods for the early detection of recurrent disease are clearly required. Wangsteen proposed the concept of "second-look" surgery in an attempt to detect early locally recurrent disease and reported that 17% of patients undergoing second look surgery could be returned to a disease free state [41,42]. Although the concept may be appealing, in reality many patients with either no recurrence or recurrence beyond resection would be required to undergo surgery for the questionable benefit of a few. The use of circulating tumour markers as a guide for those patients likely to have recurrent disease and hence to benefit from a second look laparotomy has therefore been intensively investigated [43,44]. Raised levels of circulating tumour markers, however, are usually associated with significant disease bulk and are insensitive for the detection of locally recurrent disease.

It is apparent that improvements are needed in the staging of primary cancers, their initial management including adjuvant therapy, methods for diagnosing disease recurrence and new treatment modalities. The discovery of tumour antigens, substances expressed principally on cancerous tissue, has opened the door to a new approach to the investigation and treatment of patients with colorectal cancer by the use of

polyclonal and more recently monoclonal antibodies to these tumour associated antigens. Almost every aspect of present management may be influenced with immunological methods of cancer screening, the use of radiolabelled antibodies for improved staging of primary cancers and detecting recurrences by external imaging as well as the intra-operative localisation of tumour deposits using radiation detectors. In addition further circulating tumour markers may be detected by antibodies allowing improved methods of diagnosis and follow up. The present stage in the clinical use of antibodies in colorectal cancer is reviewed in section 1.3.

1.2. THE DEVELOPMENT OF ANTIBODIES TO TUMOUR ANTIGENS

In 1953 Foley published the results of work carried out on the "antigenic properties of methycholanthrene-induced tumours in mice of strain of origin" [45]. In these studies Foley demonstrated that methycholanthrene induced tumours transplanted between syngeneic (highly inbred) mice could be rejected under appropriately controlled circumstances. Since these tumours were transplanted between genetically identical animals the rejection could only be due to an immunological response to a newly developed antigen on the surface of the tumour during neoplastic transformation. This formed the first conclusive evidence of antigens forming on chemically induced tumours which were termed tumor-specific transplantation antigens as tumour rejection was used as the reaction to detect their presence.

Although tumour antigens have now been defined on a variety of human tumour tissues none, apart from the idiotype to the surface Ig found on some B cell lymphomas[46], have been found to be entirely specific for cancer. Many tumour antigens represent only quantitative or qualitative differences from naturally occurring tissue or serum components and are considered tumour antigens only in that they are sought by antibody probes. Examples of such antigens are the paraproteins associated with multiple myeloma, the pathological elevations of human chorionic

gonadotrophin (HCG) in patients with choriocarcinoma and prostatic acid phosphatase in patients with carcinoma of the prostate. True tumour antigens are substances which appear de novo during tumour growth and include specific tumour antigens and the larger group of oncofoetal substances (carcinoembryonic antigen (CEA), alpha foetoprotein (AFP), haemoglobin F and some enzymes).

Production of polyclonal antisera

The first reports on the use of antibodies in the treatment of cancer were those of Hericourt and Richet in 1895[47] who prepared antisera to a human osteogenic sarcoma in an ass and two dogs and used it in the treatment of two patients, one with a fibrosarcoma of the chest wall and the other a gastric cancer. The initial results stimulated a more extensive study of 50 patients with cancer who similarly were felt to have benefitted from treatment whereas those given normal serum obtained no similar response [48]. Antisera raised against tumour tissue contain many clones of antibodies(polyclonal). Although many attempts to use polyclonal sera in therapy have been made since this time no convincing evidence of a therapeutic potential has been established. The presence within this mixture of antibodies against tumour tissue was demonstrated in 1954 by the radioactive labelling of polyclonal antisera with subsequent specific localisation of activity in tumour tissue [49].

It was subsequently demonstrated that not only could radiolabelled antibodies localise in tumour tissue but that a therapeutic dose of radioactivity could be delivered [50]. The obvious disadvantage of polyclonal antisera is the diverse nature of the target antigens, only some clones of antibodies being related to tumour tissue. This problem was initially tackled by removal of the cross reacting antibodies by injection into a second animal[51]. Results were greatly improved when the target antigen could be isolated, such as with CEA, and affinity purification of the polyclonal antisera was introduced[52].

Production of monoclonal antibodies

An antigen usually has several determinants, or epitopes, which can stimulate the conversion of a B lymphocyte into a plasma cell. Immune responses therefore result in the production of polyclonal antibodies. If individual plasma cells were selected and cloned antibodies with a single defined specificity could be obtained, but unfortunately plasma cells do not survive in tissue culture. In 1975 Kohler and Milstein published their work on the production of monoclonal antibodies by the fusion of sensitised lymphocytes and a myeloma cell line [53]. This resulted in the ability to produce a plentiful supply of pure antibody recognising a single epitope of any specific antigen and has had a monumental impact on the use of antibodies as both diagnostic and therapeutic tools.

1.3. THE USE OF ANTIBODIES IN COLORECTAL CANCER

New applications for the use of monoclonal antibodies in clinical medicine are being investigated at a staggering rate. In the field of colorectal cancer the main areas being explored at present include the following ;

1.3.i) Serology

1.3.i.a) Circulating tumour antigens

Tumour markers are substances secreted into the circulation by malignant tumours and which may be used to assess the presence, stage or progress of disease. The nature of these markers varies greatly, some being recognisable products of normal cell metabolism and others having no known biological function. Normal products of cellular metabolism which may be produced in excessive amounts in malignancy include the enzyme acid phosphatase whose level in the serum has proven of value in the management of prostatic carcinoma[54] and beta HCG, a hormone useful in the monitoring of germ cell carcinomas [55]. Perhaps the largest group of both established and potential tumour markers are glycoproteins, expressed by normal foetal tissues but not significantly in adult tissues unless neoplasia arises ; the onco-foetal antigens. Included in this group are AFP and CEA, which have been shown to be useful clinical markers of hepatocellular carcinomas and gastrointestinal cancers respectively [56,57]. Many

other tumour associated antigens have been described. The majority are not oncofoetal in nature but are simply expressed more strongly by malignancies than normal tissues. Some are glycoproteins although the vast majority of "new" tumour markers are carbohydrates, such as CA125[58] found in ovarian cancer and CA 19-9[59] in colonic and pancreatic cancer.

Other circulating markers of malignancy which have been investigated in patients with colorectal cancer include levels of immune complexes [60,61] and the secretory immunoglobulins IgA and IgM [62,63]. Levels of immune complex were elevated in a high percentage of patients with benign polyps and equated poorly with tumour bulk whilst secretory immunoglobulin was significantly raised only when liver metastases were present.

1.3.1.b) Carcinoembryonic antigen levels in colorectal cancer

It is now almost twenty years since CEA was first suggested as a serum marker in colorectal cancer. Since this time investigators have looked at many different applications of the serum CEA level including its use in diagnosis, staging and detecting recurrent colorectal cancer. Others have used CEA levels as a basis for second look laparotomy and to monitor patient response to chemotherapy.

The majority of patients with colorectal cancer have an elevated level of CEA pre-operatively. The degree of elevation will depend on the arbitrary cut-off level which is taken as "abnormal". In a series of 518 patients with gastro-intestinal neoplasms studied by Cooper et al in 1979[64], 78% of patients with colorectal cancer had a level greater than 2.5ng/ml. In this series, however, 56% of patients with other malignancies and almost 50% of those with benign disease had similarly elevated levels. This would not suggest a useful role for CEA levels in the screening for colorectal cancers. A correlation is generally found between the stage of the disease at presentation and the CEA level, patients with extensive disease being more likely to have elevated levels [65]. Although previously reported not to correlate with resectability [66] the pre-operative CEA level has been shown on stastistical analysis to correlate with tumour fixity [67]. The pre-operative CEA level has also been shown to have a prognostic value, a pre-operative level greater than 2.5ng/ml being associated with an increased risk of subsequent local recurrence or of later metastatic disease [68].

Although the use of CEA levels for the diagnosis or staging of colorectal cancer has proven to be disappointing it may have a role in the detection of recurrent disease following resection. Mach demonstrated that following curative resections

CEA levels return to normal and that failure to do so was suggestive of residual disease [69]. Longer follow up of this series of patients, however, revealed a group who had moderately raised and fluctuating levels of CEA but had no evidence of recurrent disease. This group did not include patients with conditions known to be associated with elevations in circulating CEA such as heavy cigarette smokers and those with liver disease [70]. Despite these difficulties a group of patients could be defined in whom a rise in CEA levels preceded the recurrence of clinically obvious disease and who may therefore have benefitted from interventional treatment. The reported "lag time" was 2-26 months. Although other authors have supported the concept of a useful lag in the time between CEA levels becoming elevated and the recurrence of clinically detectable disease [71,72] the opposite situation was found by Finlay [73], who demonstrated hepatic metastases by CT scanning a considerable period of time before the serum CEA level became elevated. Whether the finding of asymptomatic recurrent disease by elevations of post-operative CEA levels and subsequent treatment will affect the prognosis remains to be established in long term prospective studies. Despite this some surgeons have based a second-look operation on a persistently elevated level [43,74-76]. Although refinements in CEA determination eg. the frequency of serial sampling, slope analysis and CEA nomograms may increase the certainty with which recurrent disease is

diagnosed the beneficial effect of second look surgery remains to be determined.

Treatment based on elevation of CEA levels is likely to be most beneficial when recurrent disease is minimal and not detectable by conventional means. On this premise Hine and Dykes[77] prospectively randomised patients, who were asymptomatic but had persistently raised CEA levels following resection for colorectal cancer, for either chemotherapy or no specific treatment. A course of 5FU and methyl CCNU produced no significant difference between treated and untreated groups as regards disease free interval and survival. The CEA levels in these patients did, however, show an inflection at the time of chemotherapy.

The majority of patients with disseminated colorectal cancer will have elevated CEA levels, the absolute number depending on the cut off level selected as normal. Elevated levels of CEA have been used as a means of monitoring tumour bulk and hence the response of individual patients to chemotherapy. Although patients in remission have been found to have reducing levels of circulating CEA and those with progressing disease exhibit rising levels, the correlation is poor in the majority of patients [78,79]. Serum levels of CEA have also been shown to correlate poorly with radiological evidence of tumour regression [80].

1.3.1.c) Other tumour markers in colorectal cancer

The gastrointestinal cancer antigen (GICA) is recognised by a monoclonal antibody CA19-9, raised in the mouse to a human colorectal carcinoma cell line SW1116[81]. Initially the antigen was believed to be specific to the sera of patients with colorectal cancer [82] and to be onco-foetal in origin. Since this time, however, antibody CA19-9 has been shown by immunocytochemistry to bind to both pancreatic and gastric tumours [83] and elevated antigen levels in serum are to be found associated with these malignancies rather than with colorectal cancers [84]. The specificity of the CA19-9 test for diagnosing malignancy is high with less than 2% of normal people having elevated levels but its sensitivity is lower than that of CEA for colorectal cancers. An alternative role for GICA in the diagnosis and investigation of pancreatic carcinoma appears to be more promising at present [85-87]. Prospective studies are awaited to determine the value of serial CA 19-9 estimations in the routine follow up of patients following resection of a colorectal cancer.

Other carbohydrate molecules may be useful as potential tumour markers. For example Stage Specific Embryonic Antigen-1 (SSEA-1) and the Lewis^x antigen have been detected in colorectal cancer tissues using Fluorescein labelled monoclonal antibodies [88] and by RIA in the serum of patients with colorectal cancer. Low levels, however, may be present

in the serum and tissues of normal individuals and in those with non neoplastic diseases [89]. Elevated serum levels of secretory immunoglobulins have been reported in some patients with colorectal cancers, and may signify the presence of metastatic disease [62,63]. Circulating immune complexes have also been monitored in patients with colorectal cancers and following a curative resection or in normal controls the levels of immune complex are minimal. In advanced disease, however, the serum levels of immune complex vary and correlate poorly with tumour bulk[60,61].

Although individually these other markers may not be as clinically useful as CEA they may have a role in the management of patients with colorectal cancer if they can be combined into a prognostic index which accurately predicts those patients likely to develop recurrence and hence may benefit from adjuvant therapy.

1.3.ii) Immunohistochemistry

The role of circulating CEA and other tumour markers has been investigated intensively in patients with colorectal cancer because of their potential use for detecting recurrent disease. The expression of tumour antigens in excised colorectal tissues has yet to establish a definite clinical role. Several important applications are, however, being investigated. In 1984 Heyderman recommended the use of a panel of monoclonal antibodies to epithelial membrane antigen (EMA), CEA and prostatic acid phosphatase to aid in the histological determination of the tissue of origin of poorly differentiated carcinomas [90]. Of more obvious clinical benefit is the use of immunohistochemistry for the detection of micrometastases. In colorectal cancer two main studies have been carried out. In 1984 Crowson reported that 4% of patients with colorectal cancer will have micrometastases in regional lymph nodes which may be detected using anti-CEA MAb's [91]. This contrasts with a recent study suggesting that 25% of patients with colorectal cancer have micrometastases detectable by immunohistochemistry [92]. The clinical significance of these findings is great as this may allow improved staging for patients with colorectal cancer and may indicate a patient group who would benefit from adjuvant therapy.

Another important aspect of the immunohistochemical detection of tumour antigens is

their use as prognostic markers. The antigen expression of primary cancers may be categorised with standard pathological variables to produce better prognostic indicators in large bowel cancer. This may help in the selection of patients for adjuvant therapy [93,94].

The immunohistochemical staining of tumour biopsies for a range of tumour antigens may also provide a means of determining the optimum antibody for use in any individual patient for either tumour imaging with radiolabelled antibodies or for drug, radionuclide or toxin conjugated antibody therapy.

1.3.iii) Radioimmunolocalisation of colorectal cancers

1.3.iii.a) External imaging

Radio-immunolocalisation (RIL) is a term coined for the technique of localising tumours following the administration of radiolabelled antibodies, usually by imaging with an external gamma camera. Its success is dependent on the use of an antibody which is specifically or selectively taken up by tumour tissue being combined with a radioactive isotope with a satisfactory half life and energy of emissions.

Localisation experiments using animals with CEA expressing tumour xenografts implanted subcutaneously have shown that radio-iodinated anti-CEA antibodies can successfully localise these tumours [52,95,96]. On the basis of these experiments Goldenberg administered hyperimmune goat anti-CEA

antiserum radio-labelled with ^{131}I to 18 patients with diverse carcinomas (97). External gamma camera images were obtained at intervals following administration of the radio-labelled antibody. The images obtained were found to be obscured by the blood pool activity of the radionuclide but this problem could be partly overcome by computerised subtraction following the administration of Technetium oxide ($^{99m}TcO_4^-$) and ^{99m}Tc labelled human serum albumin. Of four patients with advanced colorectal cancers three had positive images of the primary tumour and the secondary deposits were identified in all. In the decade since these initial studies were reported by Goldenberg significant changes have taken place in the antibodies used for imaging, the radiolabel and the imaging techniques.

Antibody used in imaging

Early tumour localisation in animal xenografts was carried out using IgG fractions of anti-CEA serum. This serum was produced by the repeated innoculation of CEA derived from colorectal tumours into a host animal and the subsequent seperation of the IgG fraction (95). Such antisera contained many antibody species and was therefore subject to significant cross reactivity. To reduce this problem antisera were absorbed with human red blood cells and a variety of human tissues (liver, lung, spleen, kidney) conjugated to a gel matrix (Sepharose 4B). Further purification was achieved by affinity chromatography in which an isolate

of a specific antigen is used to separate specific antibodies from a mixture (antigen immuno-absorbent)[97].

Although this allowed a significant improvement in purity of the antibody the most noticeable advance in antibody production was the development of hybridoma technology by Kohler and Milstein in 1975 [53]. By the fusion of lymphocytes sensitised by CEA with a myeloma cell line a continuous culture of cells producing monoclonal anti-CEA antibodies could be established. The results achieved by affinity purified anti-CEA antisera in the localisation of colorectal cancers[97-104], however, have been similar on a numerical basis to monoclonal anti-CEA [105,106]. Most studies, however, have been neither controlled nor had any quantification of tumour uptake of radio-labelled antibody. Of the few studies which have quantified uptake the ratios of activity in the tumour to normal colon were found to range from 2 : 1 [107] to 8.6 : 1 [106]. These values may not be directly comparable, however, due to differences in the dose of antibody and radio-label administered and the interval between administration and tissue sampling.

Although anti-CEA antibodies have been the most widely used in the radio-immunolocalisation of colorectal cancers other groups have investigated the use of monoclonal antibodies to other tumour associated antigens. Monoclonal antibody 791T/36 was originally raised using cultured osteogenic sarcoma cells as

immunogen and has been shown to localise osteogenic sarcoma xenografts[108] and human malignant bone tumours [109]. It was also found to react with colorectal cancer cell lines [110] and has been used to localise human colorectal cancers [111-114]. Other antibodies which have been used for the localisation of colorectal cancers have included 17-1A [115] and 19-9 [116] which were raised to colorectal cancer cell lines. Monoclonal antibody YPC 2/12.1, raised to a membrane extract of a colorectal cancer, has been reported as being useful in tumour localisation [117], but this was subsequently disputed [118].

Radionuclides used in tumour imaging

The most widely used radionuclides for the imaging of colorectal cancers in patients are Iodine 131(^{131}I) and Indium 111(^{111}In). ^{131}I was the first radioisotope to be used, being covalently bound to the antibody molecule by either the Iodogen method [119] or the Chloramine T method [120]. There are, however, major drawbacks to the use of ^{131}I as a radionuclide for imaging. Firstly ^{131}I has a relatively long half-life of 8 days and tends to persist in the intravascular compartment. This results in a high background activity and the need to employ computerised subtraction techniques using a second radionuclide [97,111,121]. Such techniques may, however, result in the production

of false positive images [122-124]. The administration of radioiodine also requires the pre-administration of inactive iodine prior to scanning to block the uptake of radioactivity in the thyroid gland. Other drawbacks include the high energy gamma emissions of ^{131}I which are unsatisfactory for imaging techniques and the production of unwanted beta emissions. These difficulties have led some groups to use ^{111}In as the radionuclide of choice [102,105,106,114,125]. The ^{111}In is attached to the protein molecule by the use of a chelating agent as originally described by Krejcarek et al in 1977[126] and later modified by Hnatowich [127-129]. ^{111}In has the advantage of a shorter half life of 2.8 days, medium energy gamma emissions, no beta emissions and does not require subtraction techniques to allow tumour localisation. ^{111}In labelled antibodies are, however, taken up by the liver, spleen and bone marrow independent of the antigen to which they have been raised and this may seriously prohibit the localisation of metastatic deposits [130].

Imaging techniques in radioimmunolocalisation

The standard technique for gathering information following the administration of labelled tumour associated monoclonal antibodies is planar imaging using a gamma camera. From the outset of antibody imaging difficulties arose with imaging due to the high background activity and low tumour uptake of radiolabelled antibody.

With iodinated antibodies this was largely due to persisting activity in the blood pool. Methods were therefore introduced for reducing this background activity by administering a second radionuclide and by techniques for computer subtraction of images [97]. Considerable controversy persists as to the optimum method of background subtraction and whether false positive images are produced [122,123,124,131,132]. In an attempt to avoid the difficulties inherent in dual radio-nuclide subtraction techniques interest has turned to the use of ^{111}In linked to the monoclonal antibody by a metal chelating agent. Regardless of the radionuclide used, however, a low tumour uptake with a high background activity produces a planar image which is difficult to interpret. One possible solution to the problem is to clear the radiolabelled antibody from the circulation once tumour localisation has occurred. This rather novel solution was reported by Begent et al in 1982. A goat anti-CEA MAb was used in patients with colorectal cancers for localising the lesions. Liposomally packaged horse anti-goat antibody was subsequently used to clear the blood pool activity. Using this technique an improved quality of image was reported with three of five patients studied [133]. Another method employed in an attempt to improve the quality of images produced is to produce cross sectional images using single photon emission computerised tomography (SPECT), a technique analagous to CT scanning.

Originally described in 1963[134] this technique has produced an increased sensitivity and specificity in some studies [135]. This may, however, be associated with the production of false positive images as in planar imaging [135]. A further attempt at improving the interpretation of immunoscintigraphy involved the combination of the information obtained from SPECT with that of conventional CT scanning thus improving the anatomical localisation of the tumours [136]. Whether this is a practical technique for routine imaging remains to be established.

One distinct alternative to conventional gamma camera imaging techniques is the application of positron emission tomography (PET). This technique utilises the paired gamma-rays produced from positron-electron interaction. The gamma rays are emitted at 180^{o} to each other and their point of origin can be accurately located by two opposed linked detectors. The application of PET to antibody imaging has yet to be realised.

1.3.iii.b) Intra-operative tumour detection

Immunoscintigraphy using an external gamma camera has as yet a limited clinical application in the investigation of patients with cancer. Although large primary tumours which are easily localised by other modalities may be imaged, the small metastatic or recurrent deposits are rarely detected. Most investigators have reported their failure to image tumours of less than 2cm in diameter, with even poorer results if the tumour is situated deeply. The fundamental problem lies with the specificity and affinity of the radiolabelled MAb's. The uptake of activity achieved in the tumour tissue is generally only 0.01-0.001% of the injected dose and tumour to normal tissue differentials in uptake are 1.3:1 to 8:1[130] This inevitably results in few counts being emitted from small tumours against a high background activity in blood and normal tissues. With deep seated tumours scatter of activity is an additional problem as is the attenuation caused by the tissues intervening between tumour and detector. In addition to these difficulties certain organs may accumulate a large percentage of the injected radiolabelled antibody thereby obscuring antibody localised to cancers in their vicinity. Uptake by the liver is the main problem and may, with ^{111}In labelled antibodies, account for up to 30-40% of the injected dose [125].

One solution is to use a gamma detecting probe intra-operatively following administration of

radiolabelled MAb to the patient. By reducing the distance between the detector and the tumour tissue, scatter and tissue attenuation are reduced and the number of counts from the tumour will be increased as this is inversely related to the square of the distance between source and detector. In consequence smaller tumour deposits can be detected and at lower tumour to normal colon uptake ratios than would be possible by external imaging. The potential use in patients with primary colorectal cancer would be for detection of metastatic deposits outwith the operative field and in defining the adequacy of resection margins with adherent tumours. In patients with suspected recurrent disease such as those with elevated CEA levels intraoperative radioimmunolocalisation may allow the detection of occult tumour deposits.

Types of radiation detectors

There are two main types of detector which may be used for the detection of gamma emissions intra-operatively.

Scintillation detectors

The scintillation detector comprises a scintillation crystal which produces pulses of visible light on interaction with gamma rays and a photomultiplier tube (PMT) which converts and amplifies this light signal into an electrical pulse suitable for analysis and counting.

Crystalline sodium iodide (NaI) is the most commonly used scintillator for nuclear medicine applications, the crystal being "doped" with a small proportion of Thallium (NaI(Tl)) to allow it to scintillate efficiently at room temperatures. An incident gamma-ray causes ionisation and excitation of electrons within the crystal which emit photons of visible light on returning to their original state, the total intensity of light being proportional to the energy of the gamma ray detected. NaI(Tl) is an efficient detector of gamma rays but is fragile, hygroscopic and can be damaged by rapid temperature changes.

A photoemissive cathode at the front surface of the photomultiplier tube converts the light output to a pulse of electrical energy which is then amplified in many stages by a chain of further electrodes at successively higher voltages, until an output pulse of suitable size is produced. This detector system has the disadvantage in intra-operative applications that a voltage of approximately 1000V is required by the PMT which is in close proximity to the patient.

The output signal from the detector system is then analysed before display. As the height of the electrical output signal varies with the energy of the detected gamma ray and this is characteristic to any individual radionuclide a pulse height analyser allows the selection of a range of gamma energies for counting. This is commonly performed by a counter/timer

or multichannel analyser and is an important feature of both scintillation and semiconductor detecting systems as it controls the proportion of scattered gamma rays detected allowing increased spatial resolution at the expense of sensitivity.

Semiconductor detectors

These consist of a crystal of a highly purified semiconductor which is sensitive to gamma radiation. When a voltage is applied across the crystal face no current will flow until an incident gamma ray causes ionisation. The resulting charge pulse is then collected at the electrodes, its size being proportional to the energy of the detected gamma ray. This signal is then fed to a preamplifier, an amplifier and then a counter/timer for display.

The semiconductors Silicon and Germanium have low sensitivities and require to be operated at liquid nitrogen temperatures. More recently developed semiconductors such as Gallium arsenide, Cadmium telluride and Mercuric iodide, however, are more efficient at detecting gamma rays and may be operated at room temperature. Of these CdTe is the most suitable material which is commercially available. The operative probes produced with this material are compact and operate at low voltage allowing them to be portable when powered by batteries. The disadvantages are that the small crystals of CdTe which have been produced are less sensitive gamma detectors than NaI(Tl) and the

detector systems are more expensive.

Clinical studies utilising intra-operative radiation detecting probes

Gamma radiation detecting probes have been put to a variety of clinical uses in the field of nuclear medicine and novel applications continue to be described. It is now over 15 years since the first clinical studies were carried out with semiconductor detectors[137,138] whereas the design and clinical application of an intra-operative scintillation counter (CsI/Tl) had already been described by Morris et al in 1971 for use in patients with thyroid cancer. After a substantial (therapeutic) dose of 90mCi of ^{131}I sufficient radionuclide was found to accumulate in an area of previously unresected tumour which appeared macroscopically normal tissue to allow its detection with the gamma detecting probe(GDP) and subsequent surgical resection. An activity of 5microcuries was claimed to localise in a metastasis of 3mm diameter[139]. The detection of osteoid osteomas has provided another application. This rare bone tumour, which mainly affects young adults, may be difficult to detect radiologically but it avidly takes up ^{99m}Tc polyphosphate. This fact has been exploited in these patients, by the pre-operative administration of ^{99m}Tc monophosphate or polyphosphate. The adequacy of surgical resection can then be examined by mobile scintillation cameras [140] or intra-operative

scintillation probes[140-145]. Similarly Harvey et al have administered ^{99m}Tc methylene diphosphonate (MDP) and used a scintillation probe to localise the optimum site for bone or soft tissue biopsies [146].

The use of a semiconductor GDP for the localisation of radiolabelled MAb was demonstrated recently by Aitken and his colleagues [147]. They established xenografts of the CEA secreting HX12 tumour in the flank of nude mice and subsequently injected them with a polyclonal antiserum to CEA labelled with ^{131}I. Using a CdTe probe to count over the tumour bearing or the contralateral thigh they showed preferential localisation of radio-activity in the tumour by 24hrs and maximal uptake at 72hrs. At 72 hours the ratio of counts over the tumour to that of the normal thigh were 1.80+/-0.43 : 1 (mean +/-S.D.). In this report the probe was also used in a patient with a rectal carcinoma who had been injected with 1.9mCi of ^{131}I anti-CEA 3 days previously. Very low count rates were achieved in vivo (100-150 counts/min) and the ratio between tumour and normal colon was only 1.21 : 1. When tissue from the resected specimen was assessed in a gamma well counter the tumour : normal rato was considerably higher at 3.2:1. Further patient studies were carried out by the same group using the CdTe probe and ^{131}I labelled polyclonal anti-CEA. In this report patients with both primary and recurrent colorectal cancers were investigated [148]. The mean tumour count to that of normal colon found intra-

operatively was 3.97:1 in patients with primary cancers and 4.18:1 in recurrent cancers, with all tumours showing higher counts than that of normal tissue. The ratio of counts found in vivo, however, were not compared to those assessed by gamma well counting of the excised specimens. No clinical benefit of intra-operative probing was established in these patients. In 1986 the same group reported the results of an additional study using the CdTe probe. On this occasion the $F(ab')_2$ fragments or whole MAb 17-1A was labelled with the longer lived radionuclide ^{125}I (half life of 60 days). Of the 18 patients with colorectal cancer that were studied 5 had primary tumours and 13 had recurrences[149]. The mean operative tumour to normal tissue count was 3.4:1 for whole antibody and 2.3:1 for the $F(ab')_2$ fragments. In 3 of the 18 patients (17%) intra-operative probing using the GDP assisted the surgeon in localising tumour tissue. In this study a comparison of the results obtained by gamma well counting of tumour and normal colon from the resected specimen was made with intra-operative probing and a ratio of 8.67+/-4.5 : 1 as opposed to 3.46+/-1.9 : 1 was obtained. The number of counts obtained intra-operatively and the statistical significance accorded to these counts was not, however, mentioned.

A different antigen (Tag 72) was targeted in colorectal cancer patients using the same intra-operative detector in the report by Sickle-Santanello et al in 1987 [150]. The Tag 72 antigen is

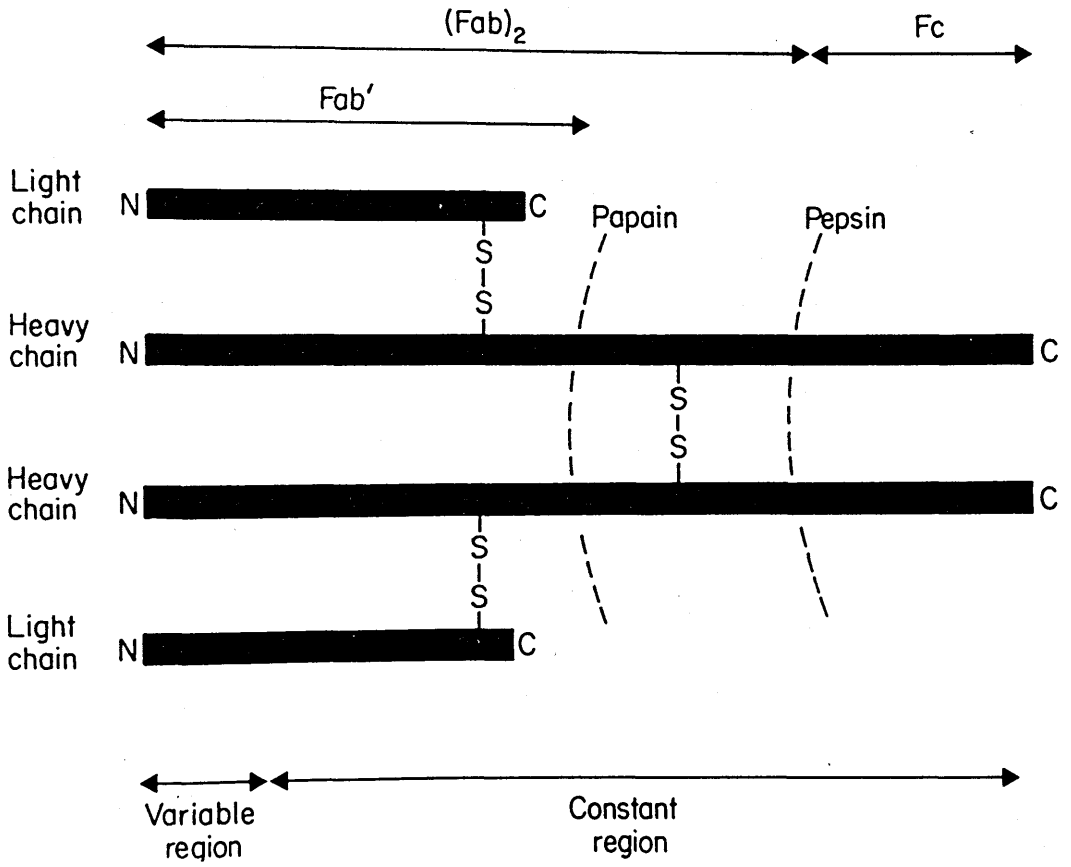

Figure 1.1. The structure of IgG and its fragments

expressed by the majority of colorectal cancers and was targeted with the ^{125}I labelled MAb B72.3. In this investigation the probe counts obtained over the tumour to that of normal colon ranged from 1.5:1 to 22:1 with a median of 11:1 but these figures were not compared with those of gamma well counted biopsies. Six of the 31 patients with recurrent cancer underwent probe directed biopsies which avoided liver resection in two, changed the operative approach in two and established subclinical lymph node recurrences in two others. Whilst this would suggest a useful application of this technique three false positive localisations were obtained in this group of patients ; one in an ischaemic ulcer, a second with an adhesive band and a third with a site on the anterior abdominal wall.

In addition to continuing their assessment of a CdTe probe for intra-operative detection of radiolabelled monoclonal antibodies in patients with colorectal cancer Martin and his colleagues have carried out further work in nude mice bearing xenografts of a CEA secreting human colon carcinoma(151). The CdTe probe was found to be more efficient at counting ^{125}I than ^{131}I labelled antibody. The uptake of activity in tumours was also found to be greater when a combination of monoclonal and polyclonal antibody was used than if either was used alone.

Although there has been a recent upsurge of interest in the use of CdTe detectors for localising iodinated antibodies in patients other uses have been suggested

and explored. Ubhi et al(1984) have reported the localisation of a mediastinal parathyroid adenoma by intra-operative probing following the administration of ^{201}Thallium Chloride [152] and the localisation of ^{99m}Tc-MDP to osteoid osteoma has been monitored using either a NaI or Cd Te detector [145]. In the latter study the CdTe system was found to be more durable, reliable and sensitive than the NaI system. This, however, contrasts with the findings of Harcke et al in which the sensitivity of the CdTe probe was found to be 2-5% of that of the NaI[153].

1.3.iv) Therapeutic aspects of monoclonal antibodies

The development of hybridoma technology by Kohler and Milstein in 1975[53] paved the way for the therapeutic applications of MAb's by allowing the production of quantities of antibodies sufficient for clinical application. Despite the ability to produce adequate amounts of specific MAb's several problems require to be resolved before the therapeutic application of MAb's is realised. Firstly the expression of an antigen by the tumour tissue is essential for the targeting of therapy. The heterogeneity of antigen expression, however, may result in some patients being unresponsive to treatment whereas in others non antigen-expressing clones of tumour cells may be selected for survival and result in tumour recurrence [154]. In addition to difference in antigen expression by cancers, normal human tissues may also express either the target antigen or a similar structure resulting in significant cross reactivity. The site and nature of the antigen itself is also of some importance. Although oncogenes have been detected in many cancers and MAb's raised to the proteins for which the oncogenes code may appear to be a logical advance for therapy this may have no useful application unless the protein is an accessible constituent of the cell membrane. Such cell membrane antigens may, however, be secreted and form a circulating complex with the MAb-therapy conjugate.

Moreover the potential of the targeted MAb may be thwarted by its removal by the reticulo-endothelial system, the instability of the complex, a lack of sensitivity of target tissues to the therapeutic agent or simply the access of a large molecule to the poorly vascularised tissue found at the core of solid tumour. Despite these potential problems a vast effort has been expended over the last ten years to define agents which may be conjugated to monoclonal antibodies and assessed for the selective destruction of cancer tissues. A brief overview is given here of some of the agents which have been considered for targeted therapy, their in vitro characteristics, and where possible the results of clinical studies. Four main groups of agents may be considered:

a) Antibodies alone

b) Antibody-drug conjugates

c) Antibody-radionuclide conjugates

d) Antibody-toxin conjugates

Antibodies against the epithelial membrane antigen are being investigated for use in the latter three applications and particular emphasis will be placed on these studies.

1.3.iv.a) Therapeutic aspects of monoclonal antibodies

Monoclonal antibody inhibition of the growth of a tumour cell line expressing the corresponding antigen in an in-vitro model has been shown with a variety of antibodies and tumour cell lines[155,156] as well as growth inhibition of tumour xenografts in nude mice [157]. Patient studies, however, have been less promising. In 1982 Sears et al reported the results of a phase 1 trial of monoclonal antibody in the treatment of patients with metastatic gastro-intestinal tumours [158] Four patients were given 15-200mg of purified MAb 17-1A [157]. All patients developed a human anti-mouse antibody (HAMA) response (Chapter 5) and one patient had an anaphylactic response to the fourth antibody injection. Only one patient was reported as showing tumour regression on serial CT scanning of liver metastases and this patient had received antibody coated leucocytes intra-arterially into the hepatic artery in addition to the intravenous administration of antibody. A further report on passive immunotherapy using the same antibody was published by the same group in 1984 [159]. A total of 20 patients were studied who had gastro-intestinal carcinomas with metastases. They received between 15 and 1000mg of antibody per patient in addition to conventional therapy consisting of a combination of surgery, chemotherapy and radiotherapy. Patients receiving lower dosages (<366mg) of antibody developed

an HAMA response whereas those receiving larger antibody dosages (>366mg) did not. Three of the group given passive immunotherapy were disease free 10,13 and 22 months following treatment. The design of this study with multiple modalities of treatment prohibits any useful conclusions to be made regarding any possible role for passive immunotherapy with monoclonal antibodies raised against a tumour associated antigen and this question therefore remains unanswered.

1.3.iv.b) Antibody-drug conjugates

The production of a composite molecule consisting of a cytotoxic drug and a tumour associated antibody for the treatment of cancer is a very appealing concept. The specificity of the antibody would allow tumour cells to be localised and subsequently destroyed by the chemo-therapeutic agent while preventing the damage to rapidly dividing tissues, notably the bone marrow, which is the major drawback to systemic chemotherapy. Investigation into this treatment modality has taken place for some thirty years [160], initially with polyclonal antisera and more recently with monoclonal antibodies. The earliest reports [160,161] concerned the use of methotrexate although many other cytotoxic agents have been used since including chlorambucil [162], adriamycin [163], melphalan [164], vindesine [165] and bleomycin [166]. The essential prerequisite for any antibody-drug conjugate is that preparation maintains both the

cytotoxic capability of the drug and the immuno-reactivity of the antibody. Even assuming that this is achieved considerable controversy remains over whether sufficient cytotoxic agent could be delivered to the tumour tissue. In an attempt to increase the delivery of the drug to tumour tissues many groups have been investigating the potential of using a drug carrier molecule which, once formed, is conjugated to the antibody. Carriers which have been investigated have included the albumins [167], agglutinins [168], polyaspartate [169] and carboxymethycellulose [170]. Antibody-drug conjugates have been shown to be both toxic and selective for tumour cell lines expressing the appropriate antigen[171-174] and have also been shown to localise in and suppress the growth of xenografts in nude mice [175,176]. To date no major therapeutic trials have been carried out in patients using antibody-drug conjugates. The biodistibution of these conjugates has been assessed, however, in patients with an ^{131}I-labelled vindesine-anti CEA conjugate [177] and ^{131}I-labelled 791T/36 MTX conjugate [178]. In the former study localisation on external imaging was achieved in only five of eight patients and the tissue uptake of activity was not measured. In the latter study the median uptake of activity was 2.9:1 as against normal tissues. Further investigation of the biodistribution in humans of antibody-drug conjugates are required along with a

method of directly assessing the drug uptake in the target tissues.

1.3.iv.c) Antibody targeted radionuclides

Evidence is accumulating from a variety of studies on different tumours and a number of different monoclonal antibodies that heterogeneity exists in the expression of all tumour associated antigens. For this reason targeted radionuclides may have an advantage over targeted drugs or toxins in that selecting a radionuclide with the appropriate emissions may allow the destruction of both the neighbouring non antigen expressing cell as well as the antigen expressing cell to which the carrier antibody is adherent.

Immunocompromised animals with tumour xenografts have been used to assess the therapeutic efficacy of radiolabelled monoclonal antibodies. Using such models ^{131}I labelled antibody has been shown to inhibit tumour xenograft growth to a greater extent than antibody alone (179) and similarly labelled specific antibody has shown greater inhibition of tumour growth than similar doses of non specific antibody (180). In these animal models, however, the tumour uptake of radio-labelled antibody as a percentage of the injected dose is many times greater than that achieved in patient studies. For example in a study on the therapeutic use of ^{131}I labelled anti-thy 1.1 monoclonal antibody 6.5% of the injected dose accumulated per gram of tumour

tissue in a lymphoma xenograft in nude mice [181], compared with the localisation achieved in patients which is rarely greater than 0.001% of the injected dose per gram [130]. The dosimetry found in animal models cannot, therefore, be extrapolated to patient studies.

A theoretical calculation of the dosimetry in humans was carried out by Vaughan et al in 1987[182]. Using data on the biodistribution of radiolabelled antibodies obtained from imaging studies they constructed a mathematical model of the radiation dose to various tissues associated with the administration of sufficient ^{131}I or ^{90}Yittrium (^{90}Y) to destroy a solid tumour. They concluded that at least a ten-fold increase in tumour uptake of the radiolabelled antibody would be required to combine tumour destruction with a survivable whole body irradiation. This study presents a plausible estimation of the effects that systemic therapy using radiolabelled antibodies would have using current techniques. Similar conclusions on the inadvisability of systemic treatment with radiolabelled antibodies have been produced by other researchers [183]. Despite this, radiolabelled antibodies have been used systemically for the treatment of metastatic melanoma [184], hepatoma [185] and cerebral glioma [186]. Interpretation of these studies is unfortunately complicated by their lack of controls and multiple concomitant therapies.

A logical solution to the dosimetry problem using radiolabelled antibodies is treatment on a loco-regional basis. This provides a high concentration directly to the tumour surface whilst avoiding the dose-limiting effects of systemic therapy. This modality of treatment has been used intra-pleurally and intra-pericardially for malignant serous effusions (187,188) and intra-peritoneally for metastatic ovarian carcinoma (189). Although of benefit in preventing the re-accumulation of malignant serous effusions the efficacy against bulky tumour was negligible. More recently studies have been carried out with a variety of monoclonal antibodies labelled with ^{131}I and used in the treatment of cerebral tumours by intra-thecal administration (190). Preliminary reports have shown evidence of tumour regression as assessed both on clinical grounds and CT findings whilst producing minimal side effects to this group of patients with a very poor prognosis.

The vast majority of patients with cancer, however, have solid tumour masses which are not amenable to regional therapy. Successful treatment of these patients using radio-labelled antibodies will require a significantly improved antibody uptake by tumour tissues or a satisfactory method of protecting radio-sensitive normal tissues.

1.3.iv.d) Antibody-toxin conjugates

A group of potent toxins derived from plants, of which the most widely known is Ricin, are capable of causing cell death at very low concentrations by the inhibition of protein synthesis at the level of the 60s subunit of intra-cellular ribosomes. Such toxins consist of two distinct subunits termed A and B. The B subunit is principally responsible for the binding of the toxin to the cell surface and the internalisation of the A unit which inhibits protein synthesis. Conjugates formed from tumour associated monoclonal antibodies and toxins have obvious appeal for the treatment of cancer and such compounds have been investigated for almost twenty years [191,192]. Unfortunately such antibody-toxin conjugates have not been found to have the expected antigen specificity due to the non specific binding of the ricin B chain to cell membranes. This binding appears to be related to sugar residues present on the B chain and may be inhibited by high local lactose concentrations [193] or by newer methods of antibody-toxin conjugation [194]. An alternative to inhibiting the non-specific binding of the B chain is to utilise the A chain alone conjugated to antibody. Unfortunately removal of the B chain although reducing the non specific uptake sometimes decreases the toxicity of the conjugate. Ricin A chains are catabolised within intracellular lysosomes and the toxicity of the A chains may be implemented in vitro by the presence of high

concentrations of ammonium chloride which alters the intra-lysosomal pH [195]. Antibody-ricin A chain conjugates have been shown to selectively inhibit the growth of, and kill, cells expressing the appropriate antigen in vitro [196,197]. Patient studies have mainly involved the treatment of acute leukaemics. In these patients marrow transplantation may be the sole method of allowing adequate drug therapy to be carried out. Autologous grafting may, however, re-introduce malignant stem cells to the host or allow the development of graft versus host disease. To circumvent these problems antibodies directed against cell surface markers on the tumour cells or expressed on normal mature T cells may be conjugated to ricin A chains and used ex vivo to purge the marrow prior to reimplantation [198,199].

Further advances in the use of antibody toxin conjugates are likely to involve the avoidance of non-specific binding of the B sub-units of whole toxin and the potentiation of the effects of A chains by inhibiting lysosomal breakdown. As in all aspects of monoclonal antibodies in therapy greater antibody specificity would allow more toxic substances to be safely administered.

1.4. EPITHELIAL MEMBRANE ANTIGEN

In section 1.3 the current and potential uses of MAb's in colorectal cancer have been outlined. These antibodies have generally been raised either to CEA or a colorectal cancer cell line. An alternative target antigen which has not previously been assessed in colorectal cancer is the epithelial membrane antigen.

1.4.i) Discovery

Normal lactating breast tissue secretes lipid which is packaged in membrane bound globules. The membrane of the milk fat globule is derived from the luminal surface of the cell during its passage from the cytoplasm to the mammary acinus. Extracts produced by the defatting of human milk consist largely of the milk fat globule membrane.(MFGM) which has been found to be a powerful immunogen. In 1977 Ceriani et al[200] described an antiserum which had been produced to defatted human cream and which was felt to be specific for an antigen expressed by the mammary membrane. A similar method of antisera preparation was used by Heyderman et al in 1979[201], emulsified membrane extracts in complete Freunds adjuvant being injected into rabbits at monthly intervals. High titre antisera were obtained 2 weeks after the fourth injection. Immunocytochemical staining was carried out on a variety of normal and neoplastic tissues using the antisera produced. Contrary to the findings of

Ceriani et al these antisera did not react solely with mammary tissues. The antigenic determinant being recognised was expressed on the luminal membrane of lactating, benign and malignant mammary tissues and in poorly differentiated carcinomas both cytoplasmic and luminal expression was present.

Normal tissues showing a positive staining reaction included the salivary gland, pancreas, stomach, bronchial mucus glands, bile ducts, endometrial and cervical glands, decidual glands and sebaceous glands. Of note was the positive staining of the distal collecting tubules of the kidney and the negative staining of the glomeruli and proximal tubules. Normal tissues of interest due to their negative reaction included the colon and ileum and the cellular elements of normal marrow and lymphoid tissues. Heyderman et al also investigated the immunocytochemical staining of a wide variety of malignant tumours using the same antisera. Adenocarcinomas were found to produce a strongly positive staining reaction including those of the stomach, colon, prostate, uterus, ovary, lung, pleura and thyroid. Tumours notable by their negative reaction included oat cell carcinomas, seminomas and non-Hodgkins lymphomas.

The unique nature of the antigen detected by these antisera to MFGM was confirmed by a comparison of tissue sections with antisera to CEA, beta oncofetal antigen, alpha lactalbumin, ferritin, lactoferrin,

secretory piece of IgA, muramidase, casein, cyst fluid protein and pregnancy specific b1 glycoprotein. In no case did the distribution of the immunocytochemical staining correspond to that of the antisera to MFGM and it was therefore concluded that a new antigenic determinant had been defined which was designated epithelial membrane antigen (EMA).

1.4.ii) Distribution

Many monoclonal antibodies have been raised to epithelial membrane antigen[202-207] and have allowed the further definition of the distribution of EMA in normal and neoplastic tissues. The findings of these studies using monoclonal antibodies have generally confirmed the findings of Heyderman using a polyclonal antisera [201]. The EMA expression of most tissues appears to be heterogenous and normal tissues which express EMA are mainly of glandular origin. Haemopoetic and lymphoid tissues were originally believed not to express EMA [201] but the antigen has subsequently been found on some non-neoplastic plasma cells [206]. A wide variety of tumours were studied on immunocytochemistry using MAb's to EMA by Pinkus and Kurtin in 1985 [207]. 90% of adenocarcinomas (breast, lung, colon, stomach, pancreas, gallbladder, prostate, endocrine, ovary, kidney and thyroid) were immunoreactive for EMA, mainly with cytoplasmic and apical luminal membrane staining. Of interest non-glandular tumours including squamous carcinomas, small cell anaplastic and mesotheliomas

were also EMA positive. A lack of immunoreactivity was noted with malignant lymphomas, endocrine neoplasms (carcinoid, medullary thyroid carcinomas, adrenocortical and phaeochromocytomas), germ cell tumours and connective tissue tumours.

The exact chemical composition and structure of EMA has yet to be established. Partial purification has been carried out, [208] the antigenic activity being associated with a wide range of molecular weights. The antigen itself is largely composed of carbohydrate with galactose and N-acetyl glucosamine as the two major sugars, the protein content of the antigen being low and of variable amino acid composition. Inorganic material was also found to be a major constituent of EMA.

1.5. CLINICAL APPLICATION OF ANTIBODIES TO EMA

1.5.i) Serological studies on EMA

Although EMA has been shown on immunohistochemistry to be expressed by a wide variety of cancers few studies have been carried out to determine if these patients have elevated serum levels of EMA. Studies have, however, been carried out in patients with breast and ovarian cancer using MAb's raised to either extracts of the MFGM or membrane enriched extracts of breast cancer metastases. In 1984 Burchell et al[209] examined the sera of 30 patients with breast cancer and 30 controls using a "sandwich" radioimmunoassay employing the MAb's HMFGM 1 and 2. The HMFG 1 antigen was found to be elevated in the serum of 30% of patients with advanced cancer and in 6% of healthy controls whereas HMFG2 was elevated in 53% of the sera from patients with advanced cancer and in 16.6% of controls. Interestingly the circulating antigen in serum recognised by these MAb's was found to be 320kD and 280kD respectively which is lower than the molecular weight of the antigen present in milk. This suggests that only a portion of the membrane antigen is shed. Antibody HMFG2 was also used by Ward for investigating patients with epithelial ovarian cancers[210], who found that 33% of stage 1 patients had elevated levels of EMA compared to 62% of patients at stage 2-4. Circulating EMA levels were also found to correlate with tumour bulk by analysis of

serum levels before and after cytoreductive surgery. In neither of these studies, however, was evidence found to suggest that HMFG2 detection of EMA levels was sufficiently sensitive for screening procedures or for the routine follow-up of patients following surgery for either breast or ovarian cancer.

Serological studies using other MAb's raised against HMFG or extracts of breast cancer tissue have given similar results, namely that patients with extensive local or metastatic breast and ovarian cancers have elevated levels whereas those with early stages of disease have serum levels similar to those of normal controls [211-213], Ashorn analysed the serum levels of HMFG antigen in patients with a variety of cancers in addition to those of the ovary and breast. Of 33 serum samples from patients with colorectal cancer only one had a level above the range of healthy controls. In patients with extensive breast cancer only 35% had elevated levels of circulating EMA and of the small number of patients examined with localised breast cancer only 22% had had above normal levels. A trend was observed, however, and patients with progressive disease had rising levels of HMFG whereas those where disease was regressing the levels were decreasing [213].

1.5.ii) Immunohistochemical studies on EMA

The use of antibodies for the detection of tissue antigens in fresh or paraffin embedded tissues is now a well established technique in histopathology for establishing the exact nature of the tissue under study. Epithelial membrane antigen (EMA) is well preserved in paraffin embedded tissues which therefore provide a suitable source of material for study. As EMA is expressed by most epithelial tumours and rarely by lymphoid neoplasms or sarcomas, an obvious role for EMA antibodies is in the differentiation of poorly differentiated carcinomas from malignant lymphomas and spindle cell sarcomas.

In 1983 Sloane et al[214] assessed the value of a polyclonal antiserum to EMA in solving diagnostic problems in tumour histopathology. Of 70 specimens examined 48 tumours were stained for the purpose of determining their histogenesis. Twenty-two of these were positive for EMA expression and twenty subsequently proved to be epithelial on follow-up studies. In six of these specimens a diagnosis of non-epithelial malignancy had been made on conventional staining. Although a role remains for the use of MAb's to EMA in determining the nature of poorly differentiated tumours EMA expression has been found on some reactive and neoplastic plasma cells, some non-Hodgkins lymphomas and Reed Sternberg cells in cases of lymphocyte-predominant Hodgkins disease[206].

On the strength of this finding Delsol [206] suggested the use of additional anti-epithelial antibodies in conjunction with anti-EMA for tumour diagnosis.

EMA antibodies may also have an important role when used in conjunction with other tumour markers for establishing the site of origin of epithelial tumours. In 1984 Heyderman et al [90] carried out an immunoperoxidase study using antibodies to EMA, CEA and prostatic acid phosphatase in prostatic, bladder and colorectal cancer. Analysing twenty tumours of each type all of the colorectal and bladder tumours and 16 of 20 prostatic tumours were positive for EMA. All 20 colorectal, 7 of 20 bladder and 5 of 20 prostatic tumours stained for CEA. Prostatic carcinomas alone were positive for prostatic acid phosphatase. It was suggested that these markers may therefore be useful in establishing the origin of pelvic tumours for which the resultant management will be markedly different.

Another major role for immunocytochemistry using EMA antibodies has been in the detection of micrometastases from breast carcinoma [215-220]. Staining of marrow aspirates with EMA antibodies by immunoflourescence in patients with clinically primary breast carcinomas allows the detection of micrometastases not apparent on conventional haematoxylin and eosin staining. This important finding has recently been supported by a follow up study

showing that those patients with EMA positive marrow smears have a poorer prognosis than those that do not [221]. Micrometastases have also been detected in the regional lymph nodes of patients with breast carcinoma using anti-EMA MAb's and this may provide a useful method of selecting patients for adjuvant cytotoxic chemotherapy [222,223].

The malignant nature of serous effusions, either pleural or peritoneal, may often be established by the cytological analysis of aspirated fluid. Occasionaly, however, although malignancy is confirmed or suspected the histological nature of the primary tumour cannot be established. This may have important implications for future management. Antisera to EMA have been shown to have an important role in differentiating the nature of such effusions [224-226].

1.5.iii) Radioimmunolocalisation

Studies have been carried out on the localisation of breast, ovarian and colonic carcinomas using antibodies raised to EMA. In 1982 Epenetos et al[227] reported the use of ^{123}I labelled EMA antibodies HMFGM 1 and HMFGM 2. These antibodies had previously been shown to react strongly with the lactating breast and epithelial tumours and only weakly with normal tissues [204,228,229]. Localisation was attempted in 20 patients with advanced malignancy, (ovarian(10), breast(6), colon(2) and two Krukenberg tumours of

colonic origin) following the intravenous administration of labelled antibody. Positive images were obtained, without subtraction techniques, at three minutes to 18 hours following injection. Of the 12 patients with ovarian tumours 15 of the 18 sites of tumour localised the radiolabelled MAB. Twelve sites of disease were present in the six patients with advanced breast carcinoma, nine of which were localised. Of the two patients with colonic carcinomas one had a negative scan and the other had incomplete scanning. The uptake of label was assessed in five patients by whole body scanning and expressed as a percentage of the injected dose, the mean tumour uptake being 0.6% of the injected dose. The site of localisation of the radiolabelled antibodies was confirmed by immunoperoxidase staining and autoradiography.

Another important study on the localisation of EMA antibodies was carried out by Rainsbury in patients with breast carcinoma [125,230]. Using the antibody LICR LON M8 (M8) labelled with ^{111}In, 29 patients with breast carcinoma were studied, 7 with primary tumours and 22 with metastases. The results of radio-localisation were compared with those of conventional X-rays and methylene diphosphonate (MDP) bone scans. Excised tumour specimens had a mean of 6.2 times greater uptake of labelled antibody than normal tissues but did not produce positive images. Skeletal metastases, on the other hand, were localised in all

sites confirmed by other means. The reasons proposed for the imaging of the bony metastases but not the primary tumour was an increased EMA expression in marrow metastases and an increased access of radio-labelled antibody due to improved vascularity. Although the vascularity of bony metastases may be greater than that of soft tissue lesions a suitable explanation for a difference in antigenic expression is not apparent.

Antibodies raised against an antigen expressed by one type of tumour may react with other types of tumour expressing the same antigen. This fact allows a single antibody to be used for the radio-localisation of more than one tumour type. Antibody 791T/36 was originally raised to an osteogenic sarcoma and has been successfully used for the localisation of both bony [109] and gastro-intestinal tumours [113,114]. Similarly the MAb M8, raised to EMA [202,203] and used in the imaging of breast carcinomas [230], was found on immunoperoxidase staining of colorectal cancers [231] to react strongly with these tumours as was the MAb 77.1. Both MAb's have been used in the imaging of colorectal cancers [232,233].

Eighteen patients were administered ^{111}In labelled M8 and scanning took place 3-5 days after administration. Thirteen of the 18 patients had positive scans(65%) and the findings were confirmed at operation. Immunocytochemistry on the excised tumours was positive in all patients with positive scans.

Eleven patients had samples of tumour and normal colon taken at the time of operation. The ratio of antibody in tumour to that of normal colon was 2.56+/-1.27 (mean+/-S.D.) and the ratio of tumour to blood activity was 11.0+/-6.2(mean+/-S.D.)

The monoclonal antibody 77.1, a mouse monoclonal antibody of IgG_{2a} class, was originally raised using a bladder carcinoma metastasis as immunogen and has anti-EMA like properties [234]. ^{111}In labelled 77.1 was administered intravenously to 14 patients. One patient had two primary tumours and two had recurrent disease. Scanning at 3-5 days following antibody administration localised ten of the fifteen tumours(64%). Positive scanning results correlated with positive immunocytochemistry. Analysis of activity in specimens resected 6 days after antibody administration revealed a mean tumour to normal colon ratio of 1.93 (range 1.0-3.26) and a mean tumour to blood ratio of 3.55 (range 1.6 to 5.07).

1.6. Conclusions and purpose of the present study

Many antibodies have been raised to tumour associated antigens which are expressed by colorectal cancers. Many of these antibodies, however, recognise different epitopes of the same antigen molecule and the number of colorectal cancer antigens which have been defined are relatively few, CEA[235], CA 19-9[236], 17-1A[155] and TAG-72[237] being most intensively investigated. In this chapter the many applications of antibodies to these antigens have been discussed. It is apparent that neither the ideal antibody or target antigen has yet been defined.

Several immunohistochemical studies have reported the marked difference in the expression of EMA by colorectal cancers and normal colon[201,207,238] and provisional studies using Indium labelled MAb's M8 and 77.1 has suggested a possible role for anti-EMA MAb's in patients with colorectal cancer [232,233]. This target antigen has therefore been investigated in greater detail. The studies were conducted in the following manner ;

An immunohistochemical study of EMA and CEA expression in normal colon and colorectal neoplasia.

No previous studies have looked specifically at EMA expression in colorectal cancer. Immunohistochemical staining of colorectal cancers using the recently developed anti-EMA MAb ICR2 was compared with an

antibody recognising CEA, the best characterised of the gastro-intestinal tumour antigens.

The anti-CEA antibody C46 was obtained from Amersham International, U.K.. It is a murine IgG which was raised to a sub fraction of CEA of high specific activity obtained from the liver metastasis of a human colorectal carcinoma (239). It was selected due to its successful use conjugated to ^{111}In in imaging human colorectal cancers and its high tumour to normal colon differential uptake in vivo(106).

The anti-EMA ICR2 has been recently developed at the Institute of Cancer Research, Sutton, Surrey. It is of the IgG_{2a} class and was raised in Chester Beatty Hooded (CBH) rats using extracts of MFGM as immunogen. Lymphocytes sensitised by repeated injections of membrane extracts in Freunds adjuvant were fused with the rat myeloma Y3 AG 1.2.3. and the resultant hybridomas were grown as ascites. The antibody was isolated by salt fractionation and ion exchange chromatography using Whatman DE52 cellulose. The reaction of this antibody with normal human tissues has been assessed (J.P. Sloane, personal communication) and the pattern of staining has been found to be similar to that of the rabbit polyclonal antiserum used for the original description of EMA (201).

Preliminary investigation was performed on randomly selected paraffin embedded colorectal tissues obtained from the Histopathology Department, University College Hospital.

To allow further comparison of CEA and EMA expression in colorectal cancers with benign adenomatous polyps and normal colon within individual patients and correlations with standard histological variables, an immunohistochemical study was subsequently carried out on tissues from a consecutive series of patients undergoing resection of their colorectal cancers.

Immunohistochemistry has been employed in the detection of micrometastases. Antibodies to CEA detected nodal metastases from colorectal cancers with considerable variations in reported frequency[91,92] while anti-EMA detection of nodal and bone marrow deposits has only been investigated in breast carcinoma [221,222]. Immunohistological assessment with both anti-CEA and anti-EMA, of lymph nodes in resected colorectal cancer specimens has therefore been investigated.

Circulating EMA levels in patients with breast and colorectal cancer.

The serological studies carried out in colorectal cancer patients have been outlined in Section 1.3.i) and those using anti-EMA antibodies in Section 1.5.i).

From these studies it would appear that only a part of the EMA molecule circulates[209] and that circulating levels will depend on the epitopes recognised by any individual antibody[211-213].

Nevertheless elevated levels have been noted in patients with breast and ovarian cancers and rarely in patients with colorectal cancer[213]. It is relevant therefore in investigating the role of EMA expression in colorectal cancer and in its potential as an antigen for antibody localisation to measure its circulating levels using ICR2 in patients with colorectal cancer and compare this to patients with breast cancer and age matched healthy blood donors.

The liver uptake of ^{111}In labelled MAb

The uptake of radiolabelled antibody in normal liver tissue is perhaps the greatest limitation to the use of radiolabelled antibodies for the detection of gastrointestinal cancers by gamma camera imaging (Section 1.3.iii). The limited organ dosimetry available would suggest that between 20% to 40% of injected ^{111}In-MAb accumulates in the liver within 24 hours[230], which obscures any uptake of activity in liver metastases or in tumours in the vicinity of the liver. The mechanism for this uptake has not been established although the activity takes the form of a low molecular weight complex within the hepatocytes [240,241]. Prior to embarking on patient studies an in-vitro model consisting of isolated hepatocytes and the EMA expressing cancer cell line MCF7 has been used to analyse factors which may reduce the hepatocytes uptake whilst maintaining the specific uptake by the tumour cell line.

Colorectal cancer imaging using the anti-EMA MAb ICR2

Localising colorectal cancers by gamma camera imaging following the administration of radiolabelled antibodies has been carried out for the last 10 years. (Section 1.3.iii). The majority of studies have used antibodies raised to CEA labelled with ^{131}I(97-101) or more recently with ^{111}In(105,106). Studies have suggested that anti-EMA antibodies are suitable for imaging colorectal cancers (232,233). The use of the anti-EMA ICR2 labelled with ^{111}In has therefore been investigated for colorectal cancer imaging.

Human biodistribution of ^{111}In-ICR2 in colorectal cancer patients.

The process whereby a radiolabelled antibody localises and allows a successful imaging of a cancer is complex, with problems at any intermediate stage affecting the eventual result. The majority of studies on radioimmunolocalisation, however, have reported very limited if any information on the biodistribution of radiolabelled antibodies in patient studies. This data is essential if improvements in the results of antibody imaging are to be achieved. The biodistribution of ^{111}In-ICR2 was therefore analysed in patients with colorectal cancer and the imaging results related to circulating antigen, circulating immune complex, clearance of activity, tumour uptake of activity and the expression of the target antigen in tumour tissue.

The effect of unlabelled antibody for the reduction of circulating immune complex and DTPA in reducing liver uptake have been explored. Previous human studies investigating cold antibody administration have failed to quantify the effect on circulating antigen formation and tumour uptake of activity [242,243]. Therefore, this is also being examined.

Intra-operative tumour detection

Tumour deposits may be detected at the time of operation using radiolabelled monoclonal antibodies and a portable radiation detector, with smaller deposits being detected than by external imaging. The potential application of this technique which was first described as recently as 1984[147] is in deciding the adequacy of resection margins and the presence of metastases with both primary and recurrent cancers. Previous studies have involved a variety of iodinated antibodies and a CdTe radiation detector [148-150]. We have utilised the ^{111}In-ICR2 administered to patients with colorectal cancer for external imaging to evaluate intra-operative tumour detection. The limitations of this technique have also been assessed using a phantom model and both CdTe and NaI detectors.

CHAPTER 2

AN IMMUNOHISTOCHEMICAL STUDY OF CARCINOEMBRYONIC ANTIGEN AND EPITHELIAL MEMBRANE ANTIGEN IN NORMAL COLON AND COLORECTAL NEOPLASIA

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2.6. DISCUSSION

2.1. INTRODUCTION

Immunohistochemistry allows the visualisation of tumour antigen expression in histological sections. In colorectal cancer these techniques have been used, mainly with antibodies raised to CEA, to identify the tissue of origin of undifferentiated cancers[90], for the detection of micrometastases in regional lymph nodes[91,92] and as one of the prognostic indices for patients undergoing resection of a colorectal cancer[93,94].

Antibodies to EMA have been used in immunocytochemistry for establishing the epithelial nature of undifferentiated tumours[214], for detecting breast cancer micrometastases[215-223] and for determining the nature of serous effusions[224-226]. The expression of EMA by colorectal cancers has previously been noted[90,207,231] but not adequately investigated. In this series of immunohistochemical studies the anti-EMA antibody ICR2 has been evaluated as a potential agent for the imaging of colorectal cancers by assessing its reaction with primary and metastatic colorectal cancers, adenomatous polyps and normal colon. A comparison has also been carried out between the expression of EMA in colorectal cancer and CEA, the best characterised of the colorectal cancer antigens. To allow a comparison to be made with standard histopathological variables the resected specimens of a consecutive series of patients with

colorectal cancer were then studied for their CEA and EMA expression.

The presence of metastatic tumour deposits in the regional lymph nodes is a major factor in predicting the eventual outcome of a particular patient. The reported mean five year survival of patients without lymph node metastases is nearly 80%, whereas for those with metastatic deposits in regional lymph nodes it is only 30% [7,8]. The accurate determination of lymph node involvement is therefore crucial. Routine Haematoxylin and Eosin (H&E) examination may not detect minute tumour deposits or groups of cells and will result in falsely optimistic tumour staging.

Colorectal cancers and their metastases have previously been shown to express both CEA and EMA [90]. Previous studies have used either anti-CEA in colorectal cancer[91,92] or anti-EMA in breast cancer[215-223] for detecting micrometastases. Controversy exists over the detection rate of colorectal micrometastases with reports of metastases present in 4-25% of patients [91,92]. A larger series of patients has therefore been studied to clarify the uncertainty in the detection rate of colorectal cancer micrometastases and to determine whether it is improved by combining anti-CEA or anti-EMA immunohistochemistry with H&E staining.

2.2. MATERIALS AND METHODS

Throughout the studies in this chapter formalin fixed and paraffin embedded tissues were used. The preliminary studies in section 2.3. used randomly selected tissue blocks. Because this did not allow the antigen expression to be compared between tissues within any individual patient or for the antigen expression to be correlated with routine pathological variables, studies in sections 2.4. and 2.5. used tissues from a consecutive series of patients. From blocks of tissue 6um sections were cut for immunocytochemistry using an MSE microtome. The monoclonal antibodies ICR2 (anti-EMA) and C46 (anti-CEA) have been described previously (Section 1.6.)

2.2.i) Indirect immunoperoxidase staining method

The slides were firstly dewaxed by washing three times in xylene (1.1.1.trichloroethane) for three minutes each time and then the xylene was removed in absolute alcohol (ethyl alcohol) x 3 for three minutes. Endogenous peroxidase activity in gastrointestinal tissue was blocked by incubating with 0.5% H_2O_2 / methanol for five minutes then the sections were washed in tap water for five minutes followed by Tris buffered saline (T.B.S.) pH 7.6 for five minutes. Suitably diluted MAb (either ICR2 or C46, both 10ug/ml) was applied to each slide(80ul) and the sections were placed in a perspex levelling tray and left for one

hour. Further washing was then carried out with T.B.S. for five minutes prior to 80ul of a 1:100 dilution of the second antibody (rabbit anti-rat for ICR2 or rabbit anti-mouse for C46, Dakopatts, Sweden) in T.B.S. being applied to each section for 30 minutes. After this period the slides were again washed for 5 minutes in T.B.S. and the sites of bound antibody revealed by application of the chromogen 3-3 diaminobenzidine tetrahydrochloride (DAB) which was made up as follows :

100mg D.A.B.

200mls TRIS/ HCL buffer pH 7.6

2mls 1% H_2O_2 =100ul H_2O_2 (30% w/v) + 2.9mls distilled water.

Slides were placed in this solution for ten minutes then washed in T.B.S. for one minute, running water for five minutes and then stained in Mayer's Haemalum (BDH chemicals, Poole) for two minutes. After placing briefly in running water the slides were cleared by dipping briefly in saturated lithium carbonate and then washed in tap water for five minutes. Sections were then dehydrated by three periods of three minutes in absolute ethanol followed by a similar period in xylene. Finally the slides were mounted in DPX (BDH, Poole, Dorset). Using this technique antigen binding sites appear brown and non antigen expressing tissues blue.

2.2.ii) System for grading antigen expression

In the preliminary studies in this chapter (2.3.i and ii) the antigen expression was simply graded as either positive if staining was present or negative if it was not. In the subsequent and more detailed studies carried out (2.4 and 2.5) immunocytochemical staining of the tissues was categorised using a system for grading similar to that of Ellis et al[244]. Using this system the percentage of cells within the histological section which express the target antigen is measured by two independent observers and classified as shown below. This system for grading antigen expression on immunocytochemical staining has been shown previously to be both consistent and reproducible when applied to breast cancer tissue stained with the MAb NCRC-11[245].

Grade 1 : < 25% cell stain
Grade 2 : 25-50% cell stain
Grade 3 : 50-75% cell stain
Grade 4 : > 75% cell stain

Controls consisted of sections known to express the target antigen (+ve control) and sections where either the first antibody or both first and second antibodies were omitted (-ve control). These controls were included with each batch of immunocytochemistry. Where two different antigens were compared in the same tissue sections the antibodies were used at the same concentration (titre) and the reaction times were standardised. The staining pattern was described as membraneous where staining was present only at the cell membrane and cytoplasmic where the body of the cell

showed positive staining. In occasional sections with both MAb's the connective tissue of the section showed faint staining. This was avoided by the application of human serum to the sections (1 in 50 dilution in phosphate buffered saline (PBS)) before applying the first antibody.

2.3. PRELIMINARY STUDIES ON CEA AND EMA EXPRESSION IN COLORECTAL NEOPLASIA AND NORMAL COLON

2.3.i) EMA expression

Immunoperoxidase staining with ICR2 was carried out on 17 primary colorectal cancers, 5 lymph node metastases, three liver metastases, 9 colonic adenomatous polyps and three sections of normal colon. The pattern of staining was membraneous and therefore more apparent in areas of gland formation within the cancers and metastases (Figure 2.1.). ICR2 reacted with 15 of the 17 primary colonic cancers(88%) and in 6 of 8 metastases(75%). No staining was noted on normal colon and in only one of the nine adenomatous polyps, this corresponded with an area of severe dysplasia.(Table 2.1.)

2.3.ii) Comparison of CEA and EMA expression

Sections of primary colorectal cancers (n=18), lymph node metastases (n=6), liver metastases (n=7), benign adenomatous polyps (n=14) and normal colon (n=17) were prepared and stained as outlined above. Consecutively cut sections were used to determine the expression of CEA and EMA and the results again classified as either positive or negative.

The results presented in Table 2.2. show that CEA was expressed by a greater number of primary cancers than EMA. Typically both antibodies gave staining of

the cell membrane which was strongest in areas of gland formation(Figures 2.1. and 2.2.). In a few specimens the primary tumours were found to give intracellular staining either alone or in addition to membraneous staining. Metastatic deposits showed a similar pattern of expression of both EMA and CEA than the primary cancers. CEA was expressed by the majority of adenomatous polyps and normal colon sections whereas EMA expression was seen only occasionally in normal colon and in adenomatous polyps only in areas showing severe dysplasia.

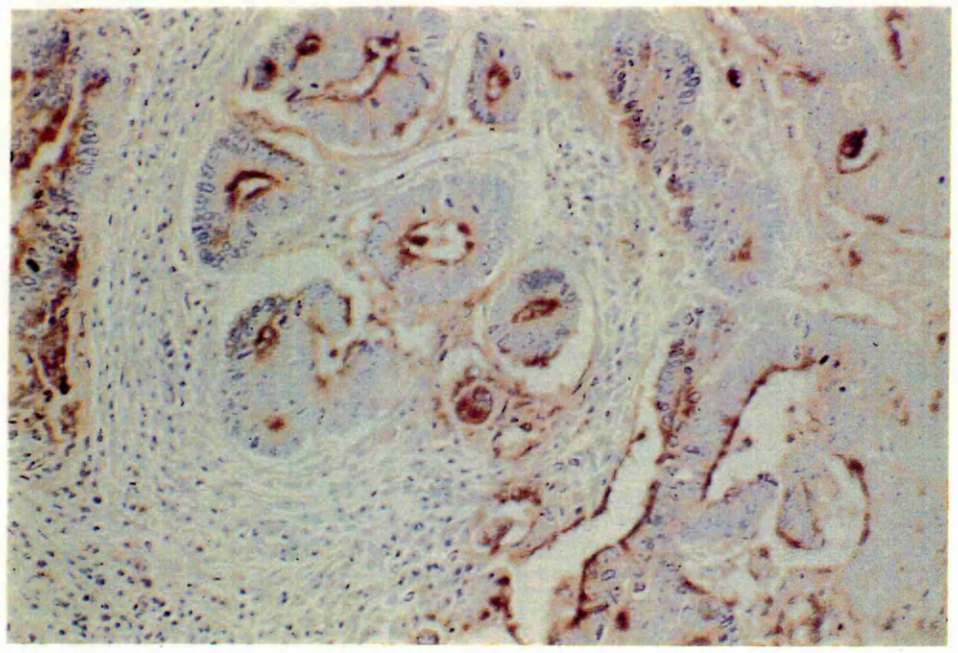


Figure 2.1. : The expression of EMA in a colorectal cancer section. Sites of antigen binding are stained brown (indirect immunoperoxidase x 200)

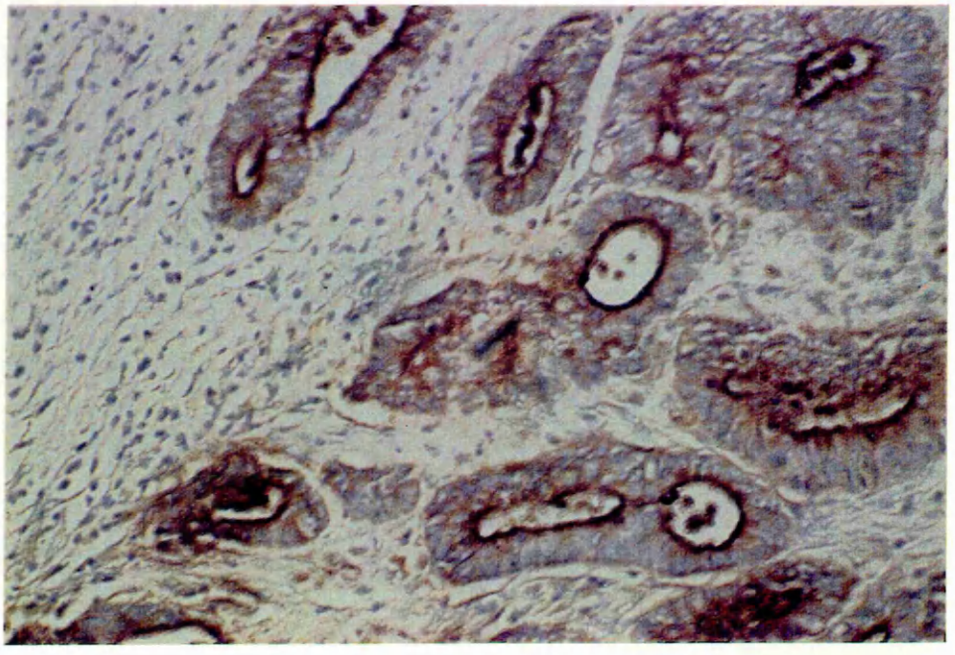


Figure 2.2. : The expression of CEA in the same colorectal cancer section. Antigen binding sites are again stained brown (indirect immunoperoxidase x 200)

Table 2.1. : **EMA expression in normal colon and colorectal neoplasia**

Tissue	No. of samples	EMA expression No. +ve	%
Carcinoma	17	15	88
Metastases	8	6	75
Polyps	9	1	11
Normal colon	3	0	0

Table 2.2. : **Comparison of CEA and EMA expression in normal colon and colorectal neoplasia**

		Staining reaction			
		EMA (ICR2)		CEA(C46)	
Tissue	Number	No. +ve	%	No. +ve	%
Carcinoma	18	15	83	18	100
Nodal metastases	6	5	83	5	83
Liver metastases	7	5	71	4	57
Polyps	14	1	7	11	79
Normal colon	17	2	12	12	71

2.4. RELATIONSHIP OF CEA AND EMA EXPRESSION WITH HISTOPATHOLOGICAL VARIABLES

Patients

Over a one year period 51 consecutive patients undergoing a potentially curative resection of a colorectal carcinoma in a single unit were studied. There were 26 males and 25 females with a median age of 68 years (range 26-90).

Specimens

All specimens had been examined conventionally with H & E staining prior to immunohistochemical examination, both procedures being carried out by independent investigators and the results subsequently compared. A total of 52 specimens of primary colorectal cancer were studied, one patient having two synchronous carcinomas. The expression of CEA and EMA was also determined in the adjacent normal colon in 45 resected specimens ; in 11 adenomatous polyps found in the resected specimens and in a total of 49 lymph node metastases present in specimens from 20 patients.

The antigen expression was measured by the indirect immunoperoxidase staining method and the staining reactions graded as described earlier (2.2.ii).

Results

Histopathological examination

Six tumours were classified as Dukes stage A, 26 stage B and 20 stage C. Twenty seven tumours were well differentiated, 22 were moderately differentiated and three were poorly differentiated. Fifteen of the primary tumours were in the right colon (caecum, ascending and right transverse), 22 were in the left colon (left transverse, descending and sigmoid) and 15 were in the rectum. The percentage of well and moderately differentiated cancers was similar in the right (47% and 40%) and left (55% and 41%) colon and in the rectum (53% and 47%)(all n.s.). Forty-nine metastatic deposits from 20 patients were found on examination of 249 lymph nodes. Of the eleven polyps six were tubular and five tubulo-villous adenomas.

Immunohistochemical examination

Primary cancers

The staining pattern was similar for both the anti-CEA and anti-EMA antibodies, being membraneous and most pronounced in areas of tumour with gland formation. A cytoplasmic staining pattern was occassionally seen with both antibodies. Some heterogeneity of antigen expression between different areas within any individual section was noted with both antibodies.

All 52 primary colorectal cancers stained positively for CEA whereas 43 of the 52 sections stained positively for EMA (83%)(Table 2.3.). The grade of immunohistochemical staining of the tumours (Figure 2.3.) was higher with the anti-CEA antibody than anti-EMA in 39 (75%), equal in 11 (21%) and less in two (4%). Tumours graded 3 and 4 for CEA expression were more commmonly well rather than moderately differentiated (23 of 27 vs 11 of 22, $p< 0.01$, X^2 test) (Figure 2.4.). No correlation was found between the staining pattern with anti-EMA and the degree of tumour differentiation (Figure 2.5.). Of the eight tumour samples with no detectable EMA expression three were moderately and five well differentiated.

The grading of CEA expression varied with the site of the primary tumour, right sided colonic tumours showing less evidence of CEA expression than left sided. Six of the 15 right sided lesions were graded 1 (<25% cell stain) whereas only two of the 37 lesions in the left colon and rectum were graded 1 ($p<0.01$,X^2 test)(Figure 2.6.). Equivalent figures for EMA expression were 3 of 15 in the right colon and 6 of 37 in the left colon and rectum (n.s.)

Lymph nodes

All nodal metastatic deposits showed staining for CEA. Forty-five of the 49 metastases from 20 colorectal cancer specimens stained positively for EMA (92%), a

Table 2.3. : **Comparison of CEA and EMA expression in tissues from a consecutive series of patients undergoing colorectal cancer resection**

Tissue	Number	EMA +ve(%)	CEA +ve(%)
Primary cancers	52	43 (83)	52 (100)
Nodal metastases	49	45 (92)	49 (100)
Adenomatous polyps	11	0 (0)	11 (100)
Normal colon	45	0 (0)	29 (64)

Figure 2.3. Immunohistochemical grading of the tumours

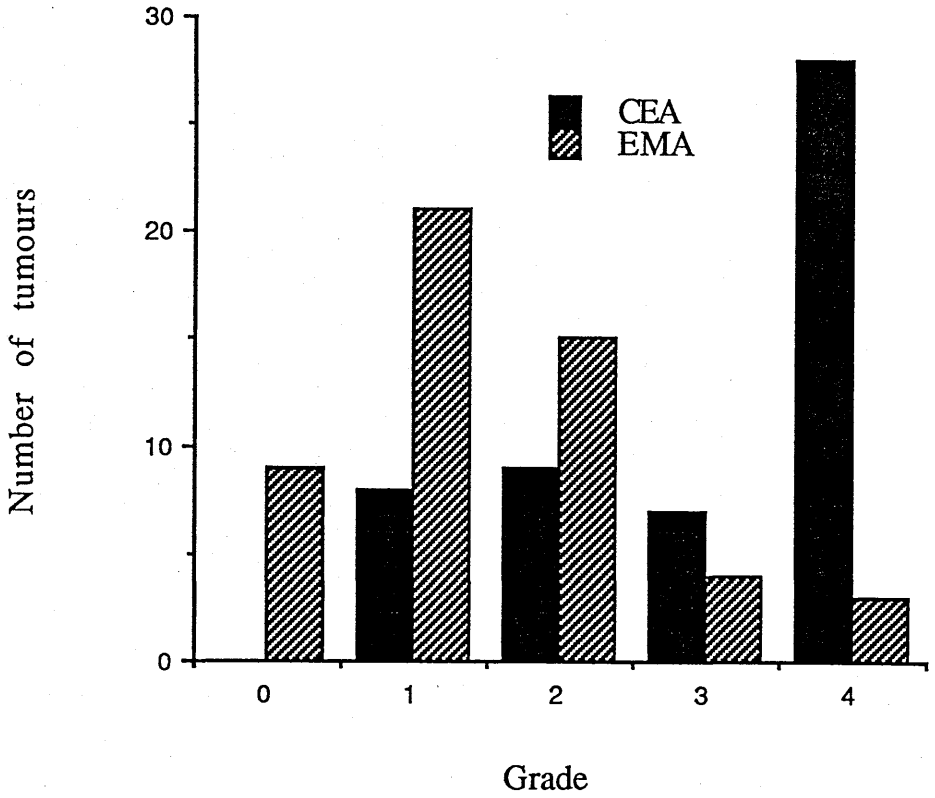


Figure 2.4. CEA expression and tumour differentiation

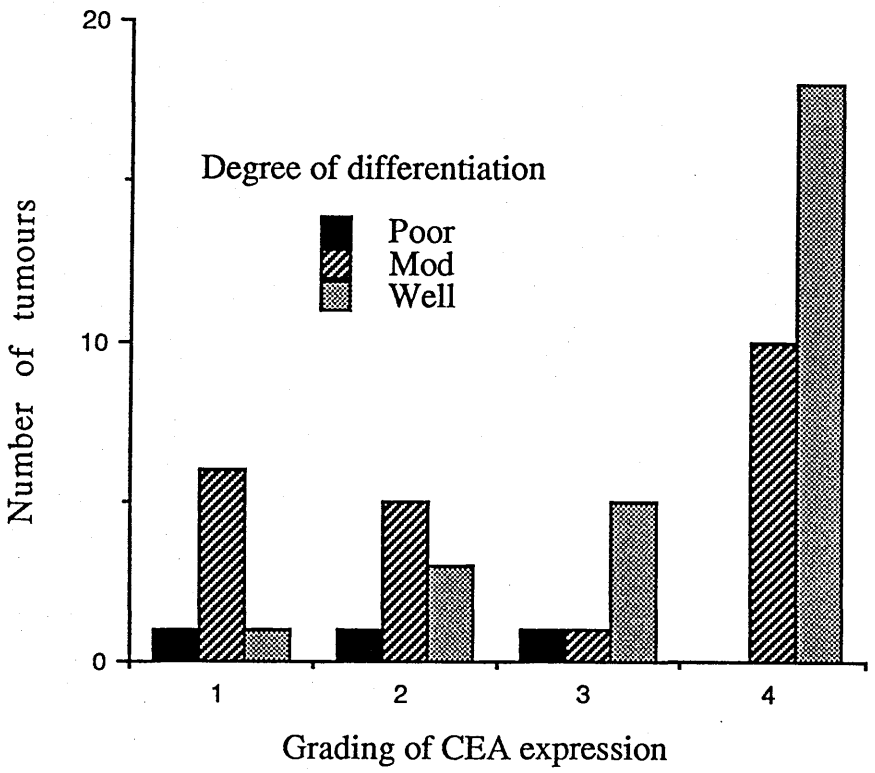


Figure 2.5. : EMA expression and tumour differentiation

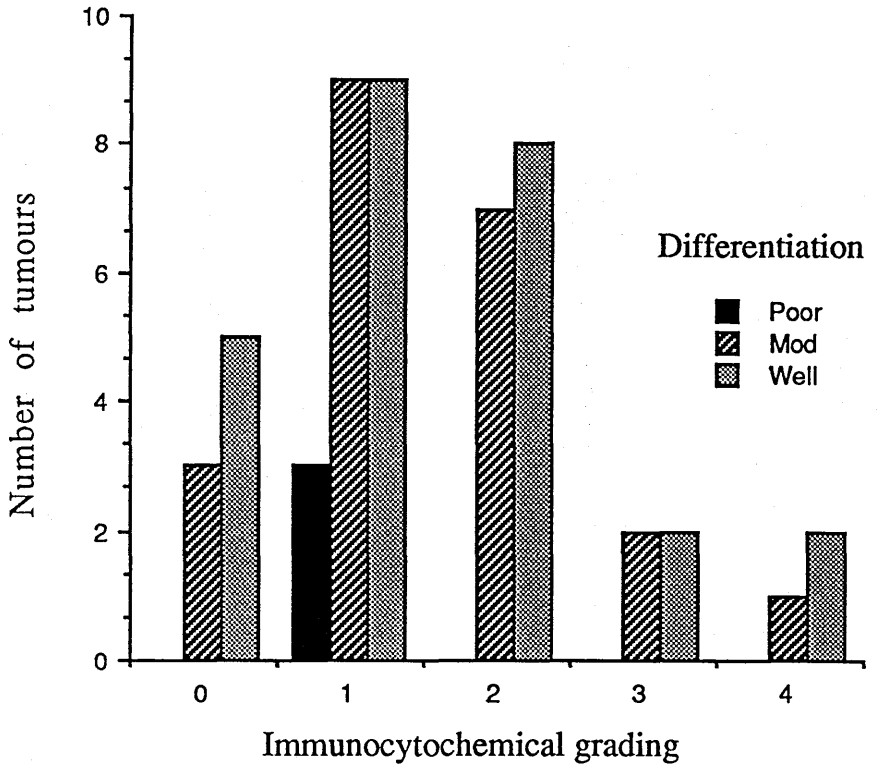
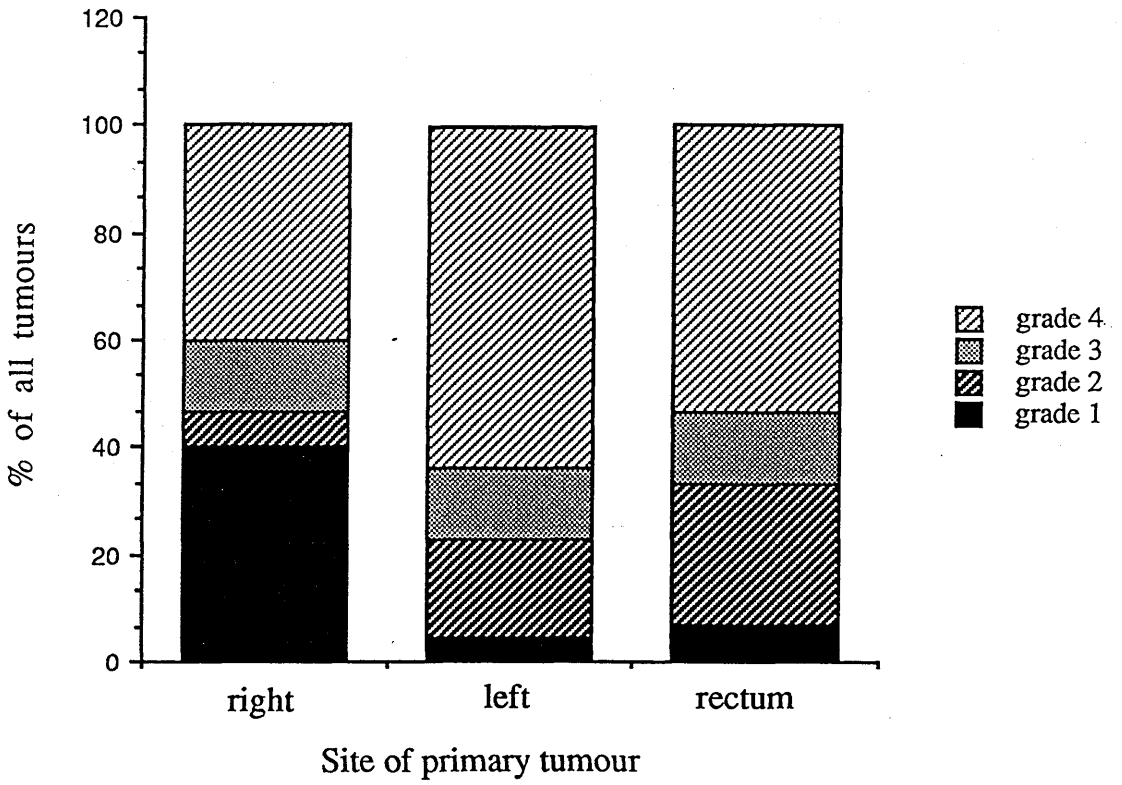


Figure 2.6. : CEA expression and tumour site



similar proportion of positive staining as was seen in the primary tumours from this group(95%).

Adjacent normal colon

A membranous pattern of staining involving the luminal surface of the colonic mucosa was found in 29 of the 45 specimens of normal colon (64%) stained with the anti-CEA antibody whereas none of the sections showed evidence of EMA expression. There was no correlation between CEA expression of normal colon and the immunohistochemical grading of the adjacent cancers. Of the 29 sections of normal colon expressing CEA, 20 of the associated cancers were graded 3 or 4 for CEA expression (69%), whilst for the 16 sections of normal colon which did not express CEA 11 of the adjacent cancers were graded 3 or 4 (69%). The group of patients with both a colonic carcinoma and adenomatous polyp, however, more commmonly expressed CEA in their normal colon than those with a carcinoma alone although this trend was not significant (8 of 9(89%) vs 21 of 36(58%), X^2 test, n.s.).

Adenomatous polyps

All of the polyps examined showed strong (grade 3-4) expression of CEA with a luminal membrane staining pattern similar to that of normal colon. None of the polyps expressed EMA.

2.5. CEA AND EMA EXPRESSION IN THE DETECTION OF OCCULT LYMPH NODE METASTASES IN PATIENTS WITH COLORECTAL CANCER

Patients and tissue sampling

The study was carried out on 47 of the 51 consecutive patients undergoing resection of colorectal carcinoma over a one year period. One patient had two synchronous carcinomas resulting in 48 specimens. The primary cancers and the regional lymph nodes were examined conventionally by an experienced histopathologist prior to immunohistochemical examination of the lymph nodes. The two procedures were carried out independently on sections from the same tissue blocks and the results compared.

Stained sections were scanned at low power(x10) and only when areas of CEA or EMA expressing cells were localised were these examined at high power(x25) in order to examine their cell morphology in more detail.

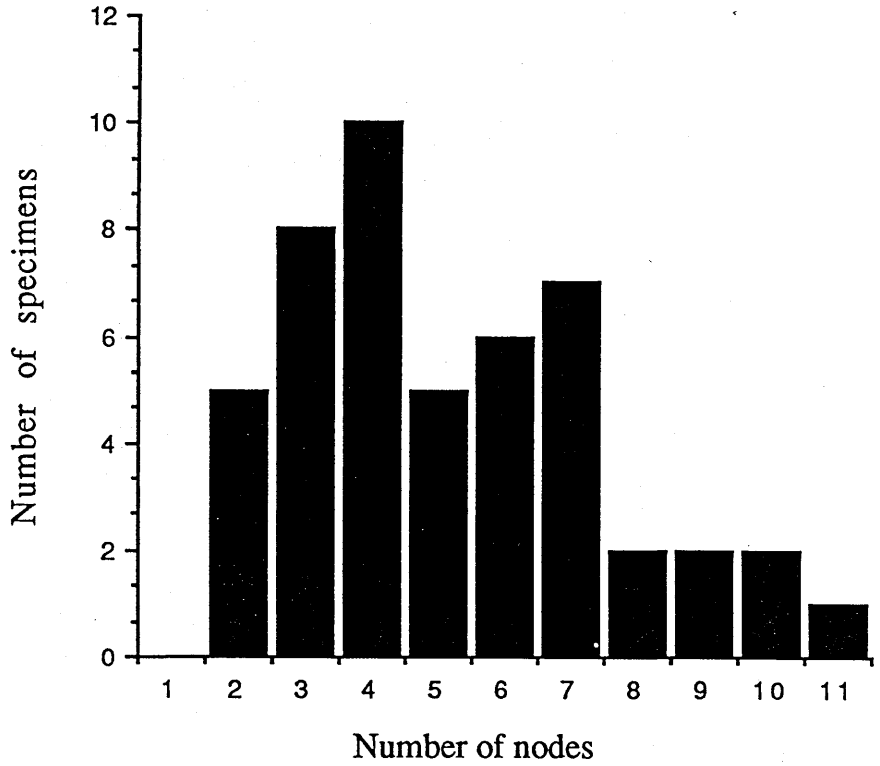
Results

Histological examination

Of the 48 colorectal cancer specimens four were staged as Dukes A, 24 Dukes B and 20 Dukes C, with 25 showing well, 20 moderate and three poor cell differentiation.

A total of 249 lymph nodes were examined by conventional light microscopy. There was a median number of five per patient (range 2-11)(Figure 2.7.).

Figure 2.7. : Number of lymph nodes examined per specimen



No significant correlation was found between the number of lymph nodes examined and the site or Dukes stage of the primary tumours (Tables 2.4. and 2.5.). Of the 249 lymph nodes examined by H & E staining, 49 (20%) contained tumour deposits and 200 were free of metastases(80%).

Immunohistochemical examination

All control sections produced the expected staining reaction or the batch of slides were restained. With both antibodies occasional plasma cells and macrophages within the regional lymph nodes showed evidence of positive staining. These were distinguished from malignant cells by their morphology.

Using C46, the anti-CEA, all metastases detected by H&E staining were shown to express CEA. Of the 200 lymph nodes reported as normal metastatic tumour was found in a single lymph node after immunocytochmical examination with anti-CEA, altering one patients Dukes staging from B to C.

On immunocytochemical evaluation with anti-EMA all but four of the lymph node metastases detected by H&E showed a positive staining reaction(92%). None of the 200 lymph nodes classified as normal on conventional light microscopy had occult metastases detected on immunohistochemical staining with anti-EMA.

Table 2.4. : Number of lymph nodes examined by tumour site

Site	Number of specimens	Number of nodes	
		Median	Range
Right colon	15	5	2-10
Left colon	19	4	2-10
Rectum	14	5	3-11

Table 2.5. : Dukes staging and the number of nodes examined

Dukes stage	Number of specimens	Number of nodes	
		Median	Range
A	4	3	2-6
B	24	5	2-10
C	20	4	2-11

2.6. DISCUSSION

In the preliminary study on the reaction of the anti-EMA antibody ICR2 with colorectal cancers (2.3.i), a similar pattern of staining was found as that previously reported with anti-EMA MAb[207] and with polyclonal antiserum[201]. Although the majority of cancer specimens examined expressed the target antigen some heterogeneity was noted between different areas within any individual specimen, a finding in common with other tumour antigens [154]. The expression of EMA by the cancers and not by normal colon suggested a possible role for ICR2 as a tumour marker in colorectal cancer and led to a comparison with CEA, the most widely investigated marker in colorectal cancer(2.3.ii). Comparing CEA and EMA in normal colon and colorectal neoplasia demonstrated that a higher percentage of colorectal cancers expressed CEA than EMA but that the pattern of staining and hence the distribution of antigen expression in the cancers was similar. Of particular interest was the lack of specificity of staining of anti-CEA in comparison to anti-EMA and the expression of EMA only in adenomatous polyps showing dysplasia.

The clinical and pathological variables in the group of patients studied in Section 2.4. are fairly comparable with those in a large prospective study on colorectal cancer[246,247]. Although the age distribution was similar the present group had a lower

male to female ratio (26 of 51 vs 506 of 709, X^2=9.3, p<0.001) and a greater proportion of cancers that were well differentiated (27 of 52 vs 203 of 709, X^2=12.4, p<0.001) and a lower proportion that were moderately differentiated. (22 of 52 vs 443 of 709, X^2=8.2, p<0.01) Otherwise there were no significant differences in the Dukes staging or the site of the cancers.

The immunohistochemistry results supported the findings of the provisional studies by the investigator [248] and others [90,207] that CEA is expressed by all primary colorectal cancers and EMA in over 80%.

The grading system employed in this study allowed cancers to be compared by their expression of tumour antigens. Although grading systems may be critisised for their semi-quantitative nature the system selected for use in the present study has been successfully applied to the antigenic expression of breast cancers [244] and has been objectively assessed and found to be reproducible [245].

A correlation between CEA expression in colonic carcinomas and their degree of differentiation has been suggested, but the evidence as to whether CEA expression is greater in well or poorly differentiated tumours is conflicting[249-252]. The grading has shown CEA expression to be greater in well rather than moderately differentiated cancers. CEA expression in the present study was also greater in distal than proximal colonic cancers, which was not a reflection of

differences in tumour differentiaton by site. A similar increase in CEA expression has been noted previously in distal compared to proximal cancers in the gastrointestinal tract [235] but scanty evidence exists of similar differences within the length of the colon. Normal tissue antigens such as mucins or blood group substances may either increase[253] or decrease [254,255] from proximal to distal. No relationship was found in the present study between the EMA expression of tumours and either their degree of differentiation or site.

CEA was expressed by all primary cancers and all nodal metastases. EMA was expressed by fewer cancers and a similar number of metastases. This is at variance with the findings of a previous study which suggested that metastases may have an enhanced expression of EMA[125].

The pattern of CEA expression found in normal colon was similar to that of adenomatous polyps with a linear staining of the luminal membranes. These results are consistent with other studies showing that CEA is a normal product of colonocyte differentiation and may be extracted from normal adult colon [256,257]. In this study EMA expression was not found in either the normal colon, which in all cases was sampled adjacent to the cancer, or in benign adenomatous polyps. This conflicts with previous results suggesting either a weak positive staining of normal colon for EMA

(238) or its expression only in normal colon adjacent to neoplasia or inflammation(201). Such differences may be explained by the cross reactivity of polyclonal antibodies used in previous studies compared with the specificity of the anti-EMA MAb used in the present study. Of interest was the increased expression of CEA by the adjacent normal colon of patients in whom both a benign adenomatous polyp and colorectal cancer were present in comparison to those with a carcinoma alone. Although not statistically significant this trend may reflect a field change in the adjacent normal colon in these patients.

The antigenic expression of tumours and their metastases is vital information if antibodies to tumour associated antigens are to be considered for use in radioimmunolocalisation or for the targeting of radionuclides, drugs or toxins for therapeutic purposes. It is well recognised that tumour antigen heterogeneity is the rule rather than the exception (154) and in this study heterogeneity of antigen expression was again noted with anti-CEA and anti-EMA within different portions of a given tumour and between primary and secondary tumours.

From this study we may conclude that CEA and EMA have a similar pattern of expression in primary colorectal cancers and their nodal metastases although EMA expression is more restricted than CEA. Normal colon and adenomatous polyps express CEA but not EMA.

This pattern of antigen expression may be of clinical significance, MAb's to CEA or EMA could be selected for use in imaging or therapy depending on the immunocytochemical staining of tumour biopsies.

In the study on the use of anti-CEA as well as anti-EMA in the detection of lymph node micrometastases the eventual tumour staging was altered in one patient by using immunohistochemistry in addition to light microscopy for the detection of lymph node metastases. This result is similar to that of a previously reported study where anti-CEA was used alone in the detection of micrometastases[91]. In contrast a recent report, again using anti-CEA, showed that metastatic deposits could be detected in the regional lymph nodes of up to 25% of the colorectal carcinoma patients examined [92].

The reason for the significantly higher rate of detection in the second study is not clear but the number of lymph nodes examined may be a crucial factor. Neither of these studies, however, appears to have employed techniques to facilitate the lymph node harvest such as mesenteric fat clearance [258]. Similarly the number of sections taken of the lymph nodes sampled may be important. In this study a single section of each lymph node was examined as is routinely practised for H&E microscopy. Sampling a single section of each lymph node, however, will result in some small metastases being missed independent of the staining method used for their detection [259].

In the present study some cross reactivity with normal plasma cells and macrophages was observed with both antibodies, a problem noted by Cutait, and others in previous immunocytochemical studies using antibodies to CEA and EMA [92,206,260,261]. Care was taken during the present investigation and that of Cutait and colleagues to confer a malignant diagnosis on antigen positive cells only if the morphology was that of neoplastic cells.

The overall results of the present study would not suggest a useful role for carrying out immunohistochemistry on lymph node sections as routinely assessed by H & E examination. In fact with EMA the diagnostic yield was worse than with H & E staining. The detection of metastatic deposits may well be significantly improved, however, by a) sampling a larger number of lymph nodes from each specimen; b) examining multiple sections of each node; c) using a panel of MAb's including those against other tumour associated antigens which have been shown to be expressed by colorectal cancers [83,88]. The detection of occult tumour deposits in the regional lymph nodes by such means in patients previously thought to have Dukes stage A or B tumours may explain relapses seen following apparently curative resections.

CHAPTER 3

CIRCULATING LEVELS OF EPITHELIAL MEMBRANE ANTIGEN IN PATIENTS WITH BREAST AND COLORECTAL CANCER

CONTENTS

3.1. INTRODUCTION

3.2. MATERIALS AND METHODS

i) Serum samples

ii) Monoclonal antibody

iii) Radioimmunoassay for EMA

3.3. RESULTS

3.4. DISCUSSION

3.1. INTRODUCTION

Whether or not a tumour associated antigen is shed into the circulation has important implications for the clinical use of MAb's to that antigen in patients with cancer. It may be useful as a circulating tumour marker if levels reflect the presence or absence of cancer. Conversely it may be a disadvantage for therapeutic use or radioimmunolocalisation if immune complexes are formed.

The role of circulating tumour markers in colorectal cancer has already been summarised (1.3.i). Post-operative levels of CEA at present is of established clinical value in the detection of recurrent colorectal cancer [57,71,72].

Circulating levels of EMA (1.5.i) have been investigated principally in patients with ovarian [210,213] and breast cancer [209,211,212,213] although a recent study has included a group of patients with colorectal cancer [213]. These studies have shown detectable levels of antigen in most normal control patients and significant elevations only in patients with advanced cancer. Of the 33 patients with colorectal cancer analysed by Ashorn and colleagues [213] only one had a level above that of controls. Although these studies have not so far established a useful clinical role for EMA as a tumour marker the circulating antigen detected by the various MAb's raised to EMA would appear to be markedly different.

Ceriani [262] isolated antigens of 46, 70 and 150kD MWt whereas Burchell [209] isolated antigens of 190, 230, 280 and 320kD. These circulating antigens are significantly smaller than the 400kD MWt EMA antigen isolated and characterised by Ormerod from human breast milk [208]. This would suggest that only a portion of the EMA molecule is shed from normal or tumour tissue into the blood. Monoclonal antibodies which recognise a single epitope on the EMA molecule may or may not, therefore, recognise all of the fragment which is secreted. This may explain the wide variation in the reported levels of circulating antigen depending on the particular anti-EMA MAb used.

In this study a competitive binding assay employing a newly developed rat antibody raised in the rat to MFGM has been employed to analyse the serum of patients with breast and colorectal cancer as well as a small group of other cancers for the presence of circulating EMA. The purpose of this investigation is to determine whether the antigen recognised by ICR2 is shed into the circulation to be of value as a tumour marker or to interfere with radioimmunolocalisation.

3.2. MATERIALS AND METHODS

3.2.i) Serum samples

Serum samples obtained from four groups of patients were studied. The groups comprised ;

A : 31 patients with colorectal cancer.

B : 52 patients with breast cancer.

C : 43 age matched controls

D : 9 patients with other forms of cancer (pancreas x 2, gastric x 1, lung x 1, oesophagus x 5).

Samples of serum were taken from patients at University College Hospital with colorectal cancer prior to undergoing surgery, samples being stored at -20^{o}C until the assay was carried out. Sera from patients with breast cancer had been snap frozen and were obtained from the liquid nitrogen bank of the Royal Marsden Hospital, Sutton, Surrey and comprised of sera taken from patients since 1974. The control sera were obtained from the National Blood Transfusion Service and consisted of Hepatitis B surface antigen and Human Immunodeficiency Virus (HIV) screened samples taken from blood donors spanning a similar age range as the cancer patients. The samples were again stored at -20^{o}C until used. The age and sex of these patients is given in Table 3.1.

3.2. ii) Monoclonal antibody

The monoclonal antibody ICR2 was used throughout these studies. It has been described previously(1.6.i)

3.2.iii) Radioimmunoassay(RIA) for EMA

A competitive binding R.I.A. was developed to detect the EMA antigen in serum using the rat anti-EMA monoclonal antibody ICR2. A purified preparation of EMA was prepared from human breast milk by the method of Ormerod et al,(208) the preparation containing 16.9ug/ml of protein. Microtitre 96 well PVC plates were coated with antigen (50ul/well of a 1/400 dilution of EMA in P.B.S. at pH 8.0) by incubation overnight at 4^{o}C, then washed and blocked by addition of 200ul/well of P.B.S. with 0.5% bovine serum albumin (PBS-BSA). Using a seperate 96 well microtitre plate doubling dilutions of duplicate 30ul aliquots of each serum sample (centrifuged after thawing to remove debris) were prepared and an equal volume (containing 4x10^{4} cpm ^{125}I) of ^{125}I-ICR2 (labelled by the Iodogen method (119) to give a specific activity of 10uCi/ug) added. Aliquots of 50ul of these mixtures were transferred to the EMA coated PVC plates, incubated for one hour at 18-20^{o}C then washed with PBS-BSA. Bound ^{125}I was determined by counting individual wells in an Innotron Hydragamma 16 well counter.

A standard curve (Figure 3.1.) was prepared using doubling dilutions of EMA (starting concentration 10ug/ml) mixed with equal volumes of ^{125}I-ICR2 and processed as described for the serum samples. Semilogarithmic plots of percentage maximum counts bound versus serum dilutions were prepared and the

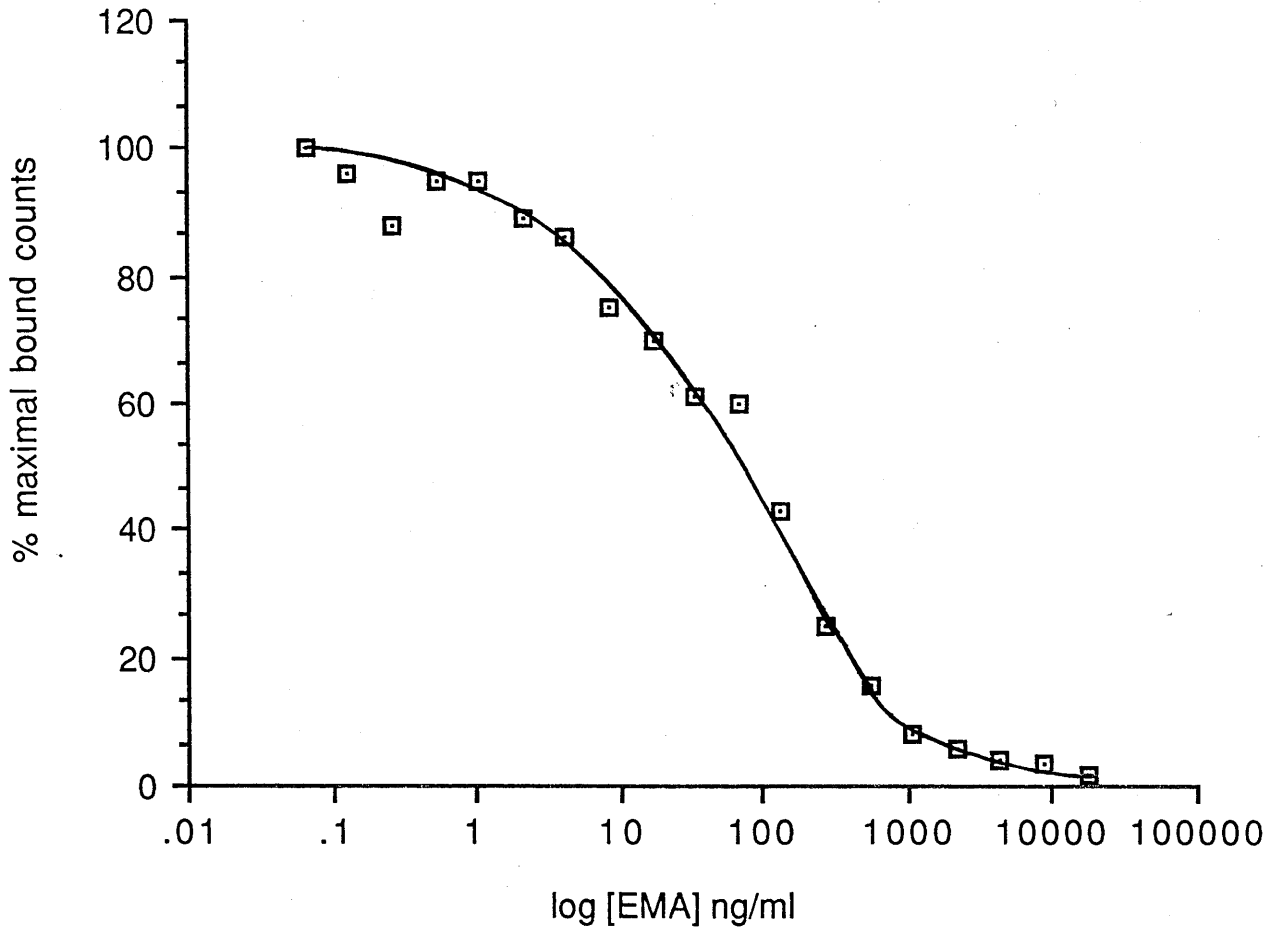


Figure 3.1. : EMA standard curve

concentration of EMA-like material present in the sera was determined from the standard curve for dilutions of serum that lay on the straight portion of the semi-log plots.

Results

The ranges of serum EMA levels in the four groups of patients is shown in Table 3.2. and depicted in Figure 3.2.

The median values of all patient groups was similar at 570-655 ng/ml. No significant differences were found between these groups using a Mann Whitney U test. EMA levels > 5000ng/ml were found in five patients, all of whom had cancer (oesophagus x 2, lung x 1, colon x 1 and breast x 1). In the control group the mean levels of EMA were found to be similar in males (540+/-380) and females (850+/-808)(mean+/- S.D.). Five patients in the control group had an EMA level >1000ng/ml of whom 4 were females (80%) whereas 19 of the 38 patients with EMA levels < 1000ng/ml were females (50%). This difference did not achieve statistical significance (X^2 = 1.74). The EMA levels are given for each patient within the groups in Appendix 3.1. and for the "other cancers" in Table 3.3.

Table 3.1. : Patient details

Patient group	Number	Age median(range)	Sex M	F
Colorectal cancer	31	74(51-87)	11	20
Breast cancer	52	59(33-81)	0	52
Controls	43	54(49-64)	21	22
Other cancers	9	72(44-82)	2	7

Table 3.2. : Serum EMA levels (ng/ml)

	Controls	Colon Ca	Breast Ca.	Other Ca.
Number	43	31	52	9
Minimum	90	60	210	120
Maximum	3240	8530	13300	67400
Range	3150	8470	13090	67280
Median	570	580	655	580
Mean	714	830	1003	9542
S.E.*	93	262	249	7312
S.D.**	609	1462	1801	21937

* Standard error of the mean

** Standard deviation

Table 3.3. : EMA levels in other cancers

Cancer	EMA level (ng/ml)
Pancreas	185
Pancreas	580
Stomach	220
Lung	9720
Oesophagus	580
Oesophagus	67400
Oesophagus	120
Oesophagus	1720
Oesophagus	5360

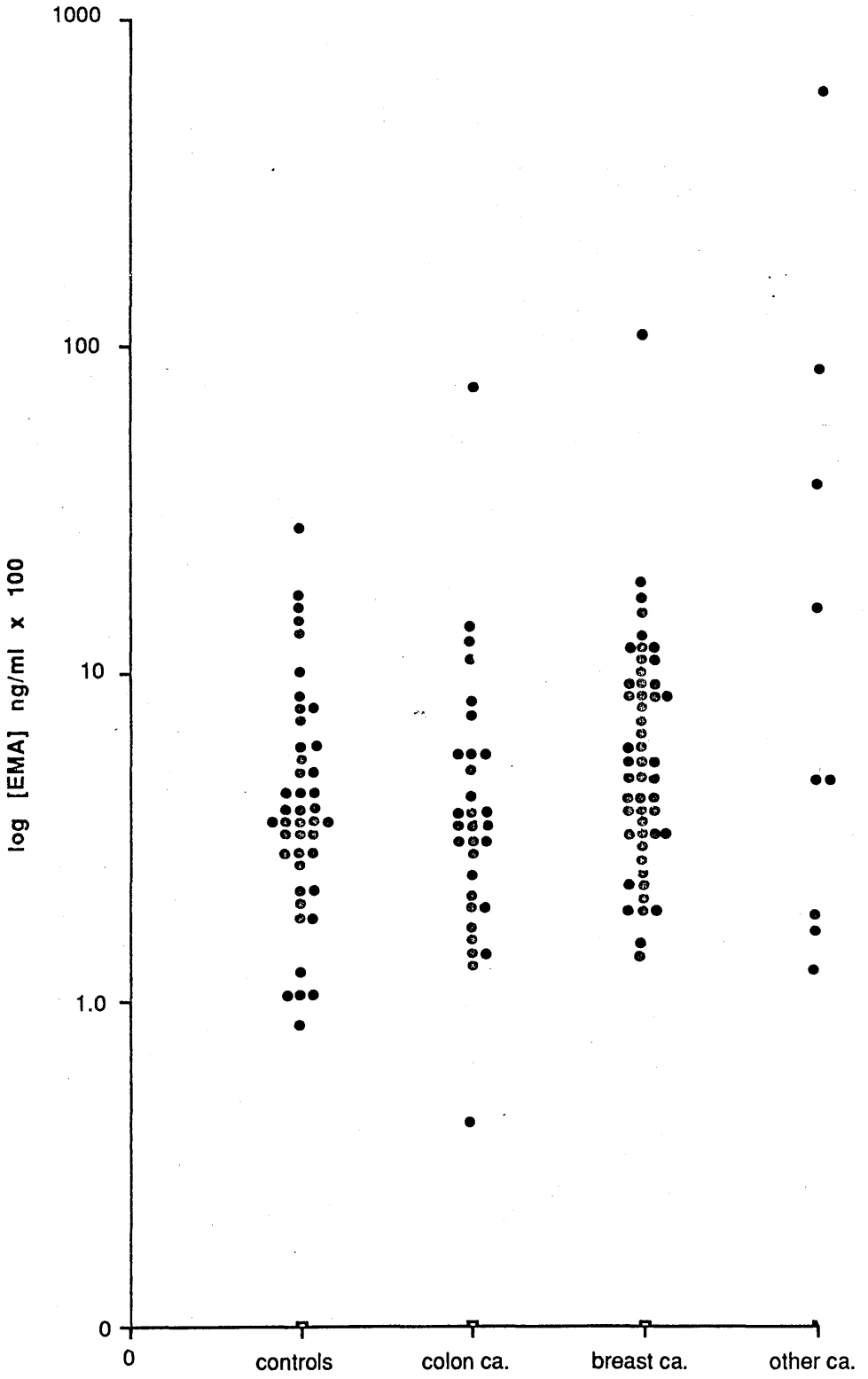


Figure 3.2. : Serum EMA levels

3.4. DISCUSSION

The normal control population in this study has been found to have detectable levels of circulating EMA which is in keeping with previous reports [262,209,213]. The range of normal levels, however, of 90-3240ng/ml is wider than those previously reported with a greater than 30 fold difference within the group. This may reflect important differences in the control group sampled. In the present study we measured EMA levels in serum samples from age matched patients obtained from the blood transfusion bank whereas Ceriani[262] selected 13 healthy subjects, Burchell[209] 30 healthy women and Ashorn[213] 52 healthy female medical students. The control group is therefore better matched for comparison with the cancer patients although occult malignancy cannot be excluded in this age group. An additional factor which should be considered is the strong expression of EMA by breast and ovarian tissues which would suggest that EMA expression may vary within the menstrual cycle or alter after the menopause. Levels of the breast cancer antigen MAM 6 have been shown to be elevated during the third trimester of pregnancy [212]. In the present investigation no significant differences were found between the levels of circulating EMA in males and females. In the control group, however, a greater number of patients with EMA levels >1000ng/ml were females compared to patients with levels <1000ng/ml. This difference, however, did not achieve statistical significance.

There was no difference in MAM 6 levels noted between males and females in the control group of Hilkens and only a small intra-patient variation in samples taken over a one year period (212).

Patients with breast cancer were studied as they have previously been shown to have elevated HMFG antigen levels (209,213,262). These studies have mainly demonstrated elevated levels only in patients with advanced disease(209) while those with localised primary disease had normal levels (213,262). In the present study the serum samples from the patients with breast cancer were taken at presentation at the hospital out-patient clinic and therefore will include both patients with primary and those with metastatic disease. In view of the similarity in EMA levels between the breast cancer patients and the controls it was not considered worthwhile to compare EMA levels with the stage of disease. Differences reported in the levels of circulating EMA between previous studies and the present investigation would be most readily explained if the majority of breast cancer patients in the present study had localised disease. The possibility remains, however, that some patients do not shed the portion of EMA recognised by ICR2 into the circulation.

Another possible cause of discrepancy between the present results and those of the previously mentioned studies is the methodology for the detection of circulating antigen. The methods usually employed

utilise MAb coated plates or beads for capturing the suspected antigen in serum which is then detected by the use of a labelled second antibody which recognises a different epitope [210,211,213,262]. These methods differ significantly from that employed in the present study in which putative antigen in serum competes with antigen in the serum for binding of applied ICR2-^{125}I. Because of differing methodologies employed the results of these serum assays may not be strictly comparable.

Despite the demonstration of the presence of EMA on the majority of colorectal cancers (Chapter 2) the sera from patients with this disease did not differ significantly in "EMA" content from those of the controls. Other studies have also suggested that circulating EMA levels are rarely elevated in patients with colorectal cancer eg. Ashorn and colleagues found an elevation in only 1 of 33 patients[213], Hilkens in 4 of 19[212] and Hayes in 1 of 48[211]. The latter group also included 25 patients with liver metastases which would suggest that the normal level is not due to the removal of antigen from the portal circulation by the liver. With CEA Tabuchi has demonstrated significantly higher levels in the portal vein than in the peripheral circulation and has shown a strong correlation between the level and degree of venous invasion of the tumour [263]. Similarly the measurement of peripheral and portal venous EMA levels would be required to establish whether the liver is

involved in EMA metabolism. It seems likely, however, that even when large bulk disease is present the amount of EMA shed by colorectal cancers is insufficient to affect the overall level in blood produced by other EMA expressing tissues (201,207).

These findings may have important implications on the use of radiolabelled MAb's to EMA for the detection of colorectal cancer and their metastases by immunoscintigraphy (Chapter 5). It would be expected that patients with high circulating levels of antigen would form immune complexes and reduce the level of radiolabelled antibody reaching the cancer. In animal models with cancer xenografts secreting CEA this has been supported with higher CEA secretion being associated with higher clearance of activity by the liver and reduced tumour uptake (264). This has been contested in patient studies in which satisfactory tumour imaging with anti-CEA has been achieved despite high circulating CEA levels (97).

The group of other cancers analysed by this assay has produced some interesting elevations in EMA levels. Four of the 9 patients in this group had EMA levels > 1000ng/ml. Of these 4 patients 3 had oesophageal and one a bronchial carcinoma. Few reports on the EMA levels in either group of patient are available. Ashorn in 4 and Hayes in 7 patients with oesophageal cancer reported no increase in EMA levels. Further investigation of EMA levels in these patient groups

would be interesting.

In conclusion some patients with breast, colonic and other forms of cancer have EMA levels above the normal range of healthy controls. The majority, however, have levels within this normal range which would suggest that EMA is unlikely to be useful as a circulating tumour marker.

CHAPTER 4

AN INVESTIGATION INTO THE LIVER UPTAKE OF INDIUM LABELLED MONOCLONAL ANTIBODIES

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4.4. DISCUSSION AND CONCLUSIONS

4.1. INTRODUCTION TO THE PROBLEM

The greatest limitation to the use of radiolabelled monoclonal antibodies for the imaging of cancers and their metastases is the level of background radio activity. This uptake by normal tissues reduces the signal to noise ratio given by the tumour and the ability to detect the cancer.

The problem is encountered independent of the antibody used[130] although different radioisotopes result in differences in the uptake of activity in normal organs [265,266].

With iodinated antibodies their persistence in the blood pool rather than in organs is the major problem and has required the use of blood pool subtraction techniques to clarify the images obtained. This may result in false positive images [122,123,131] and may explain the high incidence of tumour localisation in early imaging studies [97]. In addition, the rapid tissue dehalogenation reduces the activity persisting in the target tissues and results in a high urinary excretion of the radiolabel. Other sources of background radio-activity include the secretion of radioiodine by the gastric mucosa and uptake in thyroid tissue despite the use of blocking agents.

A satisfactory alternative to radioiodine and at present the radionuclide of choice for antibody imaging is ^{111}In. It has a more suitable energy of emissions for gamma camera imaging and forms stable complexes

with proteins via chelating agents [126-129]. A high level of non specific uptake, however, has been reported in studies carried out with ^{111}In-MAb [102,105,106,125]. The main site of uptake is the liver and to a lesser extent the spleen, bone marrow and testes. This non specific uptake is of particular relevance in the imaging of colorectal cancers. The most common site of primary neoplasms, namely the rectum and sigmoid colon, may be obscured by uptake in the bony pelvis. Nodal deposits, with a smaller uptake of radiolabel, would anatomically tend to overlie midline abdominal structures of the great vessels and lumbar spine, both of which have high non-specific activities.

By far the major contributor to background activity with ^{111}In-MAb is the liver. Organ dosimetry calculations in patients following ^{111}In-MAb administration would suggest that one third of the injected activity may have accumulated in the liver 24 hours after administration [125]. This accumulation of activity in normal liver is so considerable that the detection of liver metastases following the administration of ^{111}In-MAb is usually based on the in-filling of cold areas on early scanning images [106] or on the use of a tin colloid liver scan for digital subtraction [102]. This accumulation of activity also affects the ability to image extra-hepatic deposits if these lie near the liver or within the same imaging field and results in the liver being the dose limiting

factor for the administration of ^{111}In labelled MAb's.

The optimum radioisotope for use in antibody imaging(^{111}In) is therefore limited by its organ uptake, principally into the liver. Two mechanisms have been suggested to explain this non-specific accumulation. Firstly uptake by the macrophages of the reticuloendothelial system and secondly uptake into hepatocytes via transferrin receptors.

4.1.i) The reticuloendothelial system (RES)

The main sites of non specific uptake, namely the liver, spleen and bone marrow would suggest that the main route for the removal of radiolabelled antibodies is via the RES and that the removal of the circulating IgG is part of the body's natural defence mechanism to foreign proteins.

Macrophages of the RES have several receptors which may recognise and bind the constant region of the Ig molecule(Fc)(Figure 4.1.). This includes a receptor for the binding of monomers and complexes of IgG and another mediating endocytosis of antigen-antibody complexes. Macrophages have a variety of other receptors for complement, lymphokines, colony stimulating factors, insulin, complex carbohydrates such as asialoglycoproteins and iron containing proteins. If macrophages are important to the clearance of ^{111}In-MAb from the circulation it seems likely it would be mediated via the Fc or carbohydrate receptors.

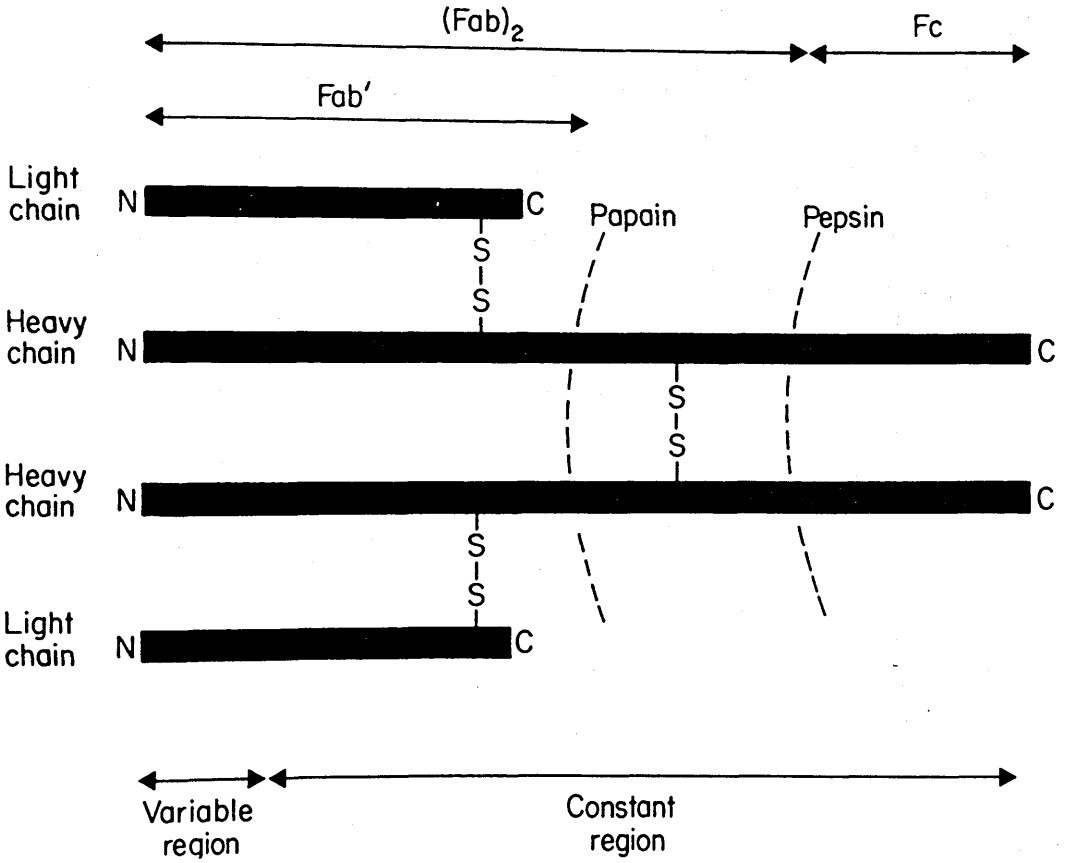


Figure 4.1. The structure of IgG and its fragments

The liver has been shown to be the principal organ responsible for the removal of immune complexes from the circulation of experimental animals [267,268]. The administration of antibodies which recognise and bind to an antigen which is secreted into the circulation may result in the formation of circulating immune complexes which are cleared from the blood by the liver via an Fc receptor mediated mechanism. In 1985 Hagan demonstrated that the concentration of ^{111}In in the liver following administration of ^{111}In labelled anti-CEA antibody to mice with CEA secreting xenografts increased as the CEA secretory state of their tumours rose. Furthermore those with a high CEA secretion had decreased uptake of radiolabelled antibody by the tumour [264]. In contrast to the findings of these animal experiments Goldenberg[97] reported that in patients undergoing imaging of colorectal cancers with anti-CEA the imaging of the tumours was satisfactory even in the presence of grossly elevated CEA levels.

The hepatic uptake of immune complexes (I.C.) is mainly thought to be a function of the hepatic macrophages or Kupffer cells which bear a surface receptor for the Fc portion of the IgG molecule [269, 270]. However, Fc receptors have also been shown to be present on the hepatocyte membrane [271] and the uptake of I.C. by hepatocytes has been demonstrated in immune animals [272]. In 1984 Sancho and colleagues assessed the binding kinetics of monomeric and aggregated IgG to Kupffer cells and the hepatocytes of mice. By isolating

mouse hepatocytes and Kupffer cells following the administration of radiolabelled human IgG to mice they demonstrated that 60-70% of the liver uptake of radioactivity was recovered in hepatocytes. In-vitro the maximum number of IgG and aggregated IgG (A-IgG) molecules bound per cell was higher with hepatocytes (mean 14 x 10^6) than on Kupffer cells (mean 2 x 10^5) although the association rate constant for aggregates was higher for Kupffer cells than for hepatocytes (273). The binding site was present on the constant region (Fc) of the antibody (Figure 4.1.) as $F(ab')_2$ fragments (Figure 4.1.) failed to inhibit labelled monomeric IgG or A-IgG. The receptor also appeared to be specific for IgG as unlabelled monomeric IgA did not inhibit the binding of labelled IgG or A-IgG.

4.1.ii) INHIBITION OF RETICULOENDOTHELIAL UPTAKE

If binding to Fc receptors is an important mechanism for the non specific clearance of MAb's this will have some important clinical implications. First it may be possible to block non-specific antibody uptake by the concomitant administration of either pooled human Ig, an antibody of the same isotype or unlabelled monoclonal antibody itself. Secondly the use of labelled $F(ab')_2$ (Figure 4.1.) fragments which do not have an Fc sequence should overcome the problem altogether.

4.1.ii.a) The effect of administering pooled IgG

The effect on non-specific uptake of administering pooled human Ig before administration of labelled MAb was monitored in patients with melanoma by Carrasquillo [243]. Two patients were given 7.5g of pooled human Ig over the one hour prior to injection of ^{111}In labelled MAb (MAb 9.2.27). Although no measurements of tissue uptake using excised tissues were carried out the data obtained for plasma clearance and for serial imaging was similar to that seen in patients given 1mg of radiolabelled antibody alone. Large amounts of radiolabel continued to accumulate in the liver, spleen and bone marrow. Pooled human Ig has previously been shown to effectively block Fc receptor binding [274] which would suggest that either the dose administered (7.5g) was insufficient to block Fc

receptor binding or that this is not an important mechanism in producing non specific uptake of radioactivity.

4.1.ii.b) Unlabelled MAb

In another group of patients with malignant melanoma Carrasquillo [243] also analysed the effects on biodistribution of giving unlabelled Ig before the administration of labelled antibody. Five patients recieved 49mg and one patient 99mg of unlabelled antibody prior to administration of 1mg of ^{111}In labelled antibody. Five control patients recieved 1mg of labelled antibody alone. No data for tissue uptake of radioactivity was presented but when unlabelled antibody was co-administered it was found to increase the half life in the blood and to reduce the uptake in the spleen and bone marrow. More importantly an increased number of tumour sites were localised. The unlabelled antibody, however, did not have a significant effect on reducing liver uptake. The effects on blood pool distribution by the injection of unlabelled antibody or giving higher antibody dosages had been noted previously and termed the "mass effect" [130]. The differences observed between the uptake in the liver and that of the spleen and bone marrow may suggest that the mechanisms of uptake vary in the different sites.

4.1.ii.c) Antibody fragments

Antibody fragments retaining the antigen binding specificity of the antibody but with loss of the Fc portion can be produced by proteolysis (Figure 4.1.). These fragments are either divalent $F(ab')_2$ or monovalent (Fab') if the disulphide bridge linking the heavy chains is reduced. It may seem an obvious solution to the problem of Fc receptor mediated uptake of MAb's to use these fragments which maintain the antibody specificity of the parent molecule without an Fc portion, but their pharmacology is quite different from that of intact immunoglobulin. While the smaller size of $F(ab')_2$ and Fab' fragments allow a more rapid uptake of antibody into the tumour tissue compared to whole antibody their half life is considerably shorter (130). Fab' fragments are excreted rapidly in the urine and are not, therefore, suitable for imaging. $F(ab')_2$ labelled with ^{111}In show a rather surprising degree of accumulation in the liver. Hnatowich(275) reported a rapid and persistent accumulation in the liver of radioactivity amounting to 20% of the injected dose in cancer patients administered ^{111}In labelled $F(ab')_2$ fragments of MAb CA19-9. Similarly Halpern and colleagues(130) noted a persistently high hepatic uptake with ^{111}In labelled $F(ab')_2$ fragments of an anti-CEA MAb in patients and no obvious difference could be detected between scintiscans obtained with fragments or whole antibody. These studies would not

suggest an important role for the Fc receptor in the clearance of ^{111}In labelled antibody.

4.1.ii.d) Isotype matched antibody

The administration of unlabelled prior to labelled antibody in the study of patients with malignant melanoma by Carrasquillo[243] increased the number of sites localised by external imaging. This would suggest that this procedure either increased uptake in tumour tissue or reduced background activity. Whether the pre-administration of MAb of the same isotype but different specificity would have a similar or greater effect has not as yet been established.

4.1.iii) The role of the hepatocyte

Transferrin(Tf) is a circulating plasma protein which has a high binding affinity for both Iron and Indium. The administration of free ^{111}In to patients results in the immediate formation of ^{111}In-Tf complex. This localises in and may be used clinically for detecting sites of malignancy[276,277]. Similarly any free Indium present in a preparation of ^{111}In-MAB would become bound to plasma Tf and be removed by hepatocytes which have high levels of surface receptors for Tf [278,279]. Instability of the MAb-chelate-^{111}In complex in vivo leading to the generation of free ^{111}In would also result in hepatocyte uptake via Tf receptors. This exchange of ^{111}In from labelled

antibody to circulating Tf has been calculated as being 1.9% of circulating activity in mice with tumour xenografts[280] but as high as 9% per day in humans[275]. Factors which may affect the stability of the complex and hence the rate of transfer from labelled antibody to Tf and liver uptake would include the number of chelate molecules bound per antibody molecule [281] and the nature of the chelating agent, since recently introduced chelating agents show a reduction in the liver uptake of activity[282].

The chelating agents commonly used for attaching metallic radionuclides to MAb's are derivatives of ethylenediamine-tetracetic acid (EDTA) and diethylene-triamine-tetracetic acid(DTPA). These compounds have binding affinities for ^{111}In which are of a similar order but slightly higher than that of Tf. Attempts have been made to reduce the transcomplexation of ^{111}In by administering additional chelating agents. Goodwin [280] demonstrated that the administration of a "chase" dose of EDTA following the administration of radiolabelled MAb to mice facilitated the urinary excretion of activity. A similar study carried out by Ward[283] investigated the effects on the biodistribution of ^{111}In-DTPA-M8 and ^{67}Ga-DFO-M8 in mice of the chelating agents desferrioxamine (DFO), diethylenetriamine pentaacetic acid (DTPA), ethylenediamine-di(O-hydroxyphenylacetic acid)(EDHPA) and 2,2' dipyridyl(DIPY). All the chelating agents

except DIPY altered the biodistribution of free ^{111}In and ^{67}Ga but did not affect the tissue uptake of the labelled antibodies.

Perhaps the most direct evidence of the importance of hepatocytes to the liver uptake of activity following ^{111}In labelled antibody administration was provided by Sands and colleagues(241), who injected mice with ^{111}In labelled antibody with or without blockade of their reticuloendothelial system (RES) and found RES blockade to make no significant difference to liver uptake. The studies of Shochat(240) have demonstrated that the activity following ^{111}In-MAB administration forms of a low molecular weight complex within the liver.

4.2. A MODEL FOR LIVER UPTAKE OF ^{111}In-MAb

Despite the major clinical problems resulting from the liver uptake of ^{111}In-MAb it is apparent from the preceding discussion that the mechanism of uptake has not been established and methods to reduce liver accumulation have not as yet been determined. The studies by Sands, Sancho and Shockat outlined above would suggest that the low molecular weight complex which accumulates in the liver following 111In-MAb administration involves hepatocytes rather than the RES (241,240,273).

To investigate ways in which the uptake of ^{111}In-MAB by liver parenchymal cells may be reduced an in-vitro

model has been developed which mimics the localisation of an intra-abdominal tumour with ^{111}In labelled MAb. This consists of freshly isolated rat hepatocytes and a cultured tumour cell line (MCF7), which expresses the specific antigen (EMA) identified by the monoclonal antibody ICR2. To these tissue cultures a variety of reagents have been applied to assess their efficacy in preferentially inhibiting the uptake of ^{111}In-MAB.

4.2.i) Materials and methods

4.2.i.a) Radiolabelled antibody

The rat MAb ICR2 was conjugated to the chelating agent DTPA using the bicyclic anhydride (ccDTPA)(Sigma, Poole, Dorset) at a molar ratio of 2:1 based on the method of Hnatowich [128]. At this ratio the conjugate retained 90% of the immunoreactivity of unconjugated ICR2. The antibody was labelled with ^{111}In (Amersham, U.K.) by the method of Carrasquillo [243] and any unbound ^{111}In separated by gel chromatography on Sephadex G25. Using this method over 95% of the radioactivity in the protein peak remained protein bound on thin layer silica gel chromatography following treatment with excess DTPA (1mmol for 15 minutes). In all experiments the antibody was reacted with ^{111}In at a ratio of 1mg antibody to 2mCi $^{111}InCl_3$, as used in patient studies. The ^{111}In-ICR2 was applied to the cells to give a final concentration of approximately 2 ug/ml. (Further description of conjugation and labelling procedures is given in Appendices 5.1.- 5.4.)

4.2.i.b) Isolation and preparation of cells

Hepatocytes were isolated from the rat liver by collagenase perfusion as follows ;

Male Wistar rats weighing less than 350g were selected. The rats were anaesthetised by an intramuscular injection of 0.4mls of diazemuls (diazepam 10mg/2 mls. Kabivitrum, Uxbridge, U.K.)

followed by 0.4mls of Hypnorm (Fentanyl citrate, 0.315mg/ml and Fluanisone 10mg/ml, Janssen animal health). An incision was made in the midline of the abdomen extending along both costal margins to fully expose the liver and abdominal organs. The bowel was retracted to one side and the portal vein exposed. Ligatures were passed round the portal vein and the vein was then cannulated (Braunule,18 gauge, B. Braun, Melsungen, Germany) the plastic sheath being held in place by the ligatures. Once completed the animal was killed by exsanguination by division of the inferior vena cava. The liver was removed suspended by the cannula in the portal vein and transferred to a perfusion cabinet which was preheated to 35^{o}C. The liver was perfused for 10 minutes with Hanks balanced salt solution (at 35^{o}C) before introducing the collagenase mixture (Table 4.1.) to which 200umol/l calcium chloride was added. After recirculation of this mixture through the liver for 20 minutes the liver was removed from the perfusion cabinet and "shaken out" to dislodge the cells. Any large aggregates of cells were divided by sharp dissection and the resultant cell mixture was then filtered through a coarse gauze into universal containers. The filtered cells were then spun in a centrifuge at 400 r.p.m. for two minutes. The supernatant was discarded and the cells which had been spun to the base of the universal container were resuspended in foetal calf serum and respun. The

Table 4.1. Composition of the collagenase perfusate

Collagenase (Type 1)(Boeringer, Mannheim)	50mg
DNAase (Type IV) (Sigma)	5mg
RNAase(Type 1-A) (Sigma)	5mg
Soya bean Trypsin inhibitor E (Sigma)	10mg

These are dissolved in 20mls of Hanks balanced salt solution.

Table 4.2. Culture medium

RPMI 1640 (Gibco)	500mls
10% heat inactivated foetal calf serum (FCS)	50mls
Streptomycin (Sigma)(final concentration 50ug/ml)	27.5mg
Penicillin(Sigma) (final concentration 50units/ml)	30mg
Insulin (Sigma)2 x 10^{-6}M	6mg

Under aseptic conditions the FCS was added to the RPMI medium by filtration through a 0.22um millipore filter(Millipore S.A., Molsheim, France). Antibiotics in the quantities shown above were added and mixed until dissolved.

procedure of resuspending and spinning the cells and discarding the supernatant was then repeated a further six times. After the second spin the cells were filtered through a fine gauze and from the fourth spin onwards the procedure was carried out under aseptic conditions. After the final spin the cells were transferred to a sterile culture flask (Falcon, Becton Dickinson) and an aliquot counted to assess cell numbers using a haemocytometer slide. After calculating the cell numbers, medium was added to the flask to produce a final cell concentration of 1 x 10^6 cells / ml. One ml of this cell suspension and 2 mls of RPMI 1640 medium (Gibco, U.K.) containing 10% foetal calf serum and antibiotics (Table 4.2.) were plated onto sterile collagen coated plates (Falcon, Becton Dickenson) and left to adhere over a two hour period.

Experiments were carried out within 24 hours of cell isolation, previous experiments having shown > 98% viability of the isolated hepatocytes within this time period (284).

4.2.i.b) Tumour cell line

The human breast cancer cell line MCF7 was used. This cell line strongly expresses the EMA antigen recognised by the MAb ICR2. It also has good growth characteristics and forms a confluent monolayer. The cell line was grown in a culture flask in RPMI 1640 medium which was enriched with 10% foetal calf serum and antibiotics (Table 4.2.). When the cells had grown

to confluence they were removed from the flask by trypsinisation, washed three times and then resuspended in sufficient medium to allow 2mls of the new cell suspension to be aliquoted into each plate. Experiments were carried out when the cells had grown to form a confluent layer in the plates, approximately 5 days from splitting the cells in the flask.

4.2.i.c) Experimental procedure

The rat hepatocytes were isolated and experiments carried out when the tumour cells had grown to form a confluent monolayer on the plates. Three plates of hepatocytes and three of MCF7 cells were used for each variable under investigation and as a control in each experiment. Prior to use the plates of cells were washed repeatedly with RPMI medium (Table 4.2.) to remove any dead and non adherent cells and 2mls of fresh medium added to all plates.

The MAb ICR2 was labelled with ^{111}In (Appendix 5.4.) at the ratio of 1mg ICR2 to approximately 2mCi $InCl_3$, the labelled activity depending on the time period between generation of the radioisotope and its use. The ICR2-^{111}In was applied to the medium in the plates of cells with the amount of labelled antibody applied being constant for all plates. The reagents being investigated were also added to the medium in the plates of cells at variable times and at different concentrations or to the labelled antibody prior to its

application to the cells. After a period of incubation at 37°C the supernatant was collected from each plate as were four washings of the cells with 2ml aliquots of PBS. In view of the rapid accumulation of activity by the liver seen in the studies with patients an incubation time of two hours was used in the majority of experiments. The cells were then removed from the plates in 2mls PBS using a rubber policeman and the supernatant, washings and cells from each plate counted in a gamma well counter (LKB 1282 Compugamma). In order to compensate for any variations in the number of either hepatocytes or tumour cells per plate the protein content from each plate was determined using the Folin-Lowry procedure [285] (Appendix 4.1.) and the radioactivity bound to the cells was expressed as the counts per minute taken up by the cells / mg cell protein.

The uptake of activity by the cells was compared between groups of at least three plates following correction for protein concentration using a students t-test.

4.2.ii) Basic properties of the experimental model

Baseline parameters were established for the uptake of ^{111}In-MAb by the hepatocytes and the tumour cell line. When the cells were incubated for two hours with approximately 3 x 10^6 cpm of ^{111}In-ICR2 in 2mls of medium, 34.3% (+/- S.D. 2.78) of the applied counts bound / mg of cell protein with the tumour cell line and 3.65% (+/- S.D. 0.67) of the applied counts bound /mg cell protein with the hepatocytes (Table 4.3. and appendix 4.2.). Incubating the cells with labelled antibody at either 4^{o}C or 37^{o}C, showed that hepatocytes bound 57% more activity at 37^{o}C than at 4^{o}C while binding to the tumour cell line was 123% greater at 37^{o}C than at 4^{o}C (Table 4.4. and Appendix 4.3.). It was concluded from these initial experiments that the binding between the labelled antibody and its specific receptor on the tumour cell line was considerably greater than with the hepatocytes and that an active and temperature dependent uptake mechanism was involved. A series of experiments were then carried out to assess the use of a variety of reagents in preferentially reducing the hepatocyte uptake of activity by the method described earlier. (4.2.i.c)

Table 4.3. Baseline uptake of ^{111}In-ICR2

Cell type	Applied cpm ($x10^6$)	Cell uptake cpm($x\ 10^4$)	Protein (mg/ml)	cpm/mg	% uptake /mg
MCF7	3.02(0.17)	26.4(2.5)	0.25(0.03)	1.06×10^6	34.3(2.78)
Heps.	2.93(0.27)	2.1(0.22)	0.20(0)	0.107×10^6	3.65(0.67)

All results are mean (S.D.) of triplicate determinations (Appendix 4.2.)

Table 4.4. The effect of temperature on cell uptake

Cell uptake of activity (% applied counts/plate)	Temperature 4° C	37° C
Hepatocytes	0.28(0.02)	0.44(0.10)
MCF 7	5.03(1.13)	11.2(0.79)

Results are mean(S.D.) of three determinations (Appendix 4.3.)

4.3. RESULTS

4.3.i) Transferrin

To assess the importance of transferrin and the transferrin receptor mechanism for uptake of ^{111}In-ICR2, hepatocytes or MCF7 cells were incubated with ^{111}In-ICR2 in the presence of either saturated or unsaturated transferrin, prepared at a final concentration of 2g/l (Appendix 4.4.). Foetal calf serum (FCS) contains transferrin and was therefore replaced by bovine serum albumin (BSA) in these experiments.

In the first experiment the influence of the iron saturation state of transferrin on the uptake of ^{111}In-ICR2 was assessed with hepatocytes alone. The hepatocytes took up approximately 0.1% of the applied counts. This value was not significantly altered by the addition of either fully saturated or fully unsaturated transferrin (ApoTf) at a concentration of 2g/l (Table 4.5. and Appendix 4.5.). For further confirmation that the transferrin saturation state was not a major influence on the cell uptake of ^{111}In-ICR2 the experiment was repeated using fully unsaturated transferrin and both hepatocytes and MCF7 cells. Again no significant differences were found in the uptake of ^{111}In-ICR2 by the presence of apotransferrin (Table 4.6. and appendix 4.6.).

Table 4.5. Tf saturation and the hepatocyte uptake of ^{111}In-ICR2

Cell group	Applied cpm(x 10^5)	Counts bound	% uptake
Control	3.56(0.40)	359(76)	0.101(0.021)
Saturated Tf	3.69(0.30)	350(49)	0.095(0.017)
Apo Tf	3.79(0.15)	363(122)	0.095(0.030)

Results are mean (S.D.) for triplicate determinations. (Appendix 4.5.)

Table 4.6. ApoTf and ^{111}In-ICR2 uptake by hepatocytes and MCF7

	MCF7		Hepatocytes	
	Control	**Apo Tf**	**Control**	**Apo Tf**
Applied (cpmx10^6)	2.45(0.01)	2.38(0.07)	2.41(0.04)	2.41(0.05)
Uptake (cpm x 10^3)	299.8(32.1)	275(35.6)	5.92(0.64)	5.51(0.92)
% uptake	12.2(1.32)	11.53(1.20)	0.25(0.02)	0.22(0.04)

Results are mean(S.D.) for triplicate determinations (Appendix 4.6.)

4.3.ii) DTPA

4.3.ii.a) Co-administration of DTPA with ^{111}In-ICR2

To assess whether the metal chelating agent DTPA may have an effect on the uptake of activity by hepatocytes the labelled antibody was applied to monolayers of both the MCF7's or hepatocytes, together with or without (control) DTPA added to produce a final [DTPA] in the tissue culture medium of 100umolar. A reduction of 13% in the uptake of activity on MCF7's was found with DTPA compared to the control group(n.s.) whereas a significant reduction of 43% was found with the hepatocytes ($p < 0.01$)(Figure 4.2., Table 4.7., Appendix 4.7.)

4.3.ii.b) Cell incubation with DTPA

Whether the reduction in hepatocyte uptake seen when DTPA was added to ^{111}In-MAb could also be achieved by pre-incubating the hepatocytes and MCF7's with DTPA was investigated by adding DTPA to the medium of both cell types at a final concentration of 100umolar for 15 minutes prior to administration of ^{111}In-MAb. After the two hour incubation period the mean uptake of activity was reduced by pretreatment and incubation in the presence of DTPA by 12.5% for the MCF7's (n.s.) and 31% for the hepatocytes ($p=0.02$)(Figure 4.3., Table 4.8., Appendix 4.8.).

Figure 4.2. The effect of DTPA co-administration

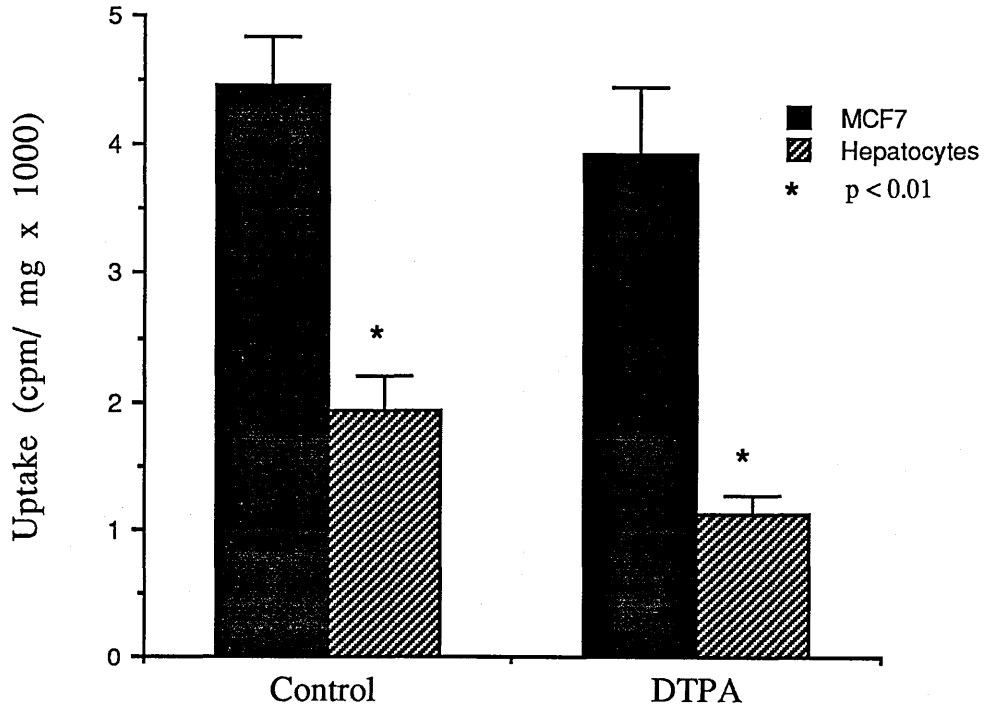


Figure 4.3. : Cell incubation with DTPA

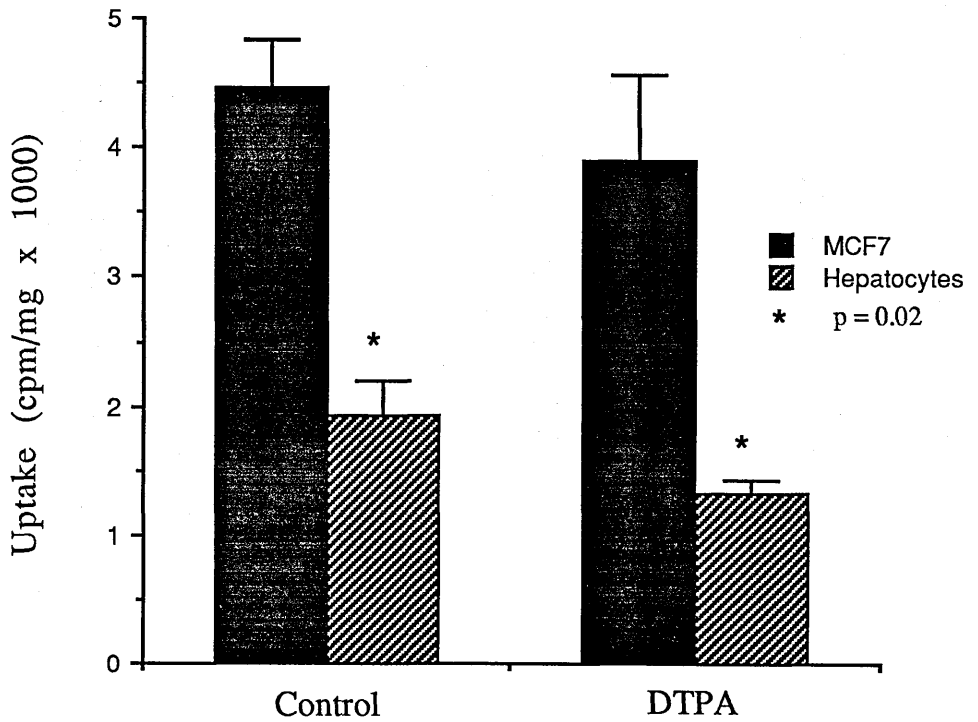


Table 4.7. Effect of DTPA co-administration

	MCF7		Hepatocytes	
	Control	DTPA	Control	DTPA
Applied (cpm x 10^5)	5.40(0.26)	5.60(0.02)	5.04(0.86)	5.71(0.14)
Uptake (cpm x 10^3)	15.9(0.94)	14.3(0.51)	7.15(1.01)	4.04(0.70)
Uptake (cpm/mg x10^3)	4.46(0.37)	3.92(0.51)	1.93(0.27)	1.11(0.16)

Results are mean(S.D.) for triplicate determinations (Appendix 4.7.)

Table 4.8. Cell incubation with DTPA

	MCF7		Hepatocytes	
	Control	DTPA	Control	DTPA
Applied (cpm x10^5)	5.4(0.26)	5.4(0.16)	5.04(0.86)	5.67(0.04)
Uptake (cpmx10^3)	15.9(0.94)	14.4(2.47)	7.15(1.01)	4.91(0.40)
Uptake (cpm/mg x10^3)	4.46(0.37)	3.90(0.67)	1.93(0.27)	1.33(0.11)

Results are mean (S.D.)for triplicate determinations. (Appendix 4.8.)

4.3.ii.c) Dose response of DTPA added to ^{111}In-MAB

Having established that DTPA, co-administered with labelled antibody or pre-incubated with the cell lines, could produce a reduction in the hepatocyte uptake of ^{111}In-MAb, experiments were carried out to determine the optimum concentration of DTPA which produced this effect. Samples of ^{111}In-ICR2 were incubated with a range of concentrations of DTPA from 100umolar to 10mmolar for one hour prior to application to the cells. The control plates of cells were incubated without chelate. Using a 100uM [DTPA] ^{111}In-MAb uptake by hepatocytes was reduced by 16% which was not statistically significant while with 1mM and 10mM [DTPA] binding was reduced by 80% and 83% respectively ($p=0.000$). At none of these concentrations of DTPA was the binding of ^{111}In-ICR2 to the tumour cell line reduced significantly (Figure 4.4., Table 4.9., Appendix 4.9.).

4.3.ii.d) DTPA incubation time and uptake of ^{111}In-ICR2

The dose response curve (Figure 4.4.) suggested that greater than 0.1mM [DTPA] was required to produce a significant reduction in the hepatocyte uptake. To investigate the optimum time for treatment with DTPA ^{111}In-ICR2 was incubated with 0.5mM DTPA for variable times before treating the cells. A significant reduction was noted at all treatment times with the hepatocytes ($p<0.05$). The binding was reduced by 32% after 5 minutes and 45% after 15 minutes. No

Figure 4.4. : The effect of DTPA concentration

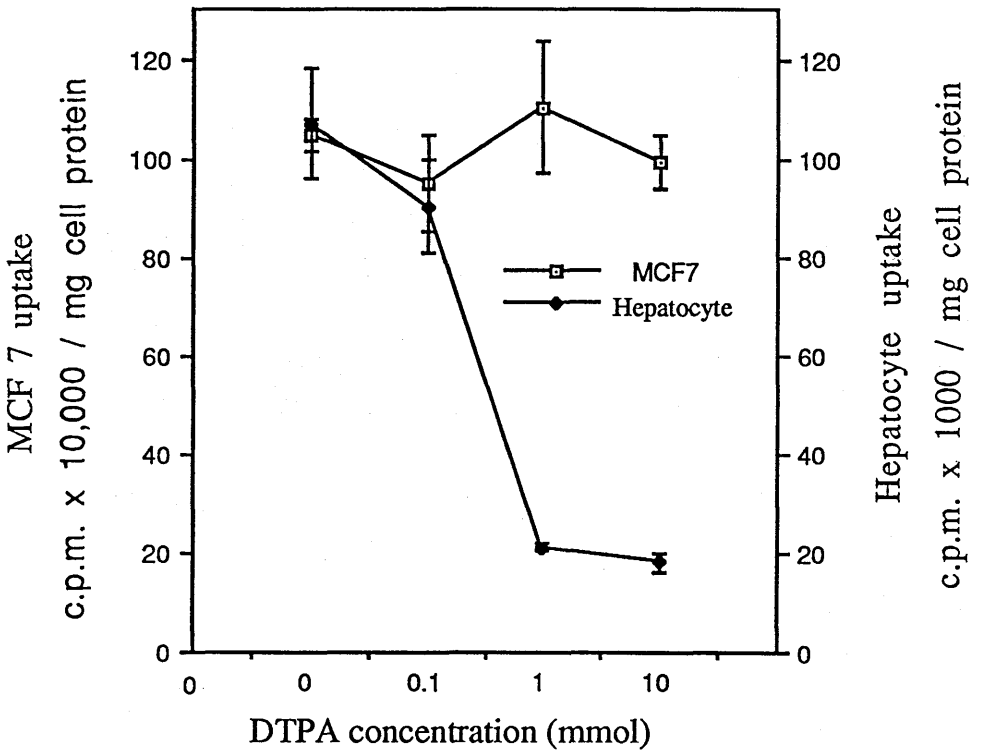


Figure 4.5. : Effect of DTPA incubation period

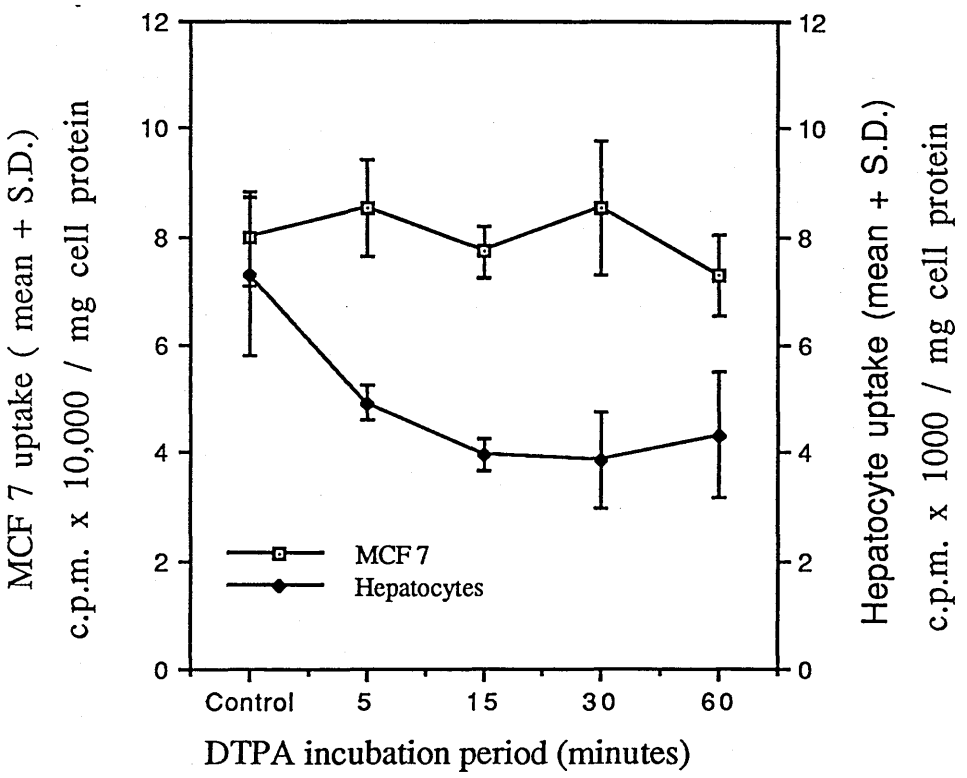


Table 4.9. DTPA concentration and the cell uptake of activity

Cell group	Applied (cpm x 10^6)	Uptake (cpm x 10^4/plate)	cpm/mg x 10^4
MCF7 control	3.02 (0.17)	26.4 (2.53)	104.5 (3.13)
MCF7 100uM DTPA	2.97 (0.09)	23.2 (4.43)	95.0 (9.60)
MCF7 1mM DTPA	2.80 (0.60)	31.2 (2.59)	110.1 (13.14)
MCF7 10mM DTPA	3.06 (0.16)	26.5 (3.28)	99.2 (5.21)
Heps control	3.10 (0.12)	2.14 (0.21)	10.7 (1.09)
Heps 100uM DTPA	2.65 (0.23)	1.89 (0.21)	9.01 (0.94)
Heps 1mM DTPA	2.95 (0.48)	0.40 (0.06)	2.1 (0.07)
Heps 10mM DTPA	3.07 (0.13)	0.39 (0.05)	1.82 (0.19)

The results are mean (S.D.) of triplicate determinations (Appendix 4.9.)

Table 4.10. DTPA incubation and cell uptake of activity

Cell group	Applied (cpm x 10^5)	Uptake cpm x 10^3/plate	cpm/mg x 10^3
MCF7 control	4.96 (0.33)	30.38 (5.52)	79.76 (8.60)
MCF7 5 mins	4.69 (0.17)	27.15 (5.90)	88.09 (8.64)
MCF7 15mins	4.56 (0.01)	24.06 (1.50)	78.58 (4.47)
MCF7 30mins	4.85 (0.10)	28.94 (4.13)	91.34 (4.68)
MCF7 60 mins	4.35 (1.04)	21.64 (1.31)	91.34 (4.68)
Heps control	5.25 (0.14)	6.97 (1.28)	7.27 (1.48)
Heps 5 min	4.76 (0.14)	4.48 (0.24)	4.93 (0.31)
Heps 15 mins	4.52 (0.21)	3.67 (0.28)	4.12 (0.64)
Heps 30 mins	4.75 (0.10)	3.72 (1.04)	3.88 (0.89)
Heps 60 mins	4.62 (0.14)	3.89 (1.46)	4.09 (1.31)

Results are mean (S.D.) of triplicate determinations.(Appendix 4.10.)

significant effect was found by pre-treatment with DTPA on the binding of ^{111}In-ICR2 to the tumour cell line. Incubation of ^{111}In-ICR2 with DTPA overnight (18 hours) did not reduce further the binding to hepatocytes. (Figure 4.5., Table 4.10., Appendix 4.10.).

4.3.ii.e) The uptake of free ^{111}In and ^{111}In-DTPA

Any instability of the ^{111}In-DTPA-MAb complex may result in the formation of either free ^{111}In or ^{111}In-DTPA. In order to determine whether the effect of DTPA on the uptake of activity by hepatocytes may be related to the presence of free or chelated ^{111}In, the effect of DTPA was assessed on the uptake of ^{111}In and ^{111}In-DTPA on hepatocytes and MCF7 cells. Two equal aliquots of 50ul of ^{111}In acetate were mixed with either 450ul of 1mM DTPA (to form stably bound ^{111}In-DTPA) or 450ul of PBS. After 30 minutes the mixtures were applied to plates of hepatocytes and MCF7 tumour cells. To seperate sets of plates DTPA (500uM) was added to the medium of the cells with the mixtures. The uptake of free ^{111}In was found to be significantly greater than that of ^{111}In-DTPA for both the hepatocytes and the tumour cell line. The co-administration of DTPA at the time of applying activity to the cell lines significantly reduced the uptake of free ^{111}In but did not reduce the uptake of ^{111}In-DTPA (Table 4.11., Apppendix 4.11.). This would suggest that the effect of DTPA on ^{111}In-MAb is not related to the presence or formation of ^{111}In-DTPA.

Table 4.11. : Cell uptake of ^{111}In and ^{111}In-DTPA

Cell group	Activity	Added DTPA	Applied (cpm x 104)	Uptake (%)	Uptake cpm/mg
MCF7	^{111}In	No	18.61 (0.13)	0.54 (0.18)	2908 (262)
MCF7	^{111}In	Yes	18.5 (0.25)	0.33 (0.07)	1712 (371)
Heps	^{111}In	No	19.1 (1.15)	0.70 (0.17)	2077 (45)
Heps	^{111}In	Yes	18.5 (0.19)	0.43 (0.04)	1150 (247)
MCF7	^{111}In-DTPA	No	19.5 (0.47)	0.27 (0.05)	1255 (369)
MCF7	^{111}In-DTPA	Yes	18.8 (0.26)	0.32 (0.03)	1866 (748)
Heps	^{111}In-DTPA	No	18.3 (0.29)	0.21 (0.06)	624 (209)
Heps	^{111}In-DTPA	Yes	18.8 (0.64)	0.25 (0.01)	713 (66)

Results are mean (S.D.) for triplicate determinations(Appendix 4.11.)

Table 4.12. : A comparison of chelators

Cell group	Applied (cpm x 10^6)	Uptake/ plate (cpm x 10^3)	Uptake/mg (cpm x 10^4)
MCF7 control	1.38 (0.05)	5.0 (1.8)	18.0 (3.2)
MCF7 EDTA	1.32 (0.08)	4.2 (2.3)	16.8 (4.7)
MCF7 DTPA	1.31 (0.04)	5.1 (1.7)	24.0 (10.9)
MCF7 ccDTPA	1.39 (0.08)	5.3 (3.0)	18.4 (5.4)
MCF7 CP040	1.27 (0.05)	4.4 (2.6)	28.2 (13.6)
Heps control	1.35 (0.03)	6.3 (0.9)	9.3 (2.1)
Heps EDTA	1.35 (0.06)	6.0 (0.2)	6.9 (1.2)
Heps DTPA	1.35 (0.06)	4.7 (0.4)	5.4 (0.6)
Heps ccDTPA	1.40 (0.14)	5.2 (1.0)	7.3 (1.9)
Heps CP040	1.57 (0.08)	14.1 (0.10)	17.3 (1.0)

Results are mean (S.D.) for triplicate determinations (Appendix 4.12.)

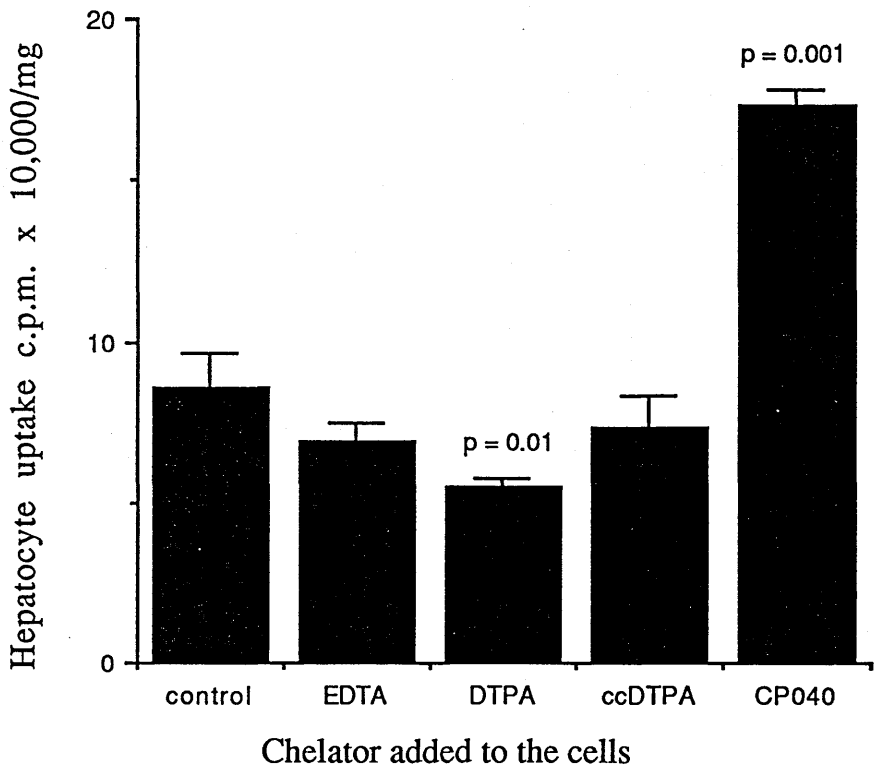
4.3.iv) A comparison of chelating agents

A comparison was carried out between four different chelating agents to assess their relative ability to inhibit the uptake of ^{111}In-MAB; the sodium salt of ethylene-diamine-tetracetic acid (NaEDTA), the sodium salt of diethylenetriamine pentacetic acid (NaDTPA), a solution of the bicyclic anhydride of DTPA (ccDTPA) and an experimental bidentate ligand CP040. ^{111}In-ICR2 was incubated with 1mM solutions of the chelates for one hour before applying the labelled antibody to either MCF7 cells or the hepatocytes and incubating for two hours. Sodium DTPA produced a significant reduction in the hepatocyte uptake of activity whereas CP040 produced a significant increase. No significant differences were found in the hepatocyte uptake between any of the other chelated groups and controls nor between any of the groups of tumour cells (Figure 4.6., Table 4.12., Appendix 4.12.).

4.3.v) The effect of pooled rat Ig

It has been suggested that non-specific antibody uptake in patients could be reduced by the administration of pooled human immunoglobulin to block Fc receptor mediated uptake. In practice this has met with little success (243). This problem has been investigated in this in vitro model using pooled rat immunoglobulin. Pooled rat IgG was mixed with ^{111}In-ICR2 at a ratio of 10:1, 50:1, 100:1 and 1000:1 then

Figure 4.6. A comparison of chelators



incubated with the tumour cells or the hepatocytes. Binding of ^{111}In-ICR2 to the cells was compared with a control group given labelled antibody alone. With the tumour cell line no significant differences were noted until a 1000 fold excess was applied at which point a reduction of 20% was achieved. No inhibition of hepatocyte binding was found with any of the concentrations of pooled Ig examined (Table 4.13, Appendix 4.13). The pooled rat IgG contains a substantial amount of rat IgG_{2a}, the same isotype as that of the radiolabelled MAb ICR2. The results would therefore suggest that binding via the Fc receptor or via carbohydrate on the Ig is not a major factor to the binding of ICR2 by hepatocytes.

Table 4.13. Pooled rat IgG and the cell uptake of activity

Cell group	Pooled Ig	Applied (cpm x 10^5)	Uptake (cpm x 10^3/plate)	Uptake/mg (cpm x 10^3)
MCF7	No	9.8 (0.1)	73.1 (2.4)	175 (20)
MCF7	x 10	10.0 (0.3)	75.6 (4.9)	207 (70)
MCF7	x 50	10.2 (0.6)	75.9 (8.8)	186 (37)
MCF7	x 100	10.2 (0.2)	61.6 (3.4)	193 (10)
MCF7	x 1000	9.5 (0.2)	44.3 (3.34)	140 (11)
Heps	No	9.1 (0.1)	3.9 (1.4)	6.6 (1.8)
Heps	x 10	9.7 (0.1)	6.3 (0.0)	11.1 (1.3)
Heps	x 50	10.1 (0.2)	5.0 (0.4)	7.3 (0.7)
Heps	x 100	11.5 (2.1)	6.0 (1.0)	9.1 (2.4)
Heps	x 1000	8.1 (2.0)	3.9 (0.5)	6.4 (0.8)

Results are mean (S.D.) of triplicate determinations (Appendix 4.13)

4.4. DISCUSSION AND CONCLUSIONS

Transferrin is the most important and prevalent circulating metal chelating agent in man. In this study therefore the effect of transferrin, both saturated with iron and fully unsaturated on the specific and non specific uptake of ^{111}In-MAb was investigated using an in vitro model. No significant differences were noted between the control group and either the saturated or unsaturated transferrin groups suggesting that transcomplexation of ^{111}In from ^{111}In-MAB to circulating transferrin is not a major cause of non specific liver uptake. These results appear to contrast, however, with studies in both animals and patients where it has been shown that 2-3% and 9% respectively of circulating activity may be found associated with circulating transferrin [280,281]. This difference may be due to other factors affecting the in vivo stability such as the pH or the time which the labelled antibody is in contact with circulating transferrin.

The chelating agent DTPA has been shown to produce significant reductions in the uptake of activity by the hepatocytes without significantly reducing tumour cell uptake. The uptake of activity, however, was far greater with the tumour cell line expressing the target antigen than with the hepatocytes and may therefore be disguising similar effects of chelates on the tumour cell line.

A reduction in the uptake of activity by the hepatocytes was found whether the chelate was added to the labelled antibody, co-administered with the labelled antibody to the cells or incubated with the cells prior to labelled antibody administration. A dose-response and time course of hepatocyte uptake with DTPA applied to the labelled antibody has also been demonstrated. The reductions in uptake of radioactivity being apparent with the hepatocytes and not the tumour cell line suggests differences in the nature of activity taken up specifically by the antigen expressing tumour cell line and non specifically by the hepatocytes. The source of the ^{111}In being affected by the chelating agent is unknown. ^{111}In bound to MAB via ccDTPA will not exchange significantly with unbound DTPA. Similarly free ^{111}In in the antibody preparation is unlikely to account for the non specific activity as this will be removed with gel chromatography and in vivo would readily complex with circulating transferrin (275). Large protein molecules may, however, bind charged metallic ions non specifically (129). This may result in a failure to remove unchelated ^{111}In on gel chromatography and its transportation to the liver. This unstably bound metal may be the target of the additional chelating agents in this study.

Further information on the possible mechanism of chelates on the liver uptake has been obtained from analysis of the cells uptake of free ^{111}In and ^{111}In-DTPA. Firstly the ratio of uptake of activity in the

tumour cell line compared to that in the hepatocytes was low compared to experiments with labelled antibody confirming that the uptake of activity in the tumours with labelled antibody is dependent on the antibody-antigen interaction. There was also significant differences with both the hepatocytes and the tumour cell line in the uptake of free ^{111}In and ^{111}In-DTPA. This would suggest that the stably chelated indium remains extracellular whereas a mechanism exists for the intracellular transport of free ^{111}In. This is supported by the reduction in uptake found when DTPA was added to the group of cells to which free ^{111}In was applied. No significant changes being found by the addition of DTPA to the culture medium after application of the activity would again suggest that the free ^{111}In is rapidly taken up by the cells and is not therefore available to extracellular chelators.

The reduction in hepatocyte uptake of activity seen in the present study when DTPA was added to either the labelled antibody or the cell lines has important practical applications. This would suggest that in patient studies with ^{111}In labelled monoclonal antibodies the uptake of activity in the liver may be reduced whilst preserving tumour uptake by either administering chelating agents to the patients or incubating the labelled antibody with chelate. The potential use of chelating agents for reducing liver uptake of ^{111}In-MAb must, however, be considered in

context. Although a significant reduction in hepatocyte uptake was found only a small percentage of the applied ^{111}In-ICR2 bound to hepatocytes. Although this may initially suggest that hepatocyte uptake is not likely to be an important factor to the overall uptake of activity by the liver these differences were based on an equivalent concentration of cell protein for hepatocytes and tumour cells. In the clinical situation, however, the liver is many times larger than the average primary colonic cancer and the number of hepatocytes is therefore likely to greatly exceed the number of tumour cells. Another factor which must be considered is the cell incubation period which was short and may overlook a continued uptake of activity. The very rapid accumulation of liver activity seen in patient studies with ^{111}In-MAb is largely due to the blood pool activity of a large vascular organ rather than specific accumulation (see Chapter 6).

The addition of chelates to the injectate has previously been carried out by some researchers for the removal of unstably bound ^{111}In, the ^{111}In-chelate complex being readily excreted by the urinary tract[243]. A rational basis for this approach has not, however, previously been established. The use of a chelating agent following labelled antibody administration has also been investigated in animals although in the few reported studies a definite role has not been established [280,283].

With a view to patient studies the optimal concentration and incubation period of DTPA with ^{111}In-ICR2 was investigated with the aim of minimising the hepatocyte binding of activity using the lowest concentration of DTPA and the shortest incubation period. This was established at 0.5mM [DTPA] for 15 minutes producing a 40% reduction in the uptake of activity by hepatocytes. Whether the remaining activity taken up by the hepatocytes may be removed by other chelating agents without affecting tumour uptake remains to be established. In an initial study comparing EDTA, NaDTPA, ccDTPA and CP040 the greatest inhibition of uptake was with Na DTPA. Of interest was the significantly increased uptake achieved with the bidentate chelate CP040, suggesting that there may be cellular uptake of this chelating agent.

Other ways of reducing the uptake of activity in the liver have been investigated and important advances have been achieved using alternatives to ccDTPA for the attachment of ^{111}In to the antibody molecule, such as bifunctional chelating agents[282,286].

Although hepatocyte uptake is the major factor in the accumulation of activity in the liver considerable activity accumulates at other sites following ^{111}In labelled antibody administration some of which is associated with the RES. A logical step for the reduction of this uptake of activity is the administration of pooled gamma globulin which has previously been shown to effectively block the Fc

receptor mechanism [274]. The results in the few patient studies, however, have shown little improvement in the results of imaging obtained [243]. This problem was investigated in the in vitro model by the application of a 10-1000 fold excess of pooled rat immunoglobulin along with the ^{111}In-MAb to the cell lines. A marginal effect being achieved only by 1000 fold excess of pooled Ig would suggest that this is not a practical method of inhibiting the non-specific uptake of activity.

CHAPTER 5

THE IMAGING OF HUMAN COLORECTAL CANCERS FOLLOWING ADMINISTRATION OF THE INDIUM LABELLED MONOCLONAL ANTIBODY ICR2

CONTENTS

5.1. A REVIEW OF PROBLEMS ASSOCIATED WITH THE RADIOIMMUNOLOCALISATION OF CANCERS.

5.2. THE LOCALISATION OF COLORECTAL CANCERS BY IMMUNOSCINTIGRAPHY WITH ^{111}IN LABELLED ICR2.

i) Introduction

ii) Patients and Methods

iii) Results

a) Interpretation of the images

b) Tumour activity and antigen expression

iv) Discussion and conclusions

5.1. A REVIEW OF PROBLEMS ASSOCIATED WITH THE RADIOIMMUNOLOCALISATION OF COLORECTAL CANCERS

Several steps are involved between the production of a monoclonal antibody or polyclonal antiserum and its successful use for the localisation of cancers. At any intermediate stage factors may affect the capacity of the labelled antibody to localise and bind to its target antigen and thereby result in a failure of imaging. Such difficulties may be due to the antibody itself, the target antigen, the radiolabel or the imaging method employed.

The antibody/antigen interaction

The ideal antibody for tumour imaging would recognise an antigen expressed exclusively by the tumour tissue. Unfortunately in man the only tumour specific antigen which has been discovered to date is the idiotype of the surface Ig on some B cell lymphomas[46]. The antigens which are targeted in tumour imaging are therefore "tumour associated" rather than tumour specific. This may take the form of over-expression of an antigen normally present on the tissue eg. epidermal growth factor receptor (EGFR)[287] or it may be an aberrant expression eg. EMA. Specific antibody binding may not, however, be essential to produce a positive image as radiolabelled MAb may accumulate in tumour tissue which does not express the target antigen. This may explain the positive

localisation of human tumours reported by Epenetos using a non-specific MAb [183]. The biodistribution of radiolabelled antibodies varies following administration to patients. This may depend on the specificity of the antibody administered, although monoclonal antibodies raised to the same target antigen may have similar[288] or markedly different biodistributions[130], or it may vary with the radiolabel being used[125]. Independant of these factors there is commonly uptake of radiolabelled antibody in the liver, spleen, bone marrow and the testicles. The reason for this distribution in normal tissues which do not express the target antigen is a major problem in immunoscintigraphy and was discussed in Section 4.1.

An additional problem in the use of radiolabelled MAb's for the localisation of cancers is the secretion of antigen from the tumour into the circulation. High circulating levels of secreted tumour associated antigens have been shown in animal models to correspond with a high level of formation and removal of circulating immune complex and a reduced uptake of radiolabelled MAb in the tumour [264]. In the patient studies by Goldenberg, however, satisfactory images were obtained of colorectal cancer using anti-CEA even when circulating CEA levels were greatly elevated [97]. These positive images may have been produced by dual isotope subtraction artefacts [122] or the uptake of soluble immune complexes by the tumour tissue.

Antigen access to tumour tissue

Larger tumours are easier to localise by external imaging because they accumulate more radioactivity than small tumours. The uptake of radioactivity per gram, however, decreases as the size of the tumour increases (264). This is probably related to a failure of the blood supply to match the growth of the tumour(289). The access of radiolabelled antibodies to large tumours is therefore poor and non uniform and the site of biopsy of tumour tissue may result in discrepancies in the reported tumour to normal tissue ratios. Although the vascularity of a tumour may be poor the capillaries are more porous than in normal tissues and therefore the access for large molecules to surface antigens on the tumour cells is facilitated (290). This extravasation is important because antibody fragments F(ab') and $F(ab')_2$ show greater accumulation in tumour than do whole antibody despite their more rapid clearance from the blood (130).

The immunoreactivity of an antibody is a measure of its binding with a specific antigen. This does not reflect on the ability of the antibody to localise tumour deposits. It does, however, allow the effect of certain manipulations eg. conjugation with DTPA, to be assessed.

The radiolabel

As mentioned in the Section 1.3.iii) the two most frequently used radiolabels in patient studies are ^{131}I and ^{111}In. With both isotopes the labelling procedures employed may lead to alterations in the immunoreactivity of the antibody, the extent of which depends on the method being used and the nature of the antibody itself. The biodistribution is also affected by the radiolabel. Labelling of the same antibody with ^{125}I and ^{111}In has been carried out in mice with colonic carcinoma xenografts. At 48 hours after injection the blood levels of activity are similar but the activity in the tumour, liver and spleen are significantly higher with ^{111}In. Whether this is a reflection of tissue dehalogenation or other factors remains to be resolved (130). These findings have, however, been supported by other authors (230,265,266).

Antibodies may be labelled with ^{111}In and a stable pharmaceutical produced. They do however, require the monoclonal antibody to be conjugated with a metal chelating agent such as DTPA or EDTA. As the chelate : MAb ratio increases the immunoreactivity of the antibody decreases. The optimum ratio may well depend on the particular Mab being used. Most authors, however, report good immunoreactivities with a 1:1 or 2:1 DTPA to MAb ratio. With conjugates prepared at these ratios, ^{111}In labelling to specific activities of approximately 150 Megabequerels per milligram (MBq / mg) can be achieved.

The nature of the chelate itself appears to have an important effect on the biodistribution of the radiolabelled antibody and newly designed chelates have been shown to reduce the uptake of radiolabel in the liver [282,291]. This may be due to a reduction in the amount of ^{111}In being lost from the labelled antibody and binding to circulating transferrin or to a reduced immunogenicity of the labelled antibody.

Imaging techniques

The imaging techniques which are commonly used, and applications which have been attempted are outlined in Section 1.3.iii.b). Whatever the method employed, satisfactory imaging is dependent on the amount of radiolabel which accumulates in the tumour as compared with that in the normal tissues. Any factor which affects the labelling efficiency, purity or immunoreactivity of the labelled antibody or its access to tumour tissue will affect the image produced. Similarly factors which increase background activity will reduce the "signal to noise ratio" and obscure the image. Possible mechanisms for reducing non specific uptake were discussed in Chapter 4. A problem commonly encountered with the imaging of colorectal cancers is that primary tumours may be obscured by uptake in the bony pelvis and hepatic metastases by uptake in normal liver.

Determination of the absolute uptake of radioactivity in the tumour from resected specimens is essential for progress to be made in imaging although few studies have reported this data. This information is also required to allow methods of reducing the background activity to be assessed.

5.2. THE LOCALISATION OF COLORECTAL CANCERS BY IMMUNOSCINTIGRAPHY WITH ^{111}In LABELLED ICR2

i) Introduction

The localisation of colorectal cancers by anti-CEA antibodies has been widely reported over the last decade (97,99,100,102,104-106,133,292-297). These studies have shown a wide variation in sensitivities for the detection of primary colorectal cancer ranging from 30% (296) to 100% (106,293). The detection of primary tumours by immunoscintigraphy may be of limited clinical value as other forms of investigation can successfully localise these tumours. Imaging for metastatic deposits which are not satisfactorily localised by conventional investigations have generally produced poorer results (104,106,292).

In an attempt to improve the results of the immunoscintigraphy of colorectal cancers MAb's to other target antigens have been investigated including 791/T36 raised to a human osteosarcoma (111), 17-1A and 19-9 raised to colorectal cancer cell lines (115,116), B72.3 raised to a breast cancer membrane extract (298) and YPC2/12.1 raised to a colorectal cancer extract (117). Imaging with MAb's to these tumour associated antigens has not, however, shown any clinical advantage over anti-CEA.

In Chapter 2 it was demonstrated that the majority of colorectal cancers and their metastases express both CEA and EMA, whilst normal colon expresses CEA but

rarely EMA. EMA is expressed more widely than CEA in non-gastrointestinal tissues [201,202] but shows a better differential expression between colonic cancers and normal colon on immunohistochemistry. MAb's to EMA have not previously been investigated for a role in the imaging of colorectal cancer. The anti-EMA MAb ICR2 labelled with ^{111}In has therefore been evaluated for the imaging of patients with colorectal cancer.

In addition to obtaining imaging information several areas have been investigated where potential may exist for improving the results obtained with immunoscintigraphy. Firstly the stability and immunoreactivity of the ^{111}In chelated antibody is known to vary with several factors including the chelate used and the number of chelate molecules substituted (Section 1). The stability and immunoreactivity of ^{111}In-ICR2 with changes in chelate : MAb ratio, pH and with storage has been investigated in order to optimise its preparation (Appendices 5.1.-5.3.)..

Another major problem in immunoscintigraphy is the formation of circulating immune complex with secreted tumour associated antigens resulting in a decreased uptake of labelled antibody in the tumour (Discussed in Chapter 6). Previous patient studies have assessed the effect of administering unlabelled antibody prior to labelled antibody on the images obtained. However the tumour uptake of radioactivity, levels of circulating immune complex and labelled antibody biodistribution

were not quantified [242,243]. To clarify the usefulness of unlabelled antibody administration a group of patients in the present study have therefore been administered unlabelled antibody prior to ^{111}In-ICR2 administration and the effect measured on imaging as well as the biodistribution of ^{111}In-ICR2.

An additional problem with immunoscintigraphy is the background uptake of activity (Sections 1.3.iii.a and 5.1.). With ^{111}In this may be due in part to unstable binding of ^{111}In to the antibody molecule at areas other than the DTPA binding site. In vivo any ^{111}In which is not stably bound to the antibody molecule will become bound to circulating transferrin and be removed from the circulation by organs expressing receptors for transferrin. Although the administration of a chelating agent to the labelled antibody has been advocated for the removal of this non specifically bound ^{111}In [243] little evidence exists for its efficacy. In Section 4.3., however, it was demonstrated that DTPA may be used to reduce the hepatocyte uptake of ^{111}In-ICR2 by its application to the injectate. Whether a similar reduction in non specific uptake may be achieved in patients has been investigated in the present study by adding DTPA to the ^{111}In-ICR2 prepared for patient administration and the effect on the images produced and the biodistribution of ^{111}In-ICR2 has been assessed. The results of these studies have been divided into imaging, presented in this chapter and biodistribution which is presented in chapter 6.

Patients and methods

All patients entering this study gave fully informed consent for the administration of ^{111}In-ICR2. The study was approved by the University College Hospital Ethics Committee and Department of Health approval was granted for the administration of the antibody.

Twenty-two patients with suspected colorectal cancer were studied (Table 5.1.). Nine were males and 13 females with a median age of 66 years (range 51-83). In twenty patients the diagnosis of colorectal cancer was based on barium enema or colonoscopy with pre-operative histological diagnosis being obtained in 8. Two patients were suspected of having recurrence of cancer following abdomino-perineal resection of the rectum, one 8 months following resection of a Dukes C lesion and the other 3 years following resection of a Dukes B lesion.

Of the three patients found on subsequent laparotomy to have liver metastases one was correctly diagnosed on pre-operative liver ultrasonography and none by ^{99m}Tc tin colloid liver scanning.

Of 20 patients with the diagnosis of primary colorectal cancers 19 underwent surgical exploration and resection. The remaining patient, a 79 year old female with a large dysplastic tubular adenoma of the rectum (No.20, D.F.) refused surgery and underwent a course of cytoreductive laser therapy.

Table 5.1. Patient details

No.	Init.	Age	Sex	Diagnosis	Site	Diff	Dukes	Operation
1.	M.D.	60	M	Ca	R/S	Mod	B	Lt. Hemi
2.	M.B.	83	F	Ca	R/S	Mod	B	Lt. Hemi
3.	R.S.	53	M	?recur	_	_	_	Laparotomy
4.	R.N.	66	M	Ca	Hep flex	Mod	B	Rt. Hemi
5.	D.N.	51	M	Ca	Hep flex	Mod	B	Rt. Hemi
6.	J.L.	71	M	Ca	Rectum	Mod	C*	Ant resection
7.	J.H.	79	F	Ca	Trans.	Signet	B	Lt. Hemi
8.	B.T.	58	F	Ca	Rect	Mod	B	Ant resection
9.	J.P.	59	M	Ca	Sigmoid	Mod	B	Lt. Hemi
10.	K.H.	65	F	?recur	_	_	_	No
11.	M.J.	67	F	Ca	Sigmoid	Mod	C	Lt. Hemi
12.	B.B.	54	F	Ca	Rectum	Mod	C	Ant resection
13.	V.D.	74	F	Ca	Sigmoid	Mod	B	Lt. Hemi
14.	F.H.	76	F	Ca	Sigmoid	Mod	B	Lt Hemi
15.	B.S.	60	F	Ca	Sigmoid	Mod	C*	Lt. Hemi
16.	R.D.	64	M	Lipoma	Sigmoid	_	_	Lt. Hemi
17.	J.P.	76	F	Ca	Spl.flex	Mod	B	Lt. Hemi
18.	E.B.	73	F	Adenoma	Caecum	_	_	Rt Hemi
19.	D.N.	73	F	Ca	Sigmoid	Well	C	Lt Hemi
20.	D.F.	79	F	Adenoma	Rectum	_	_	Laser
21.	G.X.	67	M	Ca	R/S	Mod	C*	Ant resection
22.	J.B.	56	M	Ca	Sigmoid	Mod	B	Ant resection

* with liver metastases

Pathological examination of the resected specimens confirmed colorectal cancer in 17 patients (7 rectum or rectosigmoid, 6 sigmoid, three at the flexures and one in the transverse colon). Of these one was well differentiated, one signet ring and the remainder moderately differentiated. Two patients undergoing surgery were found to have benign colonic tumours, one a large pedunculated lipoma in the sigmoid colon (No.16 R.D.) and the other a dysplastic tubulovillous adenoma in the caecum (No.18 E.B.). Of the two patients presenting with symptoms suggestive of recurrence one underwent laparotomy and was found to have dense inflammatory adhesions but no evidence of recurrent cancer and the others symptoms resolved and is being followed up in the out-patient clinic.

Preparation and administration of ^{111}In-ICR2

The production and purification of ICR2 is described in Section 1.6. and its conjugation with DTPA, labelling with ^{111}In, immunoreactivity and in vitro stability in Appendices 5.1-5.4.

To test for possible hypersensitivity a skin test was carried out on all patients prior to administration of labelled antibody. This consisted of the subcutaneous injection of 20ug of unlabelled ICR2 on the flexor aspect of the patients forearm with the skin test site being inspected after 20 minutes for evidence of an inflammatory response. If no skin reaction was noted the labelled antibody was administered intravenously in 50mls of normal saline over one minute.

Patients were divided into three groups by the nature of the administered activity. Ten patients recieved 1mg of ^{111}In-ICR2 alone (Group A), labelled as described in Appendix 5.4. A further 6 patients had their injectate reacted with DTPA (Group B and Appendix 5.4.). The final group of 6 patients also had DTPA added to their injectate but in addition had 5mg of unlabelled antibody administered intravenously in 100mls of normal saline over the 30 minutes prior to administration of labelled antibody (Group C and Appendix 5.4.).

Following the administration of the antibody patients were monitored for a hypersensitivity reaction

by recordings of temperature, pulse and blood pressure hourly for 12 hours. Full blood count (FBC), urea and electrolytes (U&E's) and liver function tests(LFT's) were compared before antibody administration and at frequent intervals over the subsequent two weeks.

External imaging

A dynamic study was carried out for the 30 minutes following injection of ^{111}In-ICR2 followed by static imaging. For the dynamic study the camera was placed anterior to the prone patient with the liver and heart included in the field of view and 20 x 3 second frames followed by 30 x 60 second frames were recorded by computer (Nodecrest, U.K.).

Static images of the anterior and posterior abdomen, anterior pelvis and pelvic outlet were acquired at 30 minutes, 24, 48 and 72 hours post injection using a 37 tube digitrac gamma camera (Siemens) fitted with a medium energy collimator. 300k counts in a 128 x 128 matrix were stored on the computer.

Interpretation of images

The static images were interpreted by a clinician with knowledge of the patients clinical and radiological findings (Observer A) and independently by an experienced nuclear medicine clinician who had no clinical details of the patients studied (Observer B). The image interpretations in this manner were

considered to represent a subjective and an objective assessment respectively and were compared.

Tumour uptake of activity and antigen expression

The uptake of radio-activity in the tumours and that in normal colon was measured in biopsies of the resected specimen. These were accurately weighed by placing them in individually weighed test tubes (Luckham, Sussex, U.K.) which were then re-weighed prior to measuring their radioactivity in a gamma well counter (LKB Compugamma). This allowed the uptake of activity in tumour to that of normal colon (T/NC ratio) to be calculated per gram of tissue. The absolute uptake of activity was calculated using a standard. This was prepared by taking a known percentage (by weight) of the injected ^{111}In-ICR2 and diluting it to 500mls in a flask using normal saline. After thorough mixing a 2 ml aliquot of this solution was then sampled and counted in a gamma well counter (LKB Compugamma) at the time of analysis of the resected tissue. The amount of activity in the standard was calculated as a percentage of the activity injected thus allowing the percentage of the injected dose per gram (% I.D./g) of tumour tissue to be calculated.

Specimens of cancer from all patients undergoing resection were also examined immunohistochemically for EMA expression by the indirect immunoperoxidase method outlined in Chapter 2, while in those patients with suspected recurrences sections were obtained from

paraffin blocks of the original tumour. Nodal metastases were also examined for EMA expression. Tumours were graded for antigen expression on a scale of 1-4 by the method of Ellis [244] (Section 2.2.ii).

Human response to rat antibody

Serum samples were collected at random from patients following administration of ^{111}In-ICR2 and analysed for a human response to the administration of rat antibody. The samples were stored at -20^{o}C until assayed and then thawed, mixed and centrifuged to remove debris before use.

Procedure

96 well PVC plates (Dynatech, Bellingshut, Sussex) were coated with the F(ab')$_2$ fraction of ICR2. These were prepared by adding 2mg of pepsin (Sigma,U.K.) to 100mg of ICR2 in a dialysis sac (Medicell Ltd). This was then placed in 0.05M acetate buffer at pH 4 and dialysis was continued until 1/3 of the optical density of the ICR2/ pepsin solution was present in the dialysis solution. The reaction was terminated by dialysis at 6^{o}C against PBS containing 0.5M saline. The F(ab')$_2$ fragments were purified by gel filtration (ACA 34) using 0.5M PBS as eluant. The F(ab')$_2$ coated plates were blocked with PBS-EDTA-coating 0.5% BSA and stored overnight at 4^{o}C. Dilutions of the serum samples were prepared in separate 96 well plates that contained 180ul of PBS-BSA in the first well and 120ul of the

same buffer in the remaining wells. Duplicate 60ul samples of each serum were added to the initial wells and then diluted serially ten times. After washing the ICR2 $F(ab')_2$ coated plates with PBS/BSA the serum dilutions were applied and incubated for one hour. The plates were then washed to remove any unbound antibody and 50ul of ^{125}I labelled rabbit anti-human immunoglobulin was applied. After a further incubation period of one hour the plates were washed x 5 and the wells cut and counted individually in a gamma well counter(Innotron Hydragamma). The ^{125}I bound to the $F(ab')_2$ fraction on the wells was plotted for each specimen against the serum dilutions. With each patient serum samples taken prior to ^{111}In-ICR2 administration were compared with those taken after antibody administration by plotting the bound activity against serum dilutions.

5.2.iii Results

5.2.iii.a) The interpretation of images

The imaging results are presented in this chapter and the analysis of ^{111}In-ICR2 biodistribution in Chapter 6.

The results are presented in Table 5.2.. In all scans a high degree of background activity was found in the liver, spleen, kidneys, bone marrow and testicles.

Of the 22 patients administered ^{111}In-ICR2 imaging was carried out pre-operatively in 20. These 20 patients included two patients with suspected recurrences and 3 with benign colonic tumours (lipoma in one and tubulo-villous adenoma in two). The remaining 15 patients had primary colorectal cancers.

Localised uptake in the region of the primary tumour was found in 12 of the 15 patients (Sensitivity for tumour detection 80% : Observer A). Figure 5.1 demonstrates the uptake of ^{111}In-ICR2 in a cancer of the rectosigmoid junction (arrowed) most obvious in the anterior pelvic view at 48 hours after administration (No.1 M.D.) and Figure 5.2. a cancer of the sigmoid colon (arrowed) which is apparent at both 48 and 72 hours (No.13 V.D.). For comparison Figure 5.3. demonstrates a normal scan from the patient with a lipoma of the sigmoid colon (No.16 R.D.). Negative scanning of a primary tumour was more common in the transverse colon or the flexures but this did not

Table 5.2. Results of immunoscintigraphy

No.	Init.	Diag	Site	Dukes	Observer A	Observer B
1.	M.D.	Ca	R/S	B	Tr +ve	Tr +ve
2.	M.B.	Ca	R/S	B	Incomplete scanning	
3.	R.S.	recurr?	_	_	F +ve	Tr -ve
4.	R.N.	Ca	Trans	B	Tr +ve	Tr +ve
5.	D.N.	Ca	Trans	B	Tr +ve	F -ve
6.	J.L.	Ca	Rectum	C*	Tr +ve	Tr +ve
7.	J.H.	Ca	Trans	B	F -ve	F-ve
8.	B.T.	Ca	Rect	B	Tr +ve	F -ve
9.	J.P.	Ca	Sigmoid	B	Incomplete scanning	
10.	K.H.	recurr?	_	_	F +ve	F +ve
11.	M.J.	Ca	Sigmoid	C	Tr +ve	Tr +ve
12.	B.B.	Ca	Rectum	C	Tr +ve	TR +ve
13.	V.D.	Ca	Sigmoid	C	Tr +ve	Tr +ve
14.	F.H.	Ca	Sigmoid	B	F -ve	Tr +ve
15.	B.S.	Ca	R/S	C*	Tr +ve	F -ve
16.	R.D.	Lipoma	Sigmoid	_	Tr -ve	Tr -ve
17.	J.P.	Ca	Spl flex	B	F -ve	F -ve
18.	E.B.	Adenoma	Caecum	_	F +ve	F +ve
19.	D.O.N.	Ca	Sigmoid	C	Tr +ve	Tr +ve
20.	D.F.	Adenoma	Rectum	_	F +ve	Tr -ve
21.	X.G.	Ca	R/S	C*	Tr +ve	Tr +ve
22.	J.B.	Ca	Sigmoid	B	Tr +ve	F -ve

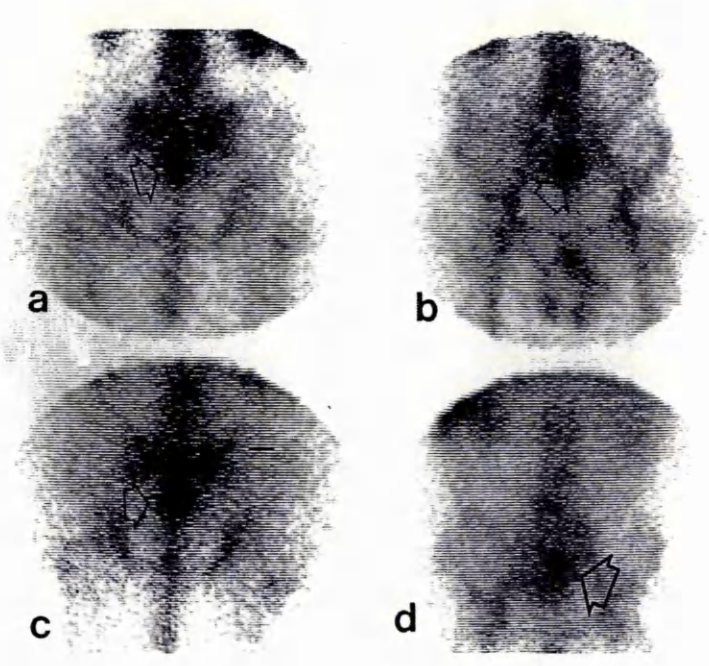


Figure 5.1. (No.1, M.D.)

The uptake of ^{111}In-ICR2 by a colorectal cancer at the rectosigmoid junction. Localised uptake can be seen (arrowed) in the pelvic outlet views at 24 (a) and 48 (c) hours following administration corresponding to that in the anterior pelvic views at similar time periods (b and d).

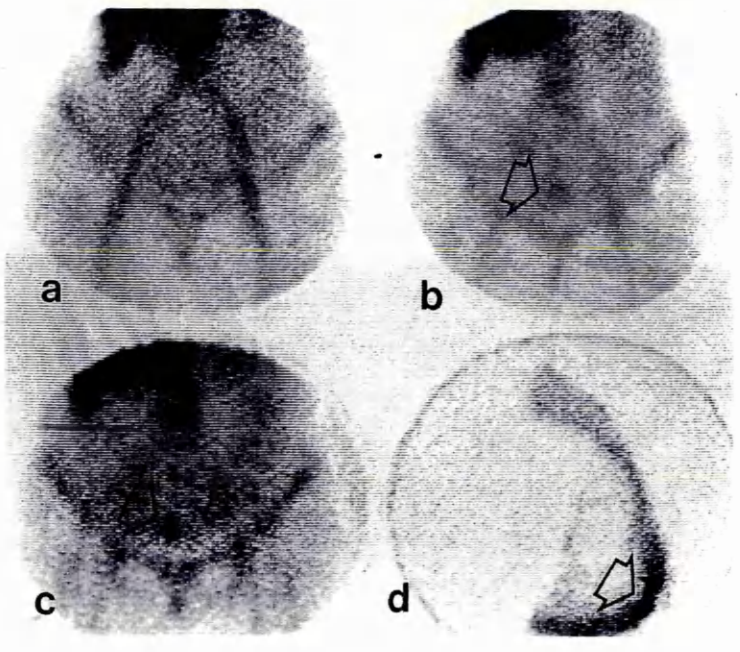


Figure 5.2. (No. 13 , V.D.)

Localisation of a sigmoid colon cancer with ^{111}In-ICR2. No tumour uptake is seen at 24 hours (a) but it becomes apparent in views of the anterior pelvis at 48 (b) and 72 (c) hours after administration. Gamma camera imaging of the resected specimen demonstrates the site of the cancer (d).

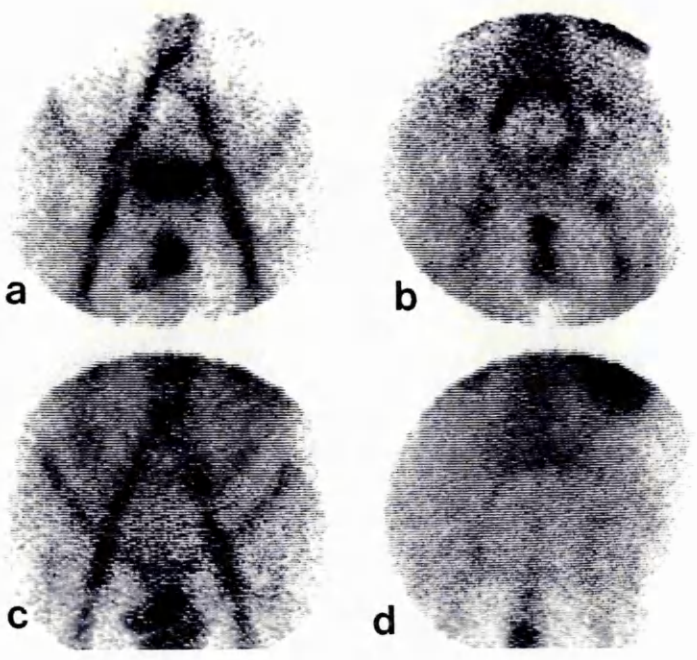


Figure 5.3. (No. 16, R.D.)

^{111}In-ICR2 scan of a patient with a lipoma of the sigmoid colon demonstrating normal blood pool activity, urinary excretion and testis/ scrotal uptake in the anterior abdomen (a) and pelvic outlet views (b) at 30 minutes and in the same views at 24 hours (c and d).

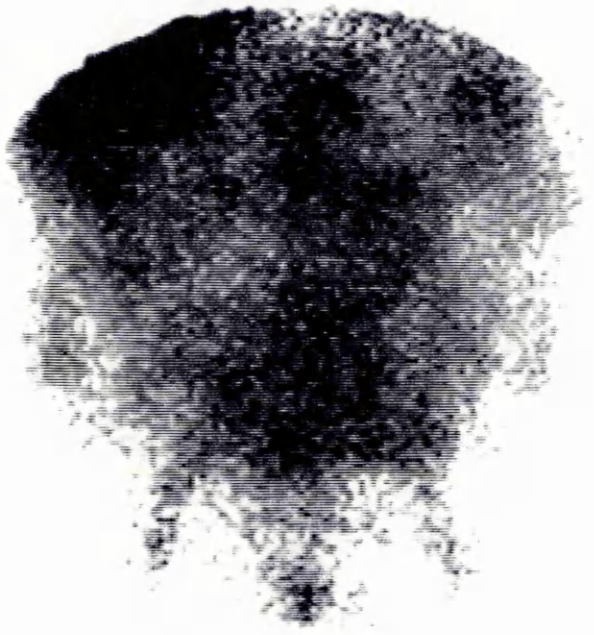


Figure 5.4. (No. 3, R.S.)

Anterior abdominal scan of a patient suspected of recurrent colorectal cancer at 24 hours after ^{111}In-ICR2 administration showing multiple focal areas of localisation.

achieve significance (2 of 3 vs 2 of 12, X^2 with yates correction, n.s.).

In the two patients with suspected recurrences localised uptake was found with both. One patient (No.3, R.S.) had multiple foci of uptake seen on an anterior abdominal scan (Figure 5.4.) which was found on subsequent laparotomy to correspond to dense fibrous adhesions and not recurrent tumour. The other patient suspected of recurrent disease (No.10, K.H.) showed symptomatic improvement and no surgery was carried out.

Three patients were subsequently found to have benign tumours. Of these two had large adenomas and false positive scans (Nos.18, E.B. and 20, D.F.) and one a true negative scan (No.16, R.D.). The overall specificity in the detection of either primary or recurrent cancer on subjective assessment is 20%.

The imaging of metastatic deposits

The results on the localisation of primary tumours are in marked contrast to those with metastases. Of the six patients with nodal metastatic deposits (Nos.6,11,12,15,19 and 21), three had accompanying liver metastases (6,15 and 21) involving both liver lobes. Nodal metastases produced positive images pre-operatively in only one patient (No.11 M.J., Figure 5.5.). None of the hepatic metastases, present in three patients, produced positive images.

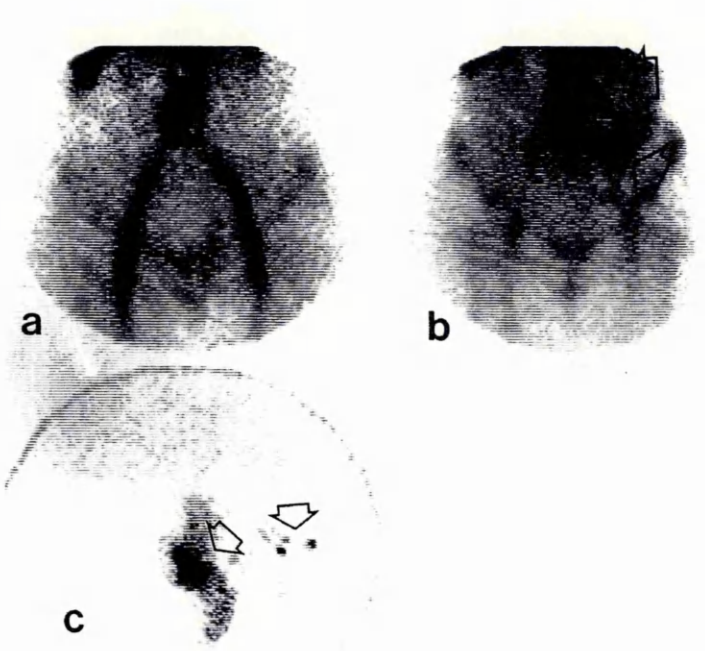


Figure 5.5. (No. 11, M.J.)

Abdominal scan of the only patient in whom nodal metastases were localised pre-operatively demonstrating a normal anterior pelvic blood pool view (a) compared with the 24 hour after administration view demonstrating both the primary tumour and the nodal metastases (b) (arrowed). Gamma camera imaging of the resected specimen (c) confirmed a hot primary tumour and regional node metastases.

Scan reporting without patient details

Of the 15 patients with primary colorectal cancers 9 were correctly identified producing a sensitivity of 60%. Two of the three patients with benign tumours had true negative scans (Nos. 16 and 20) as did one of the two patients with suspected recurrence (No. 3) resulting in a specificity of 60%. As in the subjective reporting in only one of the 6 patients with lymph node metastases were these detected pre-operatively. Of the 20 patients in whom scanning was completed the results of the subjective assessment correlated with the independent assessment in 13 (65%) and disagreed in 7 (35%).

The effect of DTPA and cold MAb administration

The effect of adding DTPA to the injectate and of administering cold MAb to the patient prior to labelled MAb was measured by comparing the sensitivity of primary cancer detection in control, DTPA and cold MAb groups. No significant differences were found between the groups on either subjective (control 5/6, DTPA alone 4/6, cold MAb 3/3) or objective assessment (control 3/6, DTPA 4/6, cold MAb 2/3) as may be expected from the small number of patients in each group. Similarly no differences were noted between the groups in the background uptake of radioactivity present on the scans either by subjective or objective reporting. The effect of these manipulations on tumour and normal organ uptake is decribed in Chapter 6.

b) Tumour uptake of activity and antigen expression

The absolute uptake of activity in the tumours as measured from gamma well counting of specimens and the prepared standard ranged from 0.0016% I.D./g to 0.016 % I.D./g., with a mean ratio in tumour to that of normal colon of 2.1 (+/- S.D. 0.915) : 1. No significant differences were found in the tumour/normal colon ratios or % I.D./g between the control patients and those in whom chelate was reacted with the injectate or cold antibody was administered (Table 5.3.)

The mean T/NC ratio for the three patients with false negative scanning was not significantly different from that in whom scanning was positive.

Of the 17 patients with primary colorectal cancer all but one tumour was found to express EMA on immunohistochemistry (Table 5.4.). This lack of EMA expression correlated with the negative imaging of a transverse colon carcinoma. Six patients had lymph node metastases on H & E microscopy all of which expressed EMA. On one of these six patients scanning pre-operatively localised both the primary tumour and nodal metastases, further confirmed by the imaging of the resected specimen (Figure 5.5.). Both patients investigated for recurrent colorectal cancer expressed EMA in their original primary tumours. The colonic lipoma showed no evidence of EMA expression (No.16 R.D.). EMA expression was found in the two tubulo-villous adenomas (No.18 E.B. and No.20 D.F.)

Table 5.3. Tumour uptake of ^{111}In-ICR2

	Patient group		
	Control	**DTPA**	**Cold MAb**
T/NC ratio	2.1+/-0.91 : 1	2.4+/-0.79 : 1	2.4+/-0.56 : 1
% I.D./g	6.1+/-3.8	10.5+/-4.7	9.7+/-2.04

All values are mean +/- standard deviation

Table 5.4. Scanning, tumour activity and antigen expression

No.	Init.	Diag	Site	Dukes	EMA grade	T/NC	% I.D./g ($x10^{-3}$)	Scan
1.	M.D.	Ca	R/S	B	2	3.2	6.5	Tr+ve
2.	M.B.	Ca	R/S	B	3	3.4	1.6	Incomplete
3.	R.S.	recurr?	_	_	_	NA	NA	F +ve
4.	R.N.	Ca	Hep flex	B	F	1.0	2.4	Tr +ve
5.	D.N.	Ca	Hep flex	B	2	1.8	4.3	Tr +ve
6.	J.L.	Ca	Rectum	C	2	2.0	6.2	Tr +ve
7.	J.H.	Ca	Trans	B	-ve	1.2	12.0	F -ve
8.	B.T.	Ca	Rectum	B	3	2.1	10.0	Tr +ve
9.	J.P.	Ca	Sigmoid	B	3	NA	NA	Incomplete
10.	K.H.	recurr?	_	_	NA	NA	NA	F +ve
11.	M.J.	Ca	Sigmoid	C	1	3.5	8.4	Tr +ve
12.	B.B.	Ca	Rectum	C	1	NA	NA	Tr +ve
13.	V.D.	Ca	Sigmoid	B	2	1.6	16.0	Tr +ve
14.	F.H.	Ca	Sigmoid	B	2	2.3	NA	F -ve
15.	B.S.	Ca	Sigmoid	B	1	NA	NA	Tr +ve
16.	R.D.	Lipoma	Sigmoid	_	-ve	1.6	6.5	Tr -ve
17.	J.P.	Ca	Spl Flex	B	1	2.3	7.2	F -ve
18.	E.B.	Adenoma	Rectum	_	Focal	1.4	6.0	F +ve
19.	D.O.N.	Ca	Sigmoid	C	3	1.9	9.0	Tr +ve
20.	D.F.	Adenoma	Caecum	_	3	NA	NA	F +ve
21.	G.X.	Ca	R/S	C	3	3.0	8.1	Tr +ve
22.	J.B.	Ca	Sigmoid	B	2	2.3	12.0	Tr +ve

corresponding to areas of tissue showing severe dysplasia. Both of these tumours produced positive images pre-operatively. In table 5.5. the EMA expression of the tumours graded by the method of Ellis is compared to the T/NC ratios of the excised tumours. Patients whose resected tumours showed no EMA expression or focal staining alone had lower T/NC ratios than those expressing EMA (1.30 +/- S.D.0.26 vs 2.45 +/- S.D.0.65, p=0.005)(Table 5.5.).

Safety of ^{111}In-ICR2 and anti-rat response

No reactions were noted to the intradermal injection of ICR2 and no acute anaphylactic reactions occurred in any of the patients as a result of the injection of the rat antibody. Similarly recordings of patients temperature, pulse and blood pressure following administration showed no obvious abnormalities nor did follow up measurement of FBC, U & E's and LFT's. One patient (No.15 B.S.) who had recieved unlabelled MAb did, however, develop a transient period of generalised malaise associated with urticarial weals around the elbows at 5 days following antibody administration. This resolved spontaneously without treatment in 48 hours.

The response to rat antibody administration was measured in 42 serum samples from 12 patients. The majority of these samples were from the first 7 days following antibody administration (Figure 5.6).and none of these samples showed any evidence of an immune

Table 5.5. EMA expression and tumour uptake of radioactivity

	Grading of antigen expression				
	-ve or focal	I	II	III	IV
T/NC ratio	1.0	2.3	3.2	3.4	
	1.2	3.5	1.8	2.1	
	1.4		2.0	1.9	
	1.6		2.3	3.0	
			1.6		
			2.3		

response to rat antibody. All three samples analysed from the second week after antibody administration, however, showed evidence of a response as demonstrated in Figure 5.7.

Figure 5.6. Human response to rat MAb

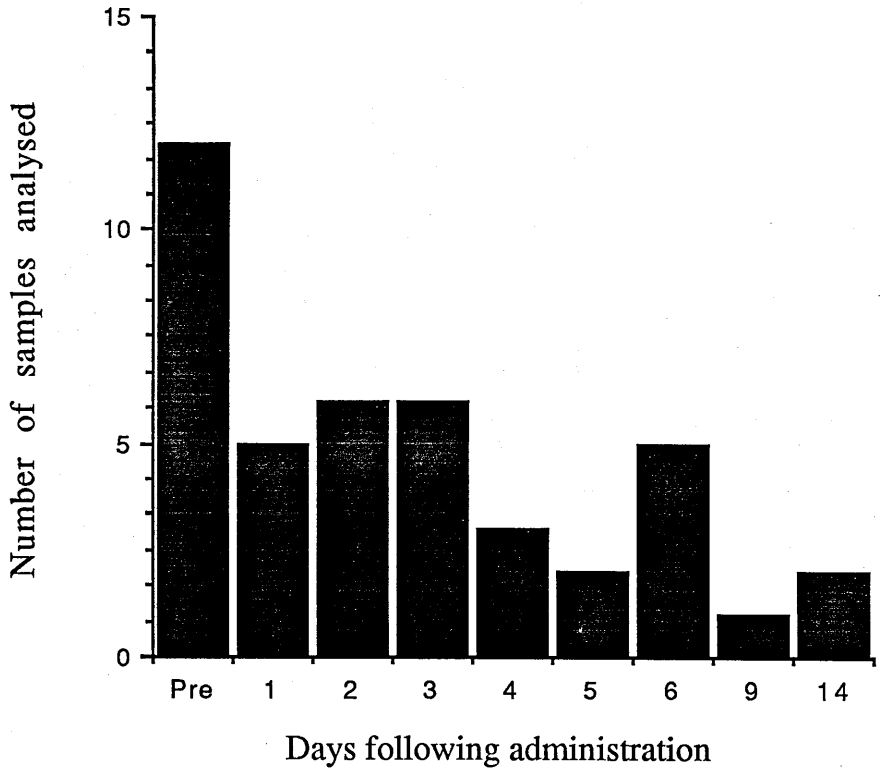
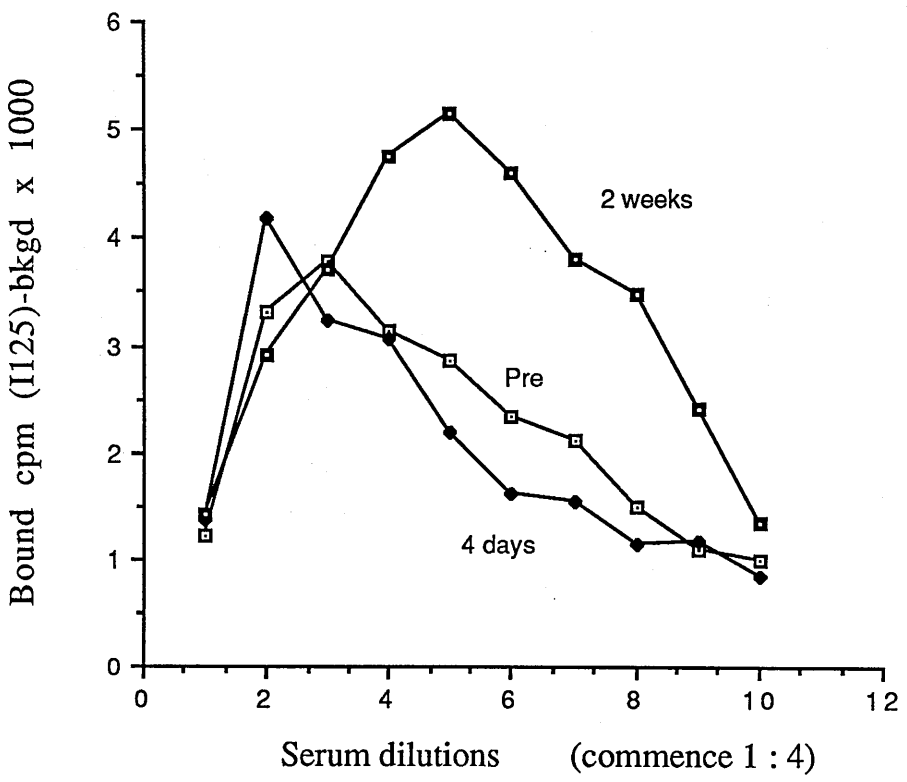


Figure 5.7. Example of human anti-rat response



5.2.iv. Discussion

Epithelial membrane antigen is expressed by a wide variety of normal tissues [201,202]. Despite this, immunoscintigraphy of colorectal cancers has been achieved in the present study due to the differential expression of EMA between colorectal cancers and normal adjacent colon [201,207,248].

The sensitivity of ^{111}In-ICR2 imaging for the detection of primary colorectal cancers was 80% when assessed with a knowledge of the clinical details, which is considerably better than some previous reports with this disease using both anti-CEA [100,104,105,292,295,296] and MAb's against other tumour associated antigens [115-117]. Some studies have been reported in which all primary cancers have been localised following radiolabelled antibody administration [106,293]. In these studies, however, it does not seem that the scans were interpreted blindly.

In the present study six of the 17 patients with colorectal cancer had metastatic deposits in the lymph nodes and three of these also had liver metastases. Imaging of these six patients localised only one metastatic site in the regional lymph nodes. These results are in keeping with other reports in which small tumour deposits fail to be localised. The inability to image small tumour deposits is due to the small absolute uptake of activity within the tumour tissue relative to background activity. ^{111}In is also rapidly taken up by normal liver and the majority of

studies claiming satisfactory localisation of liver metastases with ^{111}In labelled antibodies are based on the finding of areas of lower radioactivity within the liver substance [296] or areas of lower radioactivity on a blood pool view which later show accumulation of radioactivity [106]. This provides little advantage over conventional liver imaging with technetium colloids or by ultrasonography. The uptake of ^{111}In labelled monoclonal antibodies by normal tissues including the liver is discussed in Section 4.1. and possible strategies for reducing this uptake in the introduction to the present chapter (Section 5.2.i). Of interest regarding the general distribution of the radiolabelled antibody was the rapid uptake by the testis. This has previously been noted on antibody imaging with anti-CEA and attributed to the presence of CEA in the testis [299]. This does not, however, explain the uptake in the present study as EMA is not expressed on the testis (J.P.Sloane, unpublished observations).

Although previous studies have suggested that an increased number of metastatic tumour sites may be localised by the pre-administration of unlabelled antibody this has not been confirmed in the present study[242,243]. The previous studies have been largely based on patients with melanoma who had a large number of metastatic sites detectable by other means. In these patients the administration of cold MAb has

been shown to be associated with the detection of an increased number of metastatic sites. These studies have not been accompanied, however, with any measure of the effect on tumour uptake of the radiolabelled MAb by administering cold MAb. The significance of these results must, therefore, remain open to question.

Similarly the role of adding chelating agents to the injectate has been suggested empirically as a method of removing any non specifically bound ^{111}In. Although this again produced no significant difference to the sensitivity of cancer detection the biodistribution data presented in Chapter 6 allows its use to be evaluated.

The specificity of tumour localisation has been demonstrated in the present study by two independent analyses. Firstly a positive localisation on external imaging was only seen with primary tumours when the tumour expressed the target antigen (which included the adenomatous polyps) while the single non antigen expressing primary cancer did not produce a positive scan. Secondly a correlation was found between the antigen expression of the resected tumours as graded by immunohistochemistry and the tumour to normal colon ratios found on gamma well counting of biopsies, those tumours showing a high grade of antigen expression tending to have higher uptake ratios. Other alternative methods which have been used to prove the specificity of radiolabelled MAb uptake include double labelling

techniques in which specific and non specific antibodies are administered with different radionuclides having different energies of gamma emissions allowing their separation and flow cytometric analysis [106]. The accuracy of the former is dependent on the biodistribution of the antibodies being unaffected by the radiolabel, which is unlikely [230,265,266]. With the latter the connective tissue elements of the excised tumour may lead to considerable errors[106]. Neither technique was therefore felt likely to provide additional information.

The T/NC ratio found in the present study of 2.1 (+/- S.D.0.91) : 1 is similar to that quoted for the uptake of some MAb's by colorectal cancer [113,114,117] but significantly lower than that recently reported using an antibody to CEA [106]. This difference in uptake may explain the difference between the sensitivity of tumour detection by scanning in the latter study (100%) and that of the present study (80%).

The low "signal to noise ratio" caused by the high background activity and low uptake of radiolabelled MAb in cancers results in poor definition of the tumours on scanning. Attempts to improve this ratio have centred on clearing the activity in the blood pool after allowing time for localisation of the antibody. This has been attempted by the use of a second antibody to produce clearance by the reticuloendothelial system [133], computerised subtraction of a second circulating

radioisotope [122-124,131,132] or the use of the streptavidin-biotin complex [300-302].

Two patients in the present study had large tubulovillous adenomas which expressed EMA in areas of dysplasia and produced positive scans. The association between EMA expression of polyps and their degree of dysplasia was noted in Section 2.2. and may represent a stage in the adenoma to carcinoma sequence. Although the detection of these two benign tumours reduces the specificity of the radiolabelled MAb for cancer detection it may obviously be advantageous were scanning to localise dysplastic benign as well as malignant lesions.

As with other studies on the use of radiolabelled antibodies ^{111}In-ICR2 administration produced no harmful side effects to the patients. The finding of a primary response to rat antibody in the second week after administration, however, is of major importance. Many studies have observed the human response to mouse antibody administration and have correlated this with a reduced quality of immunoscintigraphy scans on repeated radiolabelled antibody administration [303-304]. Little information, however, is available on whether rat antibody is equally as effective at sensitising humans and further studies will be required to clarify the situation.

CHAPTER 6

THE BIODISTRIBUTION OF THE INDIUM LABELLED MONOCLONAL ANTIBODY ICR2 IN PATIENTS WITH COLORECTAL CANCER

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6.4. DISCUSSION

6.1. INTRODUCTION

Many studies over the last 10 years have demonstrated the localisation of radiolabelled tumour associated monoclonal antibodies in colorectal cancers and their imaging by a gamma camera[97-106]. The majority of studies have reported the results of imaging but given no information on the biodistribution of the labelled antibody. As the complexity of the process between radiolabeled antibody administration and the immunoscintigraphy of cancers has been identified (Chapter 5), the necessity for detailed biodistribution studies has become apparent. To this end some recent reports on patient studies with radiolabelled antibodies have concentrated on biodistribution rather than imaging [242,243]. These studies, however, have failed to explain the reason for the effective imaging that can be achieved despite the presence of high levels of circulating antigen and immune complexes. Although it has been suggested that increasing the dose of antibody administered may improve tumour imaging [184,305-308] few studies have directly explored this mechanism and most have been based principally on the quality of the images produced[242,243].

A major problem in immunoscintigraphy is the non specific uptake of ^{111}In labelled MAb's as discussed in Chapter 4. It was concluded from the study on isolated hepatocytes and the antigen expressing

tumour cell line that DTPA may have a role to play in reducing the liver uptake of radioactivity found in patient studies using ^{111}In labelled MAb's. It was also suggested that the mechanism for this effect may be the removal of weakly bound ^{111}In from the MAb molecule and its stable extracellular binding with DTPA.

In this study the biodistribution of ^{111}In-ICR2 is examined in patients with colorectal cancer whose external imaging was discussed in Chapter 5 and the effect of administering unlabelled antibody and of treating the injectate with DTPA is investigated.

The rate of urinary excretion and blood clearance of radioactivity and the specific organ uptake including the liver, spleen and bone marrow were determined as well as that of the excised tumours. The organ uptake of radioactivity was calculated by computer (Nodecrest, U.K.) by drawing a "region of interest" (ROI) around the organ being measured on the static gamma camera images. Because of the inaccuracies in this technique due to the scattering and absorption of radioactivity by normal tissues a phantom was used to calculate the percentage of the injected dose accumulating in the liver.

The level of antigen in the circulation prior to radiolabelled MAb administration was also measured as was the amount of immune complex forming after administration.

6.2. Materials and methods

In chapter 5 the results were described of external imaging with ^{111}In-ICR2 in patients with known or suspected colorectal cancer. The technique was able to localise both primary and metastatic colorectal cancers but had a low specificity if the imaging of antigen expressing dysplastic polyps was considered to be a false positive result. It was also shown that a high tumour to normal colon differential uptake of radioactivity was dependent on the antigen expression of the tumours (Table 5.5.) and that an anti-rat antibody response may occur during the second week after administration. The results of imaging were not significantly different when DTPA was added to the radiolabelled antibody or if additionally unlabelled antibody was administered prior to labelled antibody. Furthermore no significant differences were found between these patient groups in the % I.D./g in tumour tissue or the T/NC uptake ratio (Table 5.3.)

In this study the biodistribution of ^{111}In-ICR2 was analysed in the 22 patients whose imaging was described in Chapter 5. This consisted of a control group of 10 patients (Group A) to whom 1mg of labelled antibody alone was administered, a second group (Group B) of 6 patients whose injectate (1mg of ^{111}In-ICR2) was reacted with DTPA as outlined in Appendix 5.4 and a third group of 6 patients (Group C) who also had DTPA added to their injectate prior to gel chromatography but who additionaly had an infusion of 5mg of

unlabelled antibody administered in 100mls of normal saline over the one hour prior to labelled antibody administration. The nature of the injectate for each patient is shown in Table 6.1.. The number of patients on whom each variable was studied is shown in the corresponding tables as it was not always possible to study each variable on all patients.

6.2.a) Blood clearance, urinary excretion and tissue uptake

The clearance of activity from the blood was calculated by collecting 10ml samples of peripheral venous blood in heparinised bottles at 10min, 30 min, 24, 48 and 72 hours following injection, which were centrifuged at 3000 rpm for 5 minutes and the activity in paired 2ml aliquots of plasma calculated as a percentage of the activity in the 10 minute sample.

For each patient a standard was prepared to allow the percentage of the injected dose in the other samples to be calculated. At the time of administration of ^{111}In-ICR2 a weighed aliquot of the injectate was removed, diluted to 500mls in normal saline containing 0.04 molar HCl and a 2ml aliquot of this solution thus contained a known percentage of the I.D.. The counting of this 2ml standard in a gamma well counter along with the sample being measured allowed the % I.D. to be calculated. The preparation of this standard is more fully described in Chapter 5, page 160.

Table 6.1. Patients and their injectate.

No.	Init.	Diagnosis	Site	Nature of the injectate		
				^{111}In-ICR2	DTPA	Cold MAb
1.	M.D.	Ca	R/S	+	-	-
2.	M.B.	Ca	R/S	+	-	-
3.	R.S.	recurr?	_	+	-	-
4.	R.N.	Ca	Hep flex	+	-	-
5.	D.N.	Ca	Hep flex	+	-	-
6.	J.L.	Ca	Rectum	+	-	-
7.	J.H.	Ca	Trans	+	-	-
8.	B.T.	Ca	Rectum	+	-	-
9.	J.P.	Ca	Sigmoid	+	-	-
10.	K.H.	recurr?	_	+	-	-
11.	M.J.	Ca	Sigmoid	+	+	-
12.	B.B.	Ca	Rectum	+	+	-
13.	V.D.	Ca	Sigmoid	+	+	-
14.	F.H.	Ca	Sigmoid	+	+	-
15.	B.S.	Ca	R/S	+	+	-
16.	R.D.	Lipoma	Sigmoid	+	+	+
17.	J.P.	Ca	Splen flex	+	+	-
18.	E.B.	Adenoma	Caecum	+	+	+
19.	D.O.N.	Ca	Sigmoid	+	+	+
20.	D.F.	Adenoma	Rectum	+	+	+
21.	G.X.	Ca	R/S	+	+	+
22.	J.B.	Ca	Sigmoid	+	+	+

The urinary excretion of activity was calculated for the first 12 hrs and two subsequent 24 hr periods following injection of the radiolabelled MAb. Bowel preparation prior to surgery prohibited urine collection beyond this period. Prior to administration of ^{111}In-ICR2 patients were instructed to empty the urinary bladder and this voided volume was discarded. Timed urine collections were then carried out at the end of which the urinary bladder was again emptied and the volume voided added to the last 24 hour collection. The urinary excretion was calculated as a percentage of the injected dose by relating the activity in 2mls of a thoroughly mixed urine sample to that of 2mls of the standard.

The uptake of ^{111}In-ICR2 in the colonic cancer tissue was determined following resection by counting weighed samples of tumour and normal colon in a gamma well counter, results being expressed as cpm/g. These were then calculated as a % I.D. by comparison with the standard counts and as a ratio of counts per gram in tumour to that of normal colon.

Organ uptake of radioactivity

Imaging data was used to calculate the ^{111}In-ICR2 uptake in the liver, spleen, kidney and bone marrow. This was carried out using a phantom and region of interest (ROI) analysis for the liver and ROI analysis alone for the spleen, kidney and bone marrow. The latter involves the calculation of the % I.D. in the

organ by comparing the counts obtained by imaging the injectate (^{111}In-ICR2) with ROI analysis of the organs. This, however, is less accurate than the use of a phantom in a Temex trunk (see later) and ROI analysis as the phantom represents normal liver geometry, and the Temex the absorption and attenuation of normal human tissues.

Liver

Liver uptake was determined using an anthropomorphic liver phantom in a Temex trunk. The phantom consists of a hollow plastic container equivalent in size and shape to a human liver which may be filled with a radioactive solution. The Temex trunk is a rubber mould in the shape of the human trunk in which the liver phantom can be placed, and which has a density to radioactivity equivalent to that of human tissues. For each patient the phantom was filled with a known percentage of the injected dose of ^{111}In-ICR2 . Anterior and posterior images of the patients liver and the Temex trunk containing the liver phantom were acquired for 300k counts in a 128 x 128 matrix and region of interest analysis used to calculate the geometric mean of the patients liver and the liver phantom activity as cps with decay correction to injection time.

$$\text{Uptake in liver as \% I.D.} = \frac{\text{liver cps*} \times \text{C.F.} \times 100}{\text{phantom cps*} \times \text{\% I.D. in phantom}}$$

* : Geometric mean and decay corrected
C.F. : Attenuation correction factor to compensate for differences between patient and phantom antero-posterior diameter(Appendix 6.1.).

Spleen, kidney and bone marrow

Region of interest analysis was used on anterior and posterior abdominal images to obtain the geometric mean of the uptake of radioactivity (cps), decay corrected to the time of injection. Comparing this with the injected cps calculated from imaging of the injectate in its syringe allowed the % I.D. in these organs to be calculated. The injected cps was calculated by placing the injectate in its syringe on the collimator of the gamma camera and using ROI analysis to assess the counts emitted. The counts injected were calculated by subtracting this figure from the counts left in the syringe after injection.

$$\text{Organ uptake as \% I.D.} = \frac{\text{organ cps} \times \text{C.F.} \times 100}{\text{injected cps}}$$

The attenuation correction factor used in this calculation is different from that used for the liver and is described in Appendix 6.2.

Because of the small amount of radioactivity accumulating in the spleen, kidney and bone marrow

resulting in a low % I.D., data was also compared between patient groups for these organs as the counts obtained over these organs over a 5 minute period per MBq of labelled activity injected. Counts were obtained from organ regions of interest on the posterior abdominal images as these most clearly delineated the organs. The regions of interest were based on the spleen, left kidney and the fourth lumbar vertebra.

Circulating EMA levels

The circulating EMA level was calculated prior to labelled MAb administration and compared with imaging results, immune complex formation and tumour uptake of radioactivity. This was carried out in 15 of the 22 patients using the methodology outlined in Chapter 3, page 108.

Measurement of circulating immune complexes

When a solute passes down a chromatography gel its molecules diffuse both into and out of the gel matrix (stationary phase). Seperation on gel filtration depends on the different ability of various sample molecules to enter pores in the stationary phase. Very large molecules never enter the gel matrix and move rapidly through the column whereas small molecules enter the gel pores and move more slowly through the column. High performance liquid chromatography (HPLC) is therefore an accurate method of seperating molecules in order of decreasing molecular size and has been used for detecting labelled antibody and immune complex in

patient serum.

Analysis of serum samples by HPLC was carried out using a Zorbax GF250 size exclusion column which was run with 0.25M PBS pH 6.8. Serum samples were spun on a microcentrifuge (Hawksley, U.K.) to remove any clot or debris prior to 20ul aliquots being injected into the port of the HPLC. The molecular weight of eluted fractions was calculated against a commercially available standards package (Pharmacia, Upsalla, Sweden).

Serum samples were analysed both prior to and following ^{111}In-ICR2 administration. "Cold" serum was reacted with ^{125}I-ICR2 at a concentration of 0.3ug labelled antibody per ml of serum, an amount equivalent to the distribution within plasma following the administration of 1mg of labelled antibody to a patient with a plasma volume of 3 litres. The level of immune complex formed in this sample was then used as a control for each patient against which the "hot" samples following ^{111}In-ICR2 administration were compared. ^{125}I was used rather than ^{111}In-ICR2 for this purpose because of its longer half life allowing an aliquot to be stored and used on several occasions. "Hot" serum samples, taken following ^{111}In-ICR2 administration, were analysed directly without additional radiolabelling being required.

Aliquots of 20ul of serum which had either been reacted with ^{125}I-ICR2 or sampled following ^{111}In-ICR2 administration were eluted through the HPLC column with

PBS (0.25M, pH 6.8). The protein peak was charted by its U.V. absorption at 320nm and 0.5ml aliquots were collected and counted in a gamma counter (LKB Compugamma 1282, LKB Instrument, Stockholm)

Titration of unlabelled antibody

To investigate the possible effects on immune complex formation of administering unlabelled antibody prior to injection of the radiolabelled antibody plasma samples were taken from three patients and mixed with unlabelled ICR2 MAb to give a 1:1, 3:1, 10:1 and 100:1 ratio of unlabelled ICR2 to the subsequently added ^{125}I-ICR2. Following the addition of ^{125}I-ICR2 the samples were incubated for one hour then analysed by HPLC. Aliquots of 0.5ml were again collected from the column and counted in a well counter (LKB Compugamma)

HPLC analysis of patient sera

HPLC analysis of serum was carried out on 16 of the 22 patients studied with ^{111}In-ICR2. Samples taken prior to antibody administration were reacted with ^{125}I-ICR2 as were samples analysed following administration of unlabelled ICR2. Further serum analysis was carried out in patients at 10 and 30 minutes following ICR2-In^{111} administration as well as at 24 and 48 hours. The activity present as high molecular weight complex was calculated by integration of the area under the activity versus eluted volume curve.

6.3. RESULTS

6.3.a) Blood clearance, urinary excretion and tissue uptake of activity.

Blood clearance

The clearance of activity from the circulation is shown as a percentage of the circulating activity at 10 minutes after injection in Figure 6.1.. The effective half life of circulating activity was 39 hours for the control group, 33 hours for Group B(DTPA alone) and 27 hours for Group C(DTPA and cold MAb). The rate of blood clearance in Groups B and C was more rapid than for Group A, this being statistically significant for DTPA at 48 hours ($p=0.005$) and for the cold MAB at both 24 hours ($p=0.034$) and 48hrs ($p=0.0000$)(Table 6.2.and Appendix 6.3.).

Urinary excretion of activity

The mean urinary excretion of activity in group A (controls) was 16.2% of the injected dose in the first 12 hours, 3.2% for the next 24 hrs and 2.1% for the subsequent 24hrs (Table 6.3., Figure 6.2.a, Appendix 6.3.). Treatment of ^{111}In-ICR2 with DTPA reduced the mean urinary excretion of activity by 82% in the first 12 hour period following injection (16.2+/-S.D.6.94 vs 2.9 +/-S.D.1.8, $p=0.007$). No significant differences in excretion of activity were noted over the subsequent 48 hours. The administration of cold MAB in addition to DTPA had no significant effect on the urinary excretion of activity.

Figure 6.1. Blood clearance of activity

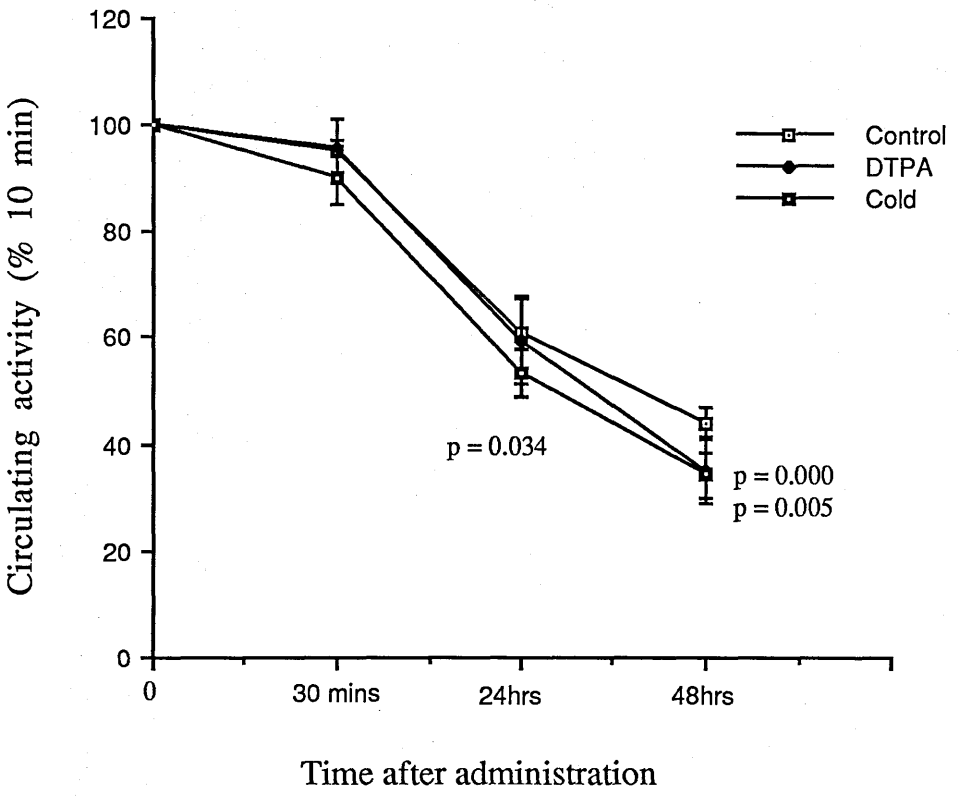


Table 6.2. Blood clearance of activity

Patient group	Time after administration 10 min	30 min	24hrs	48hrs	72hrs
Controls (n=9)	100	95+/-6	61+/-7	44+/-3	24+/-7
DTPA alone(n=5)	100	96+/-2	59+/-8	35+/-6*	26+/-4
Cold MAb(n=6)	100	90+/-5	53+/-5^{+}	34+/-4^{++}	19+/-3

Activity is shown as a percentage of that at 10 minutes following injection.

All results are mean +/- standard deviation.

Statistical comparison with controls * $p = 0.005$, $^{+}$ $p = 0.034$, $^{++}$ $p = 0.000$

Table 6.3. Urinary excretion of activity

Patient group	Time after administration 1st 12 hrs	next 24hrs	next 24 hrs
Controls(n=6)	16.2+/-6.9	3.2+/-0.9	2.1+/-1.0
DTPA alone(n=4)	2.9+/-1.8*	2.4+/-0.5	2.5+/-0.3
Cold MAb(n=6)	4.1+/-1.6^{+}	2.1+/-1.3	2.0+/-0.8

Mean % I.D.+/- S.D.

Statistical comparison with controls * $p = 0.007$, $^{+}$ $p = 0.012$

Cold MAb vs DTPA alone, n.s.

Figure 6.2.a. Urinary excretion of activity

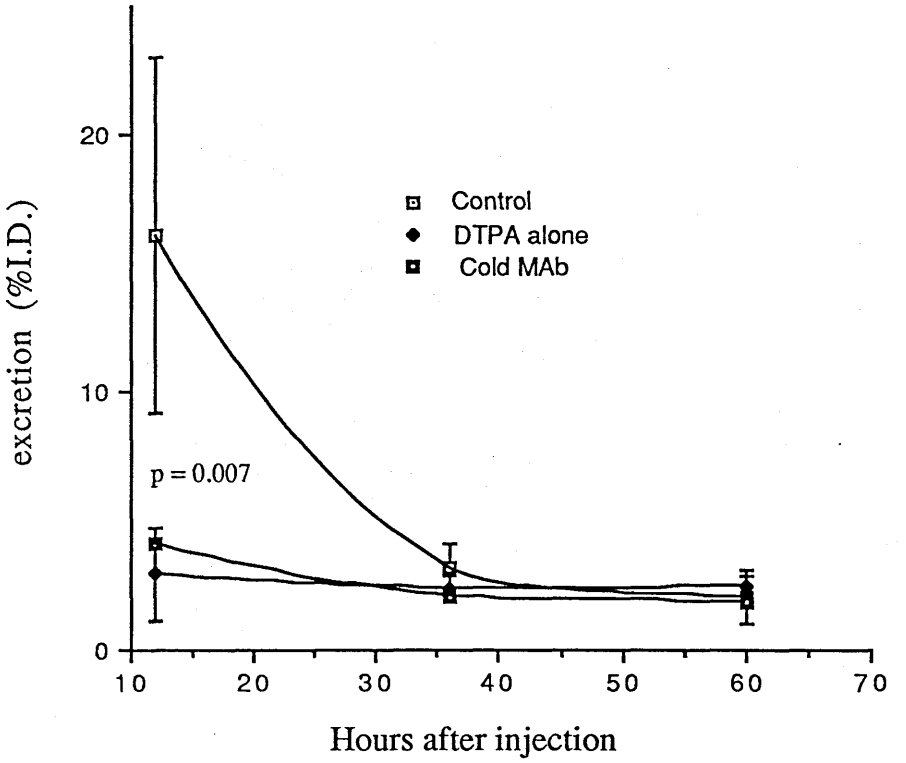
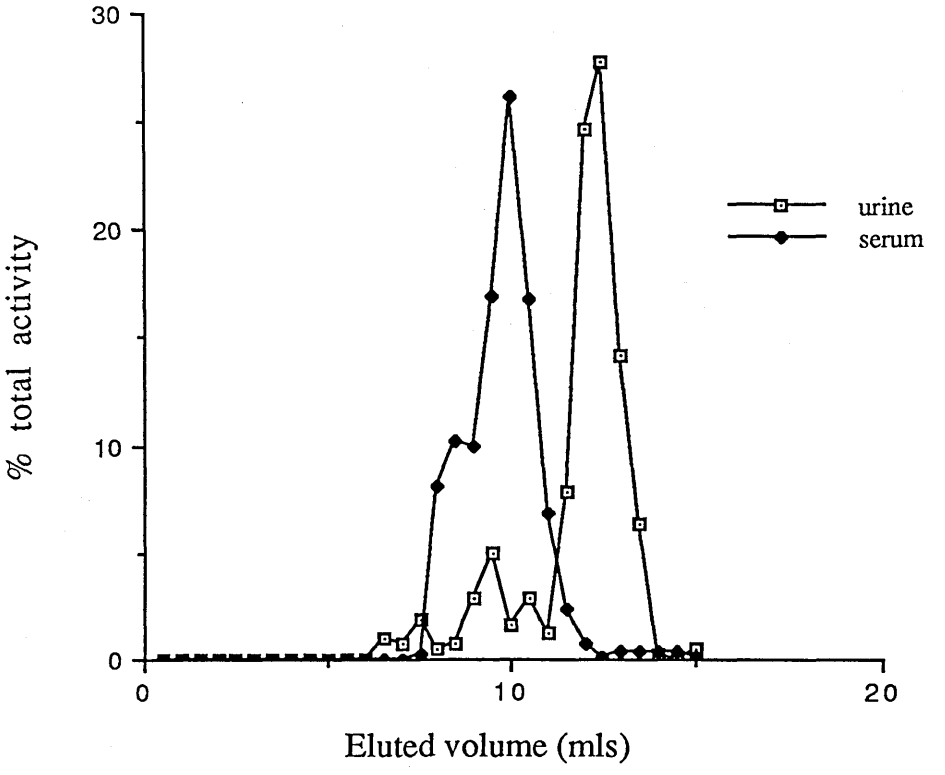


Figure 6.2.b Urine and serum HPLC



The nature of the activity in the urine was analysed in a random selection of specimens using HPLC and was found in all cases to be in a single low molecular weight form. An example is shown in Figure 6.2.b. with the HPLC of the same patients serum superimposed for comparison of the molecular weights (high M.Wt. to left of page).

Tissue uptake of activity

The uptake of activity by tumour and normal colon have been described in Chapter 5 (5.2.iii.b., page 166)

Organ uptake of ^{111}In-ICR2

Liver

The uptake of activity in the liver in the initial 30 minutes calculated from the dynamic imaging is shown compared with blood pool activity in Figure 6.3. This was measured by taking sequential ROI's of both the liver and an area overlying the heart representing blood activity. After the initial phase the blood activity gradually declines whereas that of the liver continues to rise suggesting an active uptake mechanism. Comparing this early uptake between the control, DTPA and cold MAB groups demonstrates a wide range in the rate of uptake between patients and no significant differences between the groups (Figure 6.3. and Appendix 6.4.). By 48 hours 20-30% of the I.D. has accumulated in the liver as calculated using the phantom. This was significantly higher in the DTPA

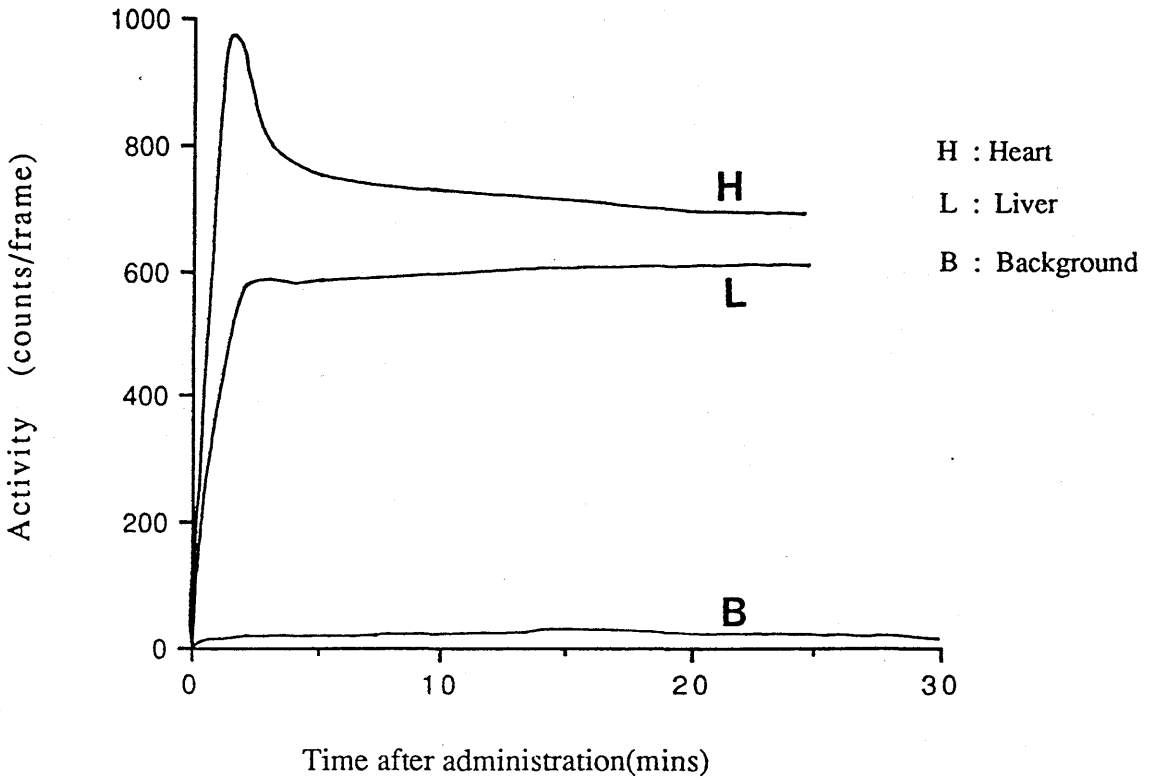


Figure 6.3.

The activity-time curve for the liver is shown for the first 30 minutes following ^{111}In-ICR2 administration and compared with blood pool activity using a region of interest centered on the heart. The background level of activity is also shown.

group than the control group at 45 minutes (p=0.049), 24 (p=0.029) and 48 hours (p=0.002). The administration of unlabelled antibody produced a significant reduction in the uptake of activity in the liver at 48 hours when compared to the group with DTPA alone (32% vs 26%, p<0.05)(Table 6.4., Figure 6.4., Appendix 6.5.).

Spleen

Within the first 48 hours following administration 2-3% of the injected dose accumulated in the spleen (2.44+/-S.D.1.4, control group). There was no significant difference in the % I.D. in the spleen between the patient groups at 45 minutes or 24 hours although by 48 hours the uptake was significantly greater for both the DTPA group (p=0.05) and cold MAB group (p=0.048) (Table 6.5., Appendix 6.6.) However, using the ROI cts / 5 min/ MBq evaluation, significant differences were noted between the control and the DTPA group at 45 minutes (p=0.05) and between controls and the cold MAB group at 45 minutes (p=0.000), 24 hours (p=0.000) and 48 hours (p=0.000)(Table 6.6., Figure 6.5., Appendix 6.7.)

Renal and marrow uptake of radioactivity

Approximately 2% of the injected dose accumulated in the kidney (left) by 45 minutes (2.04+/-S.D.0.49) and remained at a similar value for the subsequent 48 hours. No significant differences were noted between the patient groups as regards the renal or bone marrow

Table 6.4. Liver uptake of ^{111}In-ICR2

Patient group	Time after administration 45 mins	24hrs	48hrs
Control(n=9)	18.1+/-3.2	20.7+/-3.5	21.9+/-4.6
DTPA alone(n=6)	22.2+/-2.6*	26.0+/-4.9**	31.8+/-3.7***
Cold MAb (n=6)	18.7+/-6.0	22.8+/-4.4	25.8+/-4.0^{+}

Figures are the mean % injected dose +/- standard deviation.
Statistical comparison with controls * p = 0.049, ** p = 0.029, *** p = 0.002
Cold MAb vs DTPA alone, $^{+}$ p < 0.05

Table 6.5. Spleen uptake of ^{111}In-ICR2 as % I.D.

Patient group	Time after administration 45 mins	24hrs	48hrs
Control(n=8)	2.44+/-1.4	2.14+/-0.95	1.93+/-0.72
DTPA alone(n=5)	3.07+/-0.9	2.60+/-0.85	2.74+/-0.35*
Cold MAB(n=6)	3.00+/-0.7	2.97+/-0.65	2.83+/-0.66**

Figures are the mean +/- standard deviation.
Statistical comparison with controls * p = 0.05, ** p = 0.048

Table 6.6. Spleen uptake as cts/5 min/MBq

Patient group	Time after administration 45mins	24 hours	48 hours
Control (n=8)	433+/-154	470+/-159	450+/-149
DTPA alone(n=5)	603+/-124^{+}	615+/-141	526+/-76
Cold MAb(n=6)	815+/-157*	859+/-110**	800+/-143***

Results are mean +/- standard deviation
Statistical comparison with controls $^{+}$ p =0.05, * , **, *** all p = 0.000

Figure 6.4. Liver uptake of activity

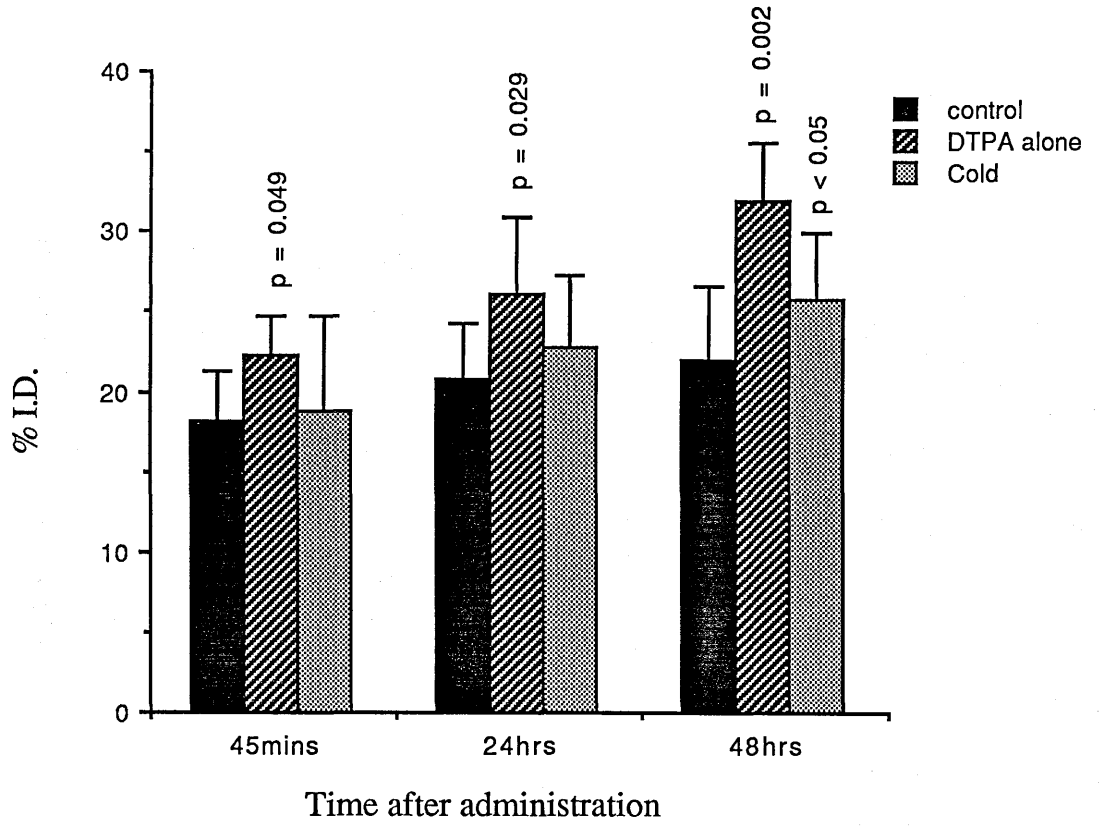
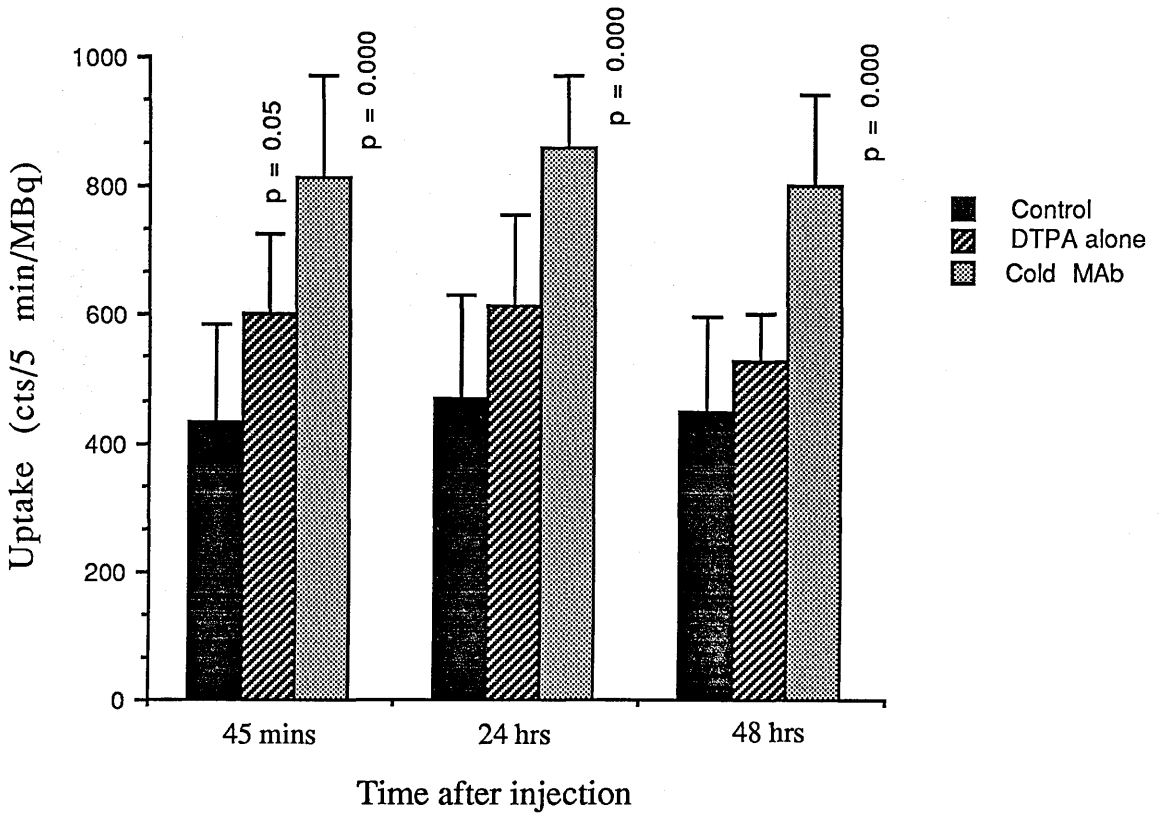


Figure 6.5. Splenic uptake of activity



uptake of activity.(Table 6.7., appendices 6.8., 6.9., 6.10.)

HPLC analysis

The nature of the labelled antibody was analysed for both the ^{125}I and ^{111}In labels and was found to elute as a single peak corresponding to a molecular weight of the standard of 150-160,000 (Figure 6.6.). When antibody with either label was combined with human serum from either cancer or normal patients two peaks eluted, the first comprising approximately 30% of total activity and the latter 70%. The former eluted with the void volume of the chromatography column and had a M.Wt. > 600,000. The latter corresponed to the 150-160,000 of IgG (Figure 6.7.).

The titration of unlabelled antibody

The results are shown for the progressive increase in cold antibody concentration in Figure 6.8.(a-d). In all of the patients serum studied the activity forming the high molecular weight complex was reduced by progressive increases in cold antibody administration. In the sample shown a decrease was achieved in the percentage of the radiolabelled activity forming immune complex from 37.6% at a 1:1 ratio of cold to labelled MAb to 26.3% at a 10:1 ratio.

Table 6.7. Renal uptake of activity

Patient group	Time after administration 45min	24hrs	48hrs
Control (n=10)	434+/-107	475+/-116	423+/-102
DTPA alone (n=5)	436+/-138	470+/-165	422+/-140
Cold MAb(n=6)	523+/-120	600+/-116	586+/-87

Results are mean counts/5 min/ MBq injected activity +/- standard deviation

Table 6.8. Immune complex formation

	Patient group	
	No cold MAb	Cold MAb
Pre MAb admin	32+/-2	33+/-5
Post cold MAb	-------	30+/-2
Post ^{111}In-ICR2		
10min	34+/-6	30+/-6
30min	31+/-6	28+/-6
24hrs	28+/-8	23+/-6

The results shown are the % of circulating activity present in the high molecular weight form. All mean +/- S.D..

Figure 6.6. : HPLC of injectate

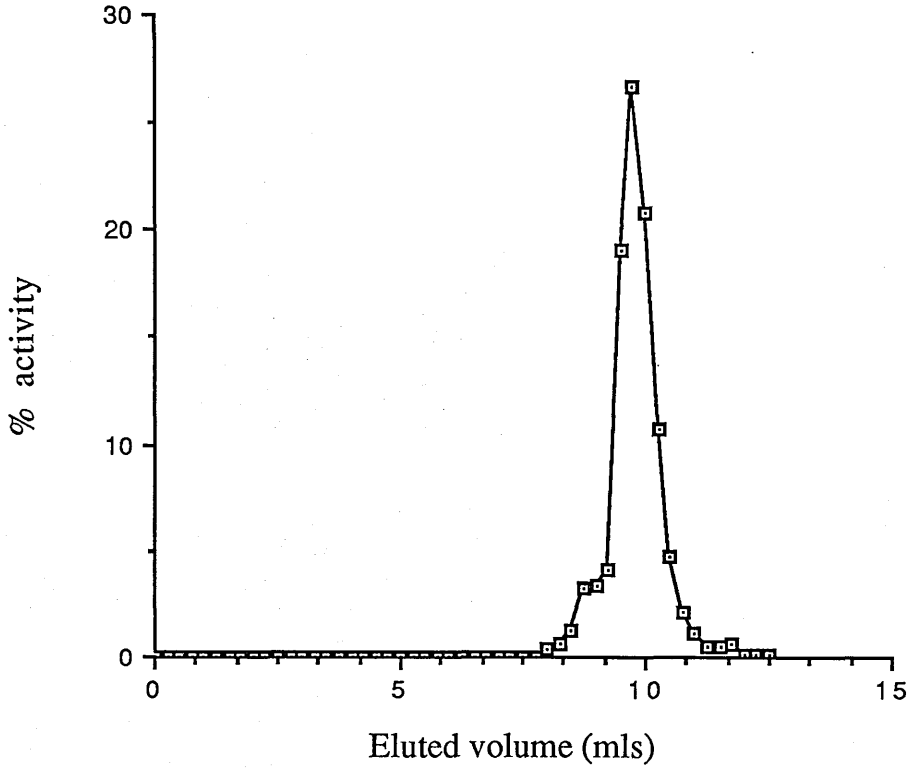


Figure 6.7. HPLC of serum with labelled MAb

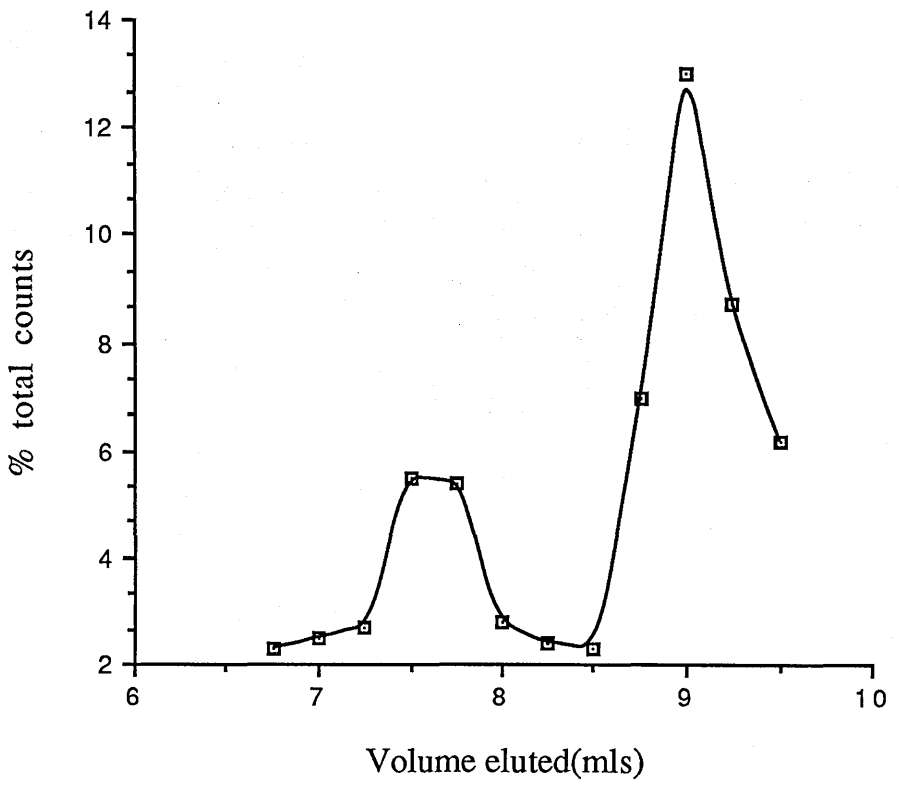


Figure 6.8. a) 1:1 cold to labelled MAb ratio

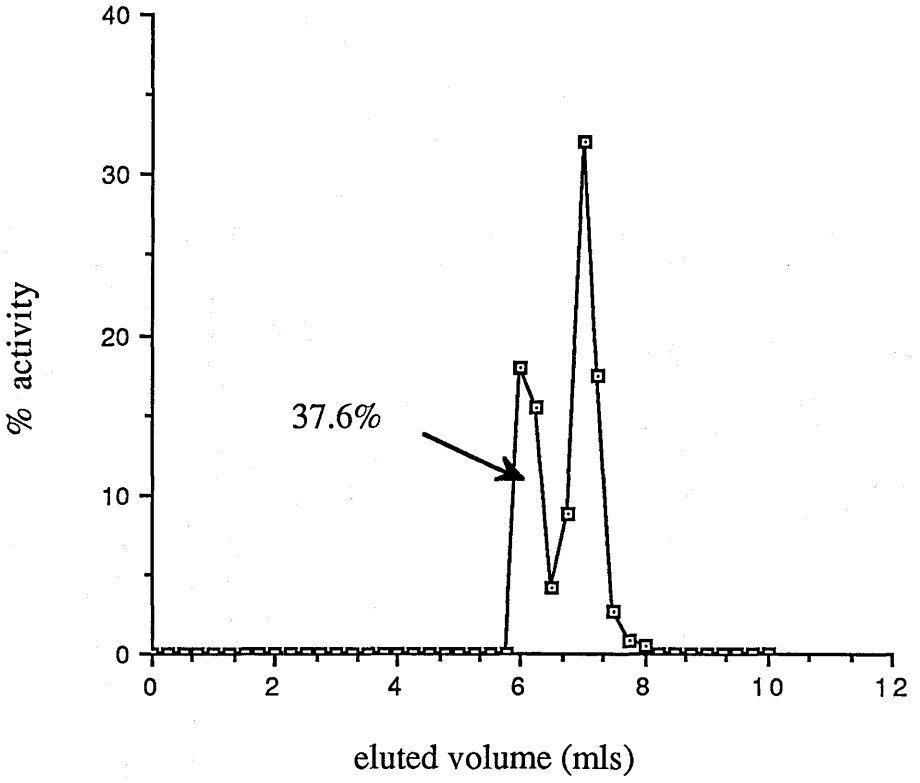


Figure 6.8.b) 3:1 cold to labelled MAb ratio

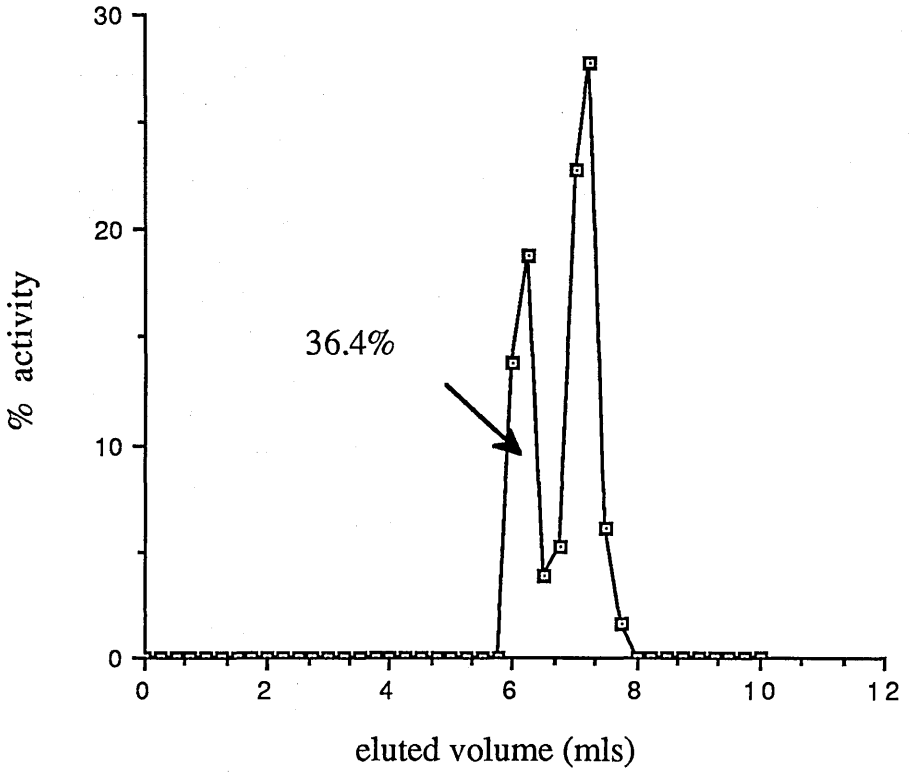


Figure 6.8. c) 10:1 cold to labelled MAb ratio

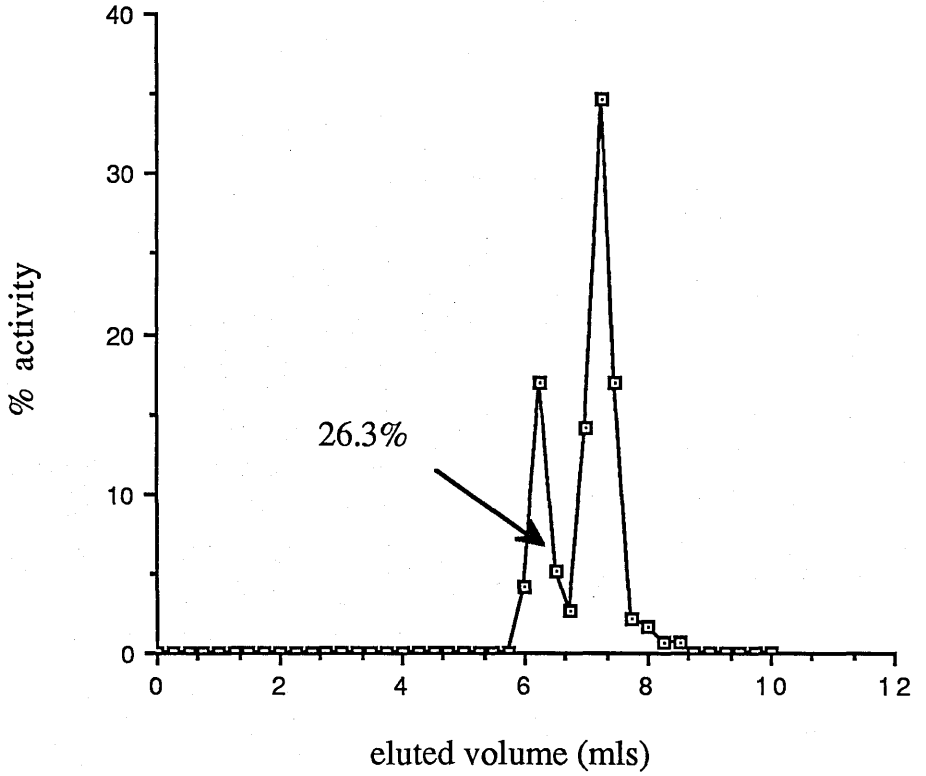
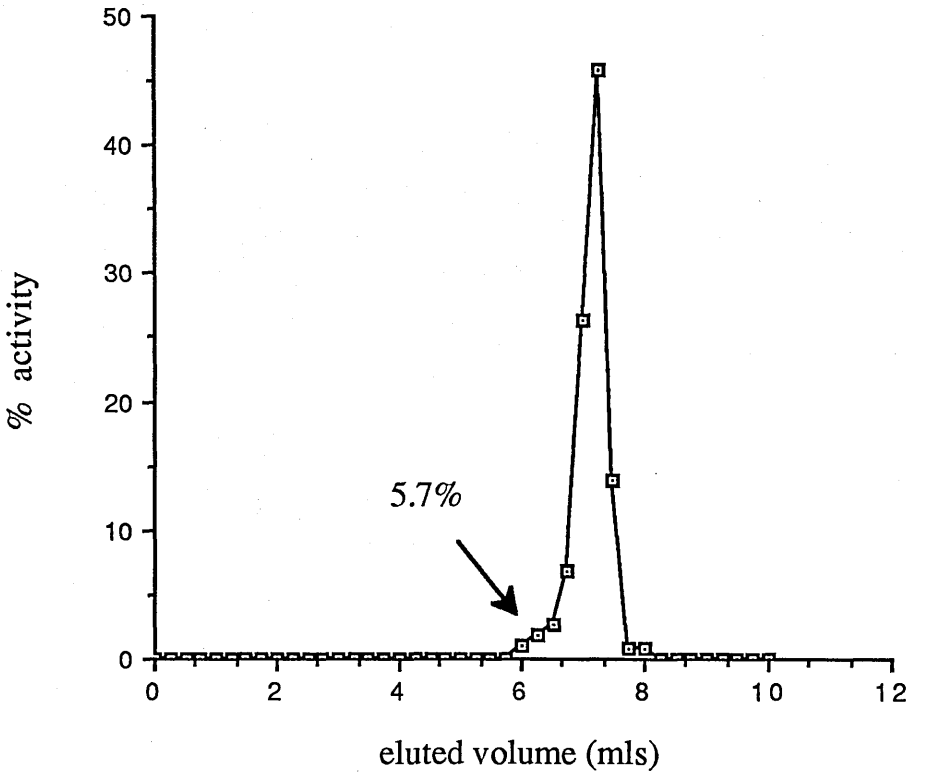


Figure 6.8. d) 100:1 cold to labelled MAb ratio



Serum analysis from imaging study patients

HPLC analysis of serum was carried out on 16 of the 22 patients studied with ^{111}In-ICR2. Prior to ^{111}In-ICR2 administration 31.5 (+/- S.D. 3.60) of the activity was associated with the high molecular weight complex. Following patient administration the activity associated with the high molecular weight peak decreased gradually with time.(Table 6.8. and Figure 6.9.) Five of the 6 patients given a 5mg dose of unlabelled antibody prior to ^{111}In-ICR2 administration had analysis of the high molecular weight complex after this step.(Figure 6.10.) Cold antibody administration reduced the mean level of high molecular weight complex from 34.2% (+/-S.D.4.66) to 30.2% (+/-S.D.1.92).

Circulating EMA levels

The mean EMA level measured in 15 of the patients in the present study of 544 (+/-S.D.340) ng/ml was not statistically different from 830 (+/-S.D.1462) and 714 (+/-S.D.609) for the colorectal cancer and control groups respectively, studied in Chapter 3. Three of the 15 patients had levels greater than 1000 ng/ml, of which one had extensive liver metastases in both lobes of the liver, and another had two synchronous cancers. Only one patient had a level less than 100ng/ml. This patient had a small tumour which failed to image with ^{111}In-ICR2 although the antigen was expressed by the tumour when examined on immunocytochemistry.

Figure 6.9. Clearance of immune complex

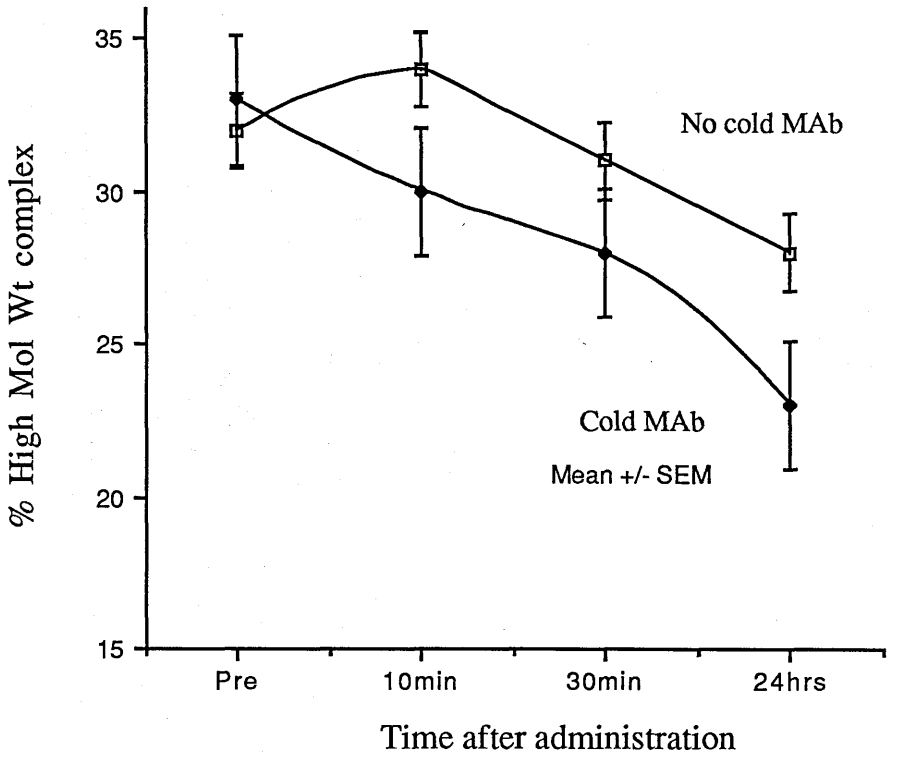
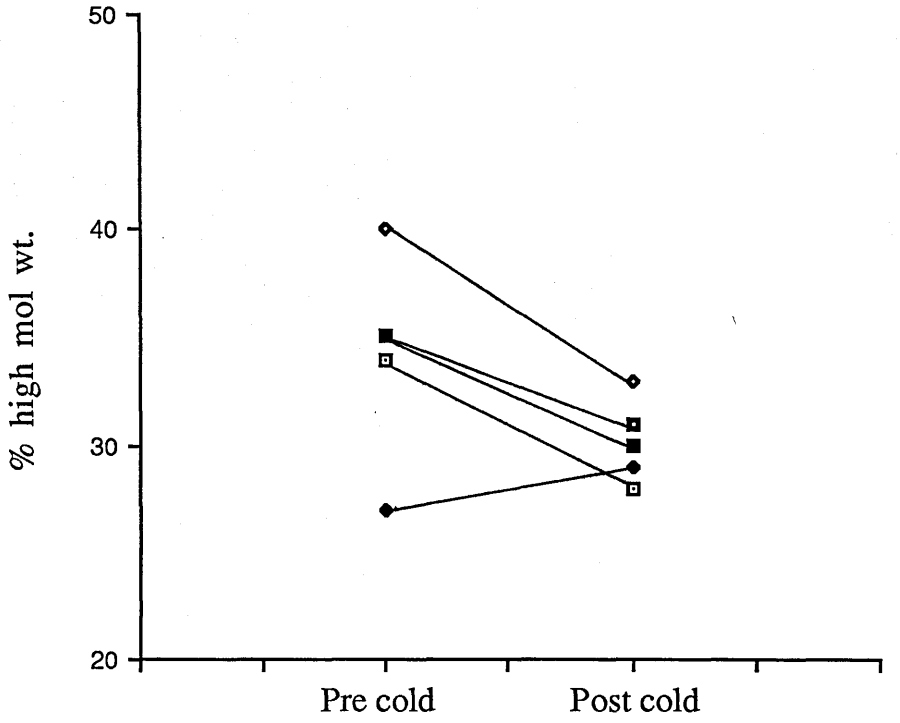


Figure 6.10. Immune complex following cold MAb



Since the % I.D. taken up in the tumours and the tumour to normal colon uptake ratios were not significantly different between the patient groups the EMA levels on all patients were pooled and compared with other measurements.

There was no significant difference in the blood clearance of activity within the first 48 hours of ^{111}In-ICR2 administration between patients with low (n=9, < 500ng/ml) or those with high (n=6, >500ng/ml) circulating EMA levels (Figure 6.11.). A positive correlation was, however, found between the EMA level prior to ^{111}In-ICR2 administration and the amount of immune complex subsequently forming in the circulation (n=11, r = 0.61, p <0.05) and the % of the injected dose taken up by the tumour (n=9, r=0.65, p= 0.05 (Table 6.9., Figures 6.12. and 6.13.).
No significant correlation was found between the circulating EMA level and the uptake of activity by either the liver or spleen (Table 6.9.).

Figure 6.11. : [EMA] and blood activity

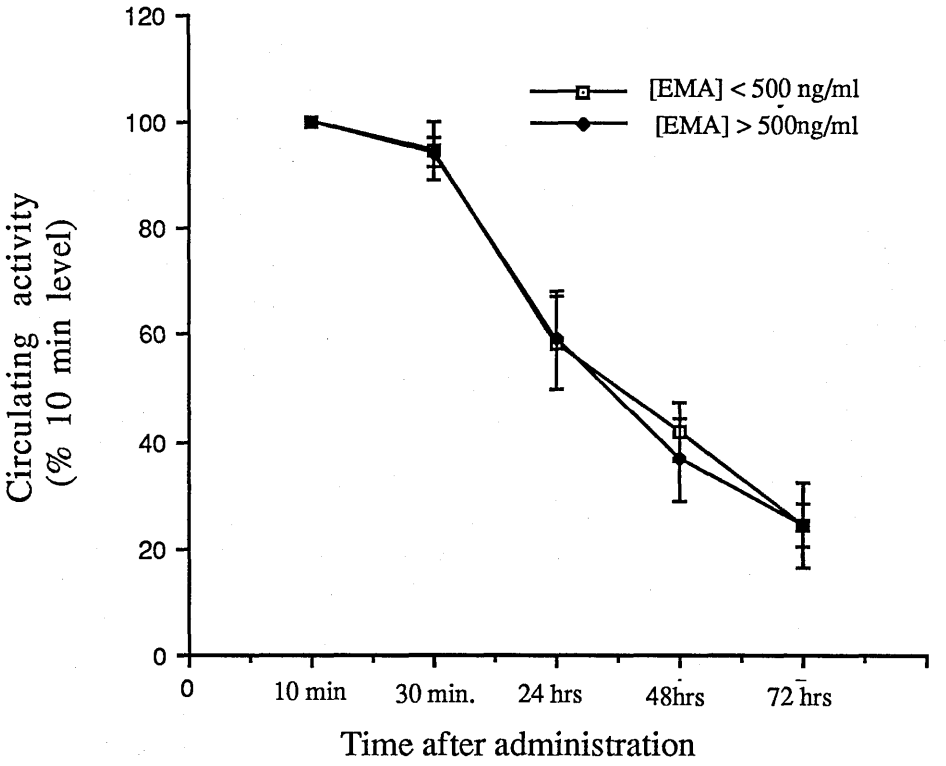


Figure 6.12. : [EMA] and immune complex formation

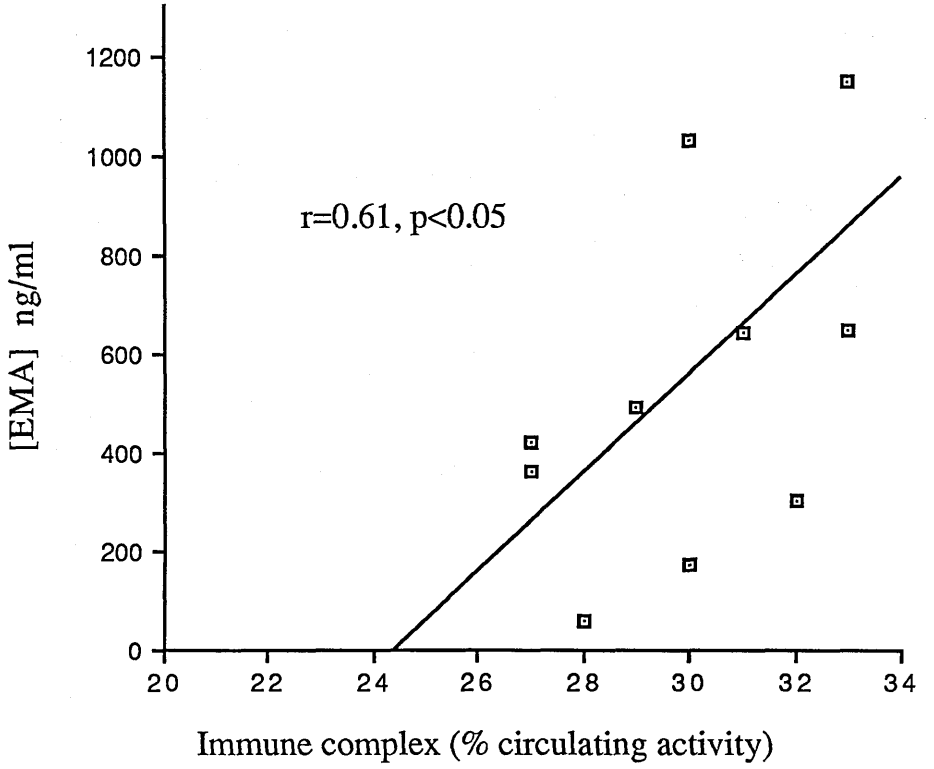


Figure 6.13. : [EMA] and tumour uptake

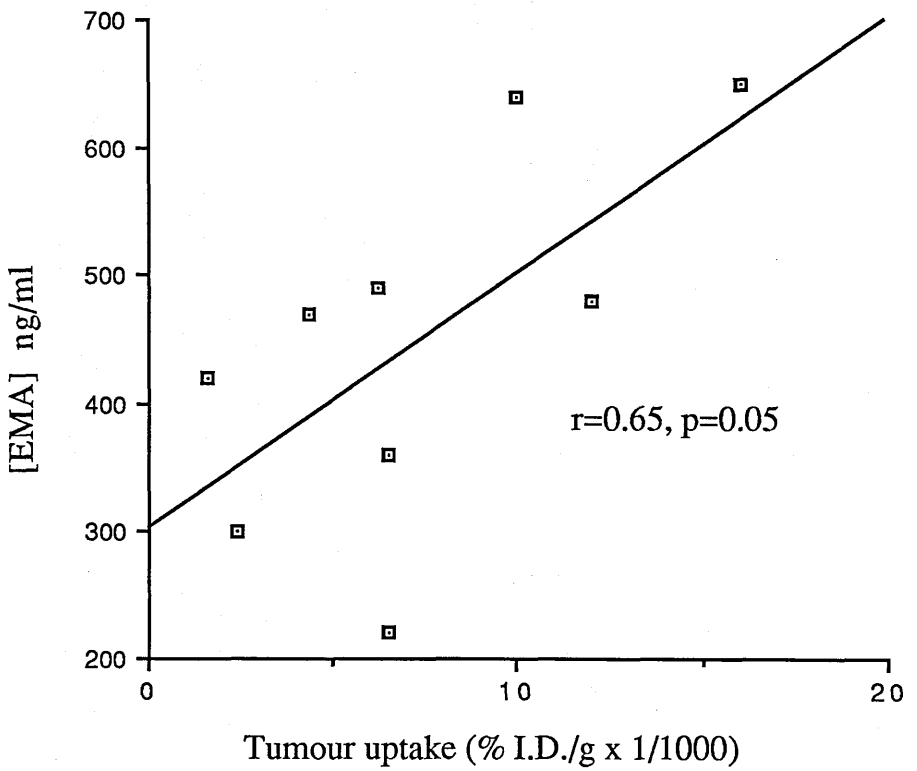


Table 6.9. Circulating antigen

No.	Initial	[EMA] ng/ml	% high mol wt complex*	Tumour %I.D./g**	Liver % I.D.	Spleen % I.D.
1.	M.D.	220	NA	6.5	25	NA
2.	M.B.	420	27	1.6	NA	NA
3.	R.S.	NA	NA	NA	23	NA
4.	R.N.	300	31	2.4	19	2.0
5.	D.N.	470	NA	4.3	18	3.7
6.	J.L.	490	29	6.2	20	1.9
7.	J.H.	480	NA	12.0	17	1.6
8.	B.T.	640	31	10.0	16	1.1
9.	J.P.	1150	33	NA	22	1.5
10.	K.H.	720	NA	NA	26	3.2
11.	M.J.	NA	NA	8.4	29	2.9
12.	B.B.	170	30	NA	23	2.6
13.	V.D.	650	33	16.0	25	2.8
14.	F.H.	60	28	NA	25	3.8
15.	B.S.	1030	30	NA	34	2.3
16.	R.D.	360	27	6.5	20	2.6
17.	J.P.	1150	33	7.2	25	1.2
18.	E.B.	NA	34	6.0	19	3.3
19.	D.O.N.	NA	27	9.0	26	2.2
20.	D.F.	NA	35	NA	27	2.4
21.	G.X.	NA	40	8.1	16	3.8
22.	J.B.	NA	35	12.0	24	3.5

* In samples taken prior to both cold and hot antibody administration.

** % I.D. x 10^{-3}/g

6.4. DISCUSSION

The patients studied in Chapter 5 (external imaging) were divided into three groups depending on whether they received 1mg of ^{111}In-ICR2 alone, had their injectate incubated with DTPA or were given, in addition, cold MAb prior to radiolabelled MAb administration. The purpose of these manipulations was to increase the tumour to normal colon ratio of uptake by reducing the administration of free ^{111}In (DTPA group) or by competitively inhibiting immune complex formation (cold MAb group) in order to improve the quality of images produced. Both the scan appearances and the tumour to normal colon ratios achieved, however, were not significantly different between the groups. In this chapter the effect was analysed of DTPA and cold MAb administration on the biodistribution of ^{111}In-ICR2 in these groups of patients.

A role for ^{111}In chelating agents for the scavenging of any free ^{111}In present with an ^{111}In labelled antibody preparation has been suggested on an empirical basis (243). Few animal studies have been carried out to investigate this problem and none which are directly relevant to the clinical situation. In 1986 Goodwin reported on the effect of the intraperitoneal injection of EDTA (5mg) commencing immediately after the administration of ^{111}In-DTPA-MAb to mice(280). This resulted in an increased urinary excretion of activity whereas in the present study the urinary excretion of activity was reduced in the patient group recieving

DTPA. This difference may be explained by the chelate being administered to the animal in Goodwin's study rather than to the labelled antibody prior to chromatography as in the present study which separated the chelated activity from the labelled antibody and allowed its removal prior to administration. The effect of administering 0.2mls of 30mmol DTPA 15 minutes before or 1 hour after administration of the labelled antibody on the normal biodistribution of ^{111}In-MAb in mice has also been investigated in a further study with no effect being found on the percentage of the injected dose taken up by the liver (283). The effect on biodistribution of the addition of chelate to the injectate prior to administration has not previously been investigated in patient studies.

In Chapter 4 a model of isolated rat hepatocytes and a tumour cell line established that the chelating agent DTPA reduced the uptake of activity from ^{111}In-MAb, a reduction in hepatocyte uptake of 40% being acheived by incubating the labelled MAb with a 0.5mM [DTPA] for 15 minutes prior to administration. This occurred without a significant reduction in uptake of activity by the tumour cell line. In the patient studies the labelled MAb was similarly incubated with 0.5mM [DTPA] for 15 minutes although prior to administration unbound activity was seperated and removed by gel chromatography.

The reduction in the early urinary excretion (first 12 hours after administration) found in the groups

receiving DTPA would suggest that a reduced amount of activity which is not firmly adherent to the antibody molecule has been administered, this activity normally being excreted in the urine. This would support the suggestion that the effects are mediated by the removal of non specifically bound ^{111}In from the MAb by the chelate.

The clearance of activity from the circulation was more rapid in the chelate group than with controls. Because of the removal of non specifically bound ^{111}In from the injectate in this group a smaller percentage of circulating activity will be in ^{111}In-transferrin form rather than stable ^{111}In-DTPA-MAb. The difference in the rates of clearance of radioactivity from the circulation may therefore reflect the relative rates of removal of labelled antibody by the reticuloendothelial system and ^{111}In-Tf by the sites of transferrin receptors.

Contrary to the result expected from the studies carried out in Chapter 4 the liver uptake as a percentage of the injected dose was significantly increased following DTPA administration to the injectate. As this group of patients will have less free activity in the circulation this would suggest that the uptake in the liver is related to the radiolabelled antibody and not the low molecular weight complex which is excreted in the urine.

The clinical findings did not correspond with the substantial reduction in the hepatocyte uptake seen in

the experimental situation (Chapter 4). This may reflect unavoidable differences in methodology. In the tissue culture studies both the chelate and the ^{111}In-MAb to which it had been added were subsequently incubated with the cells. In patient studies, however, gel chromatography was carried out after application of DTPA to the labelled antibody as explained previously. The presence of the chelate in the medium surrounding the cells may be required to produce a significant reduction in the cell uptake of radioactivity. This is likely to be the case if a constant dissociation of ^{111}In from the antibody bound chelate is occurring, a phenomenon which has been described as "chelate leak"(280). An additional factor making a comparison difficult is that the method for measuring the liver uptake of activity in patient studies gives the total uptake of activity. The presence of blood pool activity in addition to cellular uptake of activity may result in the differences in cellular uptake being disguised if blood pool activity is high.

The uptake of activity by the spleen was also significantly greater in those patients in whom DTPA was administered. This would suggest that this effect, as with the liver, is related to a reduction in the urinary excretion of activity rather than an increased uptake per se.

The significance of circulating antigen levels and the formation of immune complex on the quality and effectiveness of radiolabelled antibody imaging has not

been investigated adequately in human studies. Earlier reports suggested satisfactory imaging could be obtained with anti-CEA in the presence of high circulating levels of the antigen[97]. Many antibodies presently used for immunoscintigraphy are directed against secreted tumour associated antigens [235,309-312] and many studies have demonstrated that a large proportion of injected radiolabelled antibody forms immune complexes in the circulation [100,261,313,314]. The dose dependent effects on biodistribution and improved tumour targetting reported with the use of higher antibody dosages [305-307,315] may be related to the formation of immune complexes with the radiolabelled MAb. This relationship between tumour antigen, circulating antigen, immune complex formation and tumour uptake of activity appears to be complex. In animal xenografts Martin demonstrated an increased CEA secretory rate and serum CEA with increasing size of the tumour with the tumour CEA content and secretory rate per gram remaining constant[316]. In contrast Philben reported no difference in circulating CEA levels with tumour size and CEA content again using a mouse xenograft model [317] whilst in human studies a poor correlation between tumour CEA content and the level of plasma CEA was reported by Wagener[318].

It would appear that the correlation between tumour antigen expression and the circulating level is variable even for a particular antigen and may

therefore depend on the epitope being measured. An apparent contradiction also exists when considering the association between the level of circulating antigen and the tumour uptake of radioactivity following radiolabelled MAb administration. In the present study high levels of circulating EMA were shown to be associated with increased immune complex formation and increased uptake of radioactivity in the tumour. This has previously been reported in some studies investigating tumour CEA expression [317,319] but contradicts the findings of Hagan [264] in which high levels of circulating antigen were associated with increased levels of circulating immune complex but reduced tumour uptake of radioactivity. The latter study also demonstrated a high liver uptake of activity which was explained by the clearance of increased immune complex by the reticuloendothelial system. These differences in biodistribution may be explained by variation in the immunogenicity of the immune complex formed and hence the likelyhood of its clearance by the RES. Why tumours with high antigen content, secretion and circulating levels should image satisfactorily in the presence of high levels of immune complex may be explained if the images produced are related to a sequestration of complex in the tumour or an exchange of radiolabelled MAb between the circulating immune complex and the tumour bound antigen.

The rationale behind the administration of cold MAb prior to labelled MAb injection may at first appear

logical although the interactions involved are poorly understood and have scarcely been investigated. Cold MAb will bind with circulating antigen and reduce the immune complex formed with radiolabelled MAb allowing more of the labelled antibody to be available for binding with the target antigen on the tumour tissue. On this rationale Carrasquillo and his colleagues [243] pre-administered varied doses of cold MAb to patients with melanoma prior to administration of radiolabelled MAb and reported improvements in the quality of pictures obtained when higher dosages of cold MAb were administered. This observation was not, however, supported by any objective evidence that a higher percentage of the injected radioactivity was present in the tumour tissue or that the background uptake of radioactivity was reduced. Caution is required to ensure that the cold antibody is not only preventing immune complex formation with the radiolabelled antibody but blocking its binding to the target antigen on tumour tissue.

The HPLC analysis on cold serum in the present study suggested that immune complex formation could indeed be inhibited with the addition of cold MAb and the specificity of the antibody / antigen reaction was demonstrated by the inability of a isotype matched antibody to effect its formation (Personal communication, J Babich, Institute of Cancer Research, Sutton , Surrey). The dose of cold MAb administered in the patient studies (5mg) was an

arbitrary choice which was considered likely to produce a significant and analysable effect on biodistribution without swamping the antigen expression of the tumours. In contrast to the results of Carrasquillo [243] these patients showed no obvious improvement in the quality of their scans. In addition no significant difference was found in the tumour to normal colon ratio in these patients when compared to those of controls.

With a 5mg dose of cold antibody a mean reduction of only 4% in the amount of circulating immune complex was achieved and no significant difference in the rate of blood clearance of activity was established. The reduction of RES uptake of radioactivity which would have been expected due to the receptor mediated removal of immune complex was supported by a significant reduction in the liver uptake of activity at 48hrs although surprisingly the spleen uptake of radioactivity has increased rather than decreased. This may suggest that rather than avoiding the formation of circulating immune complex and hence RES uptake of radioactivity that the cold MAb has in fact "primed" the RES system to clear immune complex.

In conclusion the analysis of biodistribution in these patients would suggest that tumour uptake of radioactivity is increased by high levels of circulating immune complex and that this is directly related to the level of circulating antigen. Although DTPA treatment of the injectate reduces the amount of free ^{111}In in vivo and hence the urinary excretion of

activity this is not reflected by an improvement in scan quality or in a reduction in the uptake of radioactivity by the liver. Cold MAb administration prior to radiolabelled MAb has been shown to produce a reduction in the level of circulating immune complex although large doses would be required to prevent its formation. The reduction in immune complex produced in the present study was not, however, associated with an increased uptake of activity in tumour tissue or tumour to normal colon ratio.

CHAPTER 7

INTRA-OPERATIVE RADIOIMMUNOLOCALISATION

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c) Results

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7.2. THE LIMITATIONS OF INTRA-OPERATIVE RADIOIMMUNOLOCALISATION

a) Introduction

b) Materials and Methods

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7.1. : PATIENT STUDIES ON INTRA-OPERATIVE RADIOIMMUNOLOCALISATION

a) Introduction

In Chapter 1 (Section 1.3.iii.a) the use was described of a gamma radiation detecting probe(GDP) for the intra-operative(I/O) radioimmunolocalisation(RIL) of colorectal cancer deposits following their uptake of radiolabelled antibodies.

The feasability of this technique was originally described by Aitken and his colleagues in 1984 (147) who used a CEA secreting xenograft and an ^{131}I labelled anti-CEA antibody to demonstrate that elevated counts could be recorded over the tumour with the gamma detecting probe. Having established the technique with the CEA secreting xenograft Aitken carried out I/O probing in a patient with a colorectal cancer following the administration of ^{131}I labelled anti-CEA. Unfortunately the counts detected over the tumour were low as was the tumour to normal colon (T/NC) ratio.

A series of patients with both primary and recurrent colorectal cancers was then investigated using ^{131}I labelled anti-CEA . In this study higher T/NC ratios were obtained with a mean T/NC ratio of 3.97:1 in primary and 4.18:1 in recurrent cancers. These figures were not compared, however, with the actual uptake as measured by the gamma well counting of biopsies and no clinical benefit to these patients was established (148).

It was apparent from these preliminary studies that the results were limited by a high background radioactivity and low T/NC ratio of counts. O'Dwyer attempted to overcome these problems by the use of the MAb 17-1A, an alternative target antigen expressed by colorectal cancers. In addition the use of $F(ab')_2$ fragments was explored in an attempt to overcome the problem of Fc receptor mediated clearance of radiolabelled antibody and an alternative radiolabel, ^{125}I, due to its long half life(60 days) which allows time for the clearance of background radioactivity prior to probing. Despite these modifications the mean T/NC ratio obtained with the probe in this study was only 3.4:1 with whole antibody and 2.3:1 with fragments, values which were significantly less than those found from gamma well counting of biopsies. Despite these problems results obtained using the GDP contributed to the intra-operative management in three of the 18 patients studied [149]. Similarly use of the GDP altered the management in 6 of the 31 patients studied by Sickle-Santanello and colleagues using ^{125}I labelled MAb B72.3 [150], which recognises the Tag 72 antigen. Other recent reports on I/O probing using ^{125}I labelled B72.3 have shown that the intra-operative findings may be correlated with the results obtained by gamma well counting of excised tissue [320] and that the same technique may be applicable to other cancers [321]. Furthermore the use of I/O probing following administration of ^{125}I-B72.3 has been shown to localise

colorectal cancer deposits not detectable by other means including barium studies, CT scanning and exploratory laparotomy (322).

The best results obtained so far with I/O RIL have employed the MAb B72.3 which recognises the target antigen Tag 72. This antigen is expressed by the majority of adenocarcinomas and by a variety of normal tissues, particularly the breast. The similarity of the distribution of this target antigen with EMA would suggested that ICR2 may be suitable for the I/O RIL of colorectal cancers.

The radionuclides ^{131}I and ^{125}I have been used in previous clinical studies. Because of its favourable energy of gamma emissions, lack of beta emissions and suitable half life, ^{111}In, a radionuclide frequently used for external immunoscintigraphy but not previously investigated for I/O radioimmunolocalisation has been selected for antibody labelling in this study.

The two main types of radiation detector available for intra-operative use have been investigated in both the clinical and experimental evaluation of I/O RIL. These probes, which have been described in Section 1.3.iii.b), utilise crystals of either NaI or CdTe.

The clinical study carried out in part 1 of this chapter investigates patients with suspected primary or recurrent colorectal cancer.

Patients, materials and methods

Sixteen of the patients with primary colorectal tumours on whom external imaging had been carried out following the administration of ^{111}In-ICR2 (see Chapter 5) were investigated using the GDP at the time of surgery . The clinical details of the patients and the findings on external imaging are shown in Table 7.1. The radiolabelled antibody was given 3-6 days prior to surgery and was prepared and administered as described in Appendix 5.4.

Intra-operative probes

Two types of radiation detector were used for the intra-operative detection of gamma emissions following the administration of ^{111}In-ICR2. A NaI(Tl) scintillation detector with a crystal (Harshaw) 26mm diameter and 20mm thick connected to a photomultiplier tube (PMT, EMI) and collimator designed in-house for the intra-operative detection of ^{111}In was used in 11 patients with colorectal cancer (Figure 7.1.). Subsequently a CdTe semiconductor probe designed for surgical use was used in a further five patients (Radiation Monitoring Devices, Watertown, Massachusetts. Figure 7.2.)

Intra-operative counting

At the time of surgical resection, counting was carried out with the gamma detecting probe in a sterile instrument drape. After opening the abdomen but prior

Table 7.1. Patient details and results of external imaging

No.	Init.	Age	Sex	Diagnosis	Site	Dukes	Imaging
1.	M.D.	60	M	Ca	R/S	B	+
2.	M.B.	83	F	Ca	R/S	B	N.A.
5.	D.N.	51	M	Ca	Hep flex	B	+
6.	J.L.	71	M	Ca	Rectum	C	+
7.	J.H.	79	F	Ca	Trans	B	-
8.	B.T.	58	F	Ca	Rectum	B	+
11.	M.J.	67	F	Ca	Sigmoid	C	+
12.	B.B.	54	F	Ca	Rectum	C	+
13.	V.D.	74	F	Ca	Sigmoid	B	+
14.	F.H.	76	F	Ca	Sigmoid	B	-
16.	R.D.	64	M	Lipoma	Sigmoid	-	-
17.	J.P.	76	F	Ca	Splen flex	B	-
18.	E.B.	73	F	Adenoma	Caecum	-	+
19.	D.O.N.	73	F	Ca	Sigmoid	C	+
21.	G.X.	67	M	Ca	R/S	C	+
22.	J.B.	56	M	Ca	Sigmoid	B	+

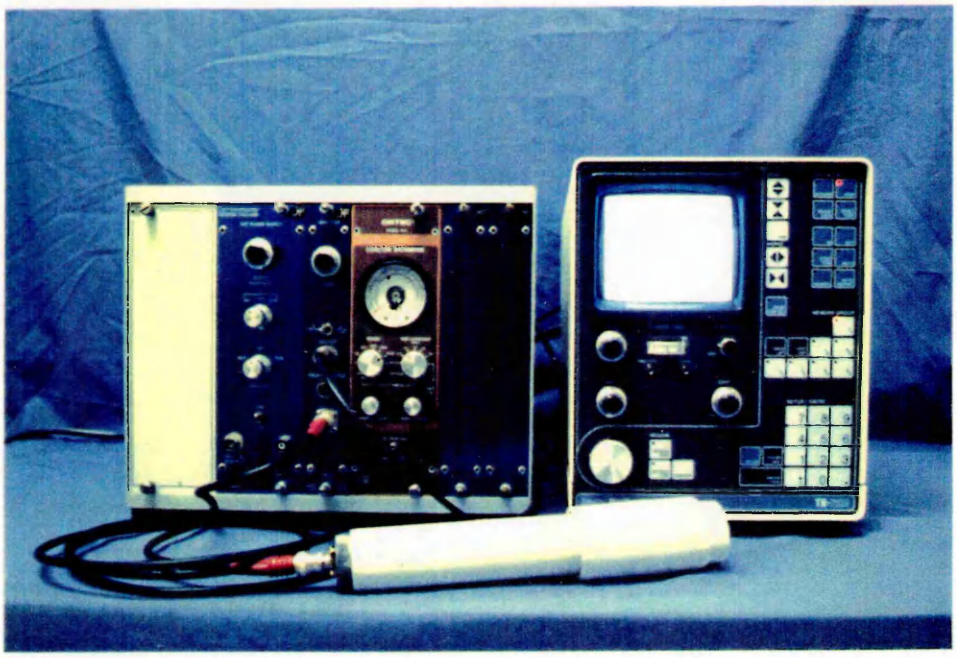
R/S : rectosigmoid

Hep flex : hepatic flexure

Splen flex : splenic flexure

Trans : transverse colon

Figure 7.1. Sodium Iodide (NaI) probe



The Na I probe is shown connected to a multichannel analyser via a power supply and signal amplifier.

Figure 7.2. : The Cadmium Telluride (CdTe probe)

The CdTe probe is shown with interchangeable collimators and ratemeter.

to mobilising the tumour, counts were taken for twenty seconds and repeated three times on the right and left sides and anterior to the tumour. The probe was held stationary, flush with the tumour and at right angles to the surface. The twenty second count period could be preset automatically with both detector systems and the number of gamma emissions recorded over this period displayed numerically.

Two seperate sites were used for the counting of normal colon, three counts being recorded at both sites for a period of twenty seconds. Having mobilised the primary tumour on its mesentery this was palpated for the presence of enlarged lymph nodes suspicious of containing metastatic tumour. Any nodes palpated were counted with the gamma detecting probe, again triplicate counts being taken over a twenty second period. Lastly the liver was probed with triplicate 20 second counts being recorded over the right and left lobes.

Probing the resected specimen

In order to quantify the effect that background and specific organ uptake had on the measurements obtained by intra-operative probing, the specimen of tumour and normal colon was again probed after it had been removed from the patient.

Immediately after resecting the specimen it was washed to remove blood and any remaining bowel contents then orientated anatomically on a flat surface. The

sites at which the tumour was scanned intra-operatively were then located and re-counted for three 20 second periods.

In four patients the resected specimens were probed at identical sites with both the NaI and CdTe probes. Triplicate counts over a twenty second period were taken using each detector to allow the results obtained to be compared directly.

Comparison of probe results with other data

The results obtained on intra-operative probing were expressed as the ratio of the mean counts obtained over the tumour to the mean counts measured over normal colon (T/NC ratio). Similarly the counts obtained over the tumour and the normal colon for the resected specimen were expressed as a ratio of the mean counts (T/NC ratio). These results were compared, for each patient, with the ratio of uptake calculated from the counts obtained per gram of resected tissue by weighing and gamma well counting biopsies from both the tumour and the normal colon. A comparison was also carried out between the intra-operative T/NC ratios and the % I.D./g in tumour tissue calculated using a standard as described in Chapter 5 (5.2.iii.b, p160) . Finally the T/NC ratios obtained were compared with the antigen expression of the resected tumours as measured by immunocytochemistry. Statistical comparisons were made by the students t test.

Results

To allow the tumour to normal colon (T/NC) uptake ratios for both the NaI and CdTe probes to be grouped together for comparison with other data the results were compared between the two groups of patients. No significant differences were found in the T/NC ratios found at operation, on the resected specimen or on the gamma well counting of biopsies. In addition when the same resected specimens were probed with both the CdTe and NaI probe the counts obtained with the latter were significantly higher than with the former (151+/-S.D.60 against 1215+/-S.D.602, p=0.03) but the T/NC ratios were not significantly different (Table 7.2, 7.3., 7.4.) T/NC ratios for both probes was therefore considered together for comparison with other data.

Of the 16 patients studied 14 had primary colorectal cancers, two with liver metastases and two had benign colonic tumours, a large sigmoid lipoma in one patient and a large dysplastic tubulovillous adenoma of the caecum in the other. The intra-operative T/NC ratio was greater than 1.5:1 in 9 of the 14 patients with cancer (64%), in whom the mean ratio was 1.83 (+/-S.D.0.21) : 1 (Table 7.2.). The two patients with benign tumours showed no preferential localisation of activity with T/NC ratios of 0.96:1 and 1.06:1.

A poor correlation was found between intra-operative tumour to normal colon ratio of counts and those obtained by gamma well counting, although patients with high intra-operative ratios tended to have higher well

Table 7.2. Results of intra-operative probing

No.	Init.	Probe	T/NC ratios			% I.D. /g
			I/O	Specimen	Well count	x 10^{-3}
1.	M.D.	NaI	1.66	4.50	3.2	6.5
2.	M.B.	NaI	1.98	5.13	3.4	1.6
5.	D.N.	NaI	NA	1.23	1.0	NA
6.	J.L.	NaI	1.64	4.00	2.0	6.2
7.	J.H.	NaI	1.81	2.73	1.2	12.0
8.	B.T.	NaI	1.66	4.45	2.1	10.0
11.	M.J.	NaI	2.13	5.87	NA	NA
12.	B.B.	NaI	2.00	6.82	3.5	8.4
13.	V.D.	NaI	0.90	4.22	1.6	16.0
14.	F.H.	CdTe	1.00	6.25	2.3	NA
16.	R.D.	CdTe	0.91	3.40	1.6	6.5
17.	J.P.	CdTe	1.32	4.90	2.3	7.2
18.	E.B.	CdTe	1.06	2.70	1.4	6.0
19.	D.O.N.	CdTe	2.06	3.35	1.9	9.0
21.	X.G.	NaI	1.57	5.15	3.0	8.1
22.	J.B.	NaI	1.07	5.69	2.3	12.0

T/NC ratios : Ratio of mean counts from tumour to that of normal colon

I/O : intra-operative

Table 7.3. Comparison of T/NC ratios in probe groups

	T/NC ratio by probe utilised		
	NaI probe(n=10)	CdTe probe(n=5)	Difference
Well counting	2.33+/-0.90 :1	1.9+/-0.41 : 1	n.s.
I/O probing	1.64+/-0.39 :1	1.27+/-0.47 : 1	n.s.
Excised specimen	4.53+/-1.54 :1	4.12+/-1.44 : 1	n.s

Table 7.4. Dual probing of the same resected specimens

	CdTe probe(cpm)			NaI probe(cpm)		
Specimen No.	Tumour	Colon	T/NC*	Tumour	Colon	T/NC*
1	150	24	6.3	1176	180	6.5
2	78	22	3.5	603	237	2.5
3	225	43	5.2	2040	420	4.9
4	162	15	10.8	1041	183	5.7

Probe counts are the mean values of at least three determinations

* Paired t test, p=0.302, n.s.

counting ratios. This is shown diagramatically in Figure 7.3. Of the four patients who had I/O probing carried out at six days after ^{111}In-ICR2 administration two showed no preferential uptake (T/NC of 0.90:1 and 1.00:1) whereas only one of the 12 patients probed at three days showed no preferential uptake (1.07:1). This difference did not, however, achieve significance.

In all patients a higher T/NC ratio was obtained on probing the resected specimens than that found intra-operatively with a mean T/NC for the resected specimen of 4.44 (+/-S.D.1.37) : 1 against 1.51 (+/-S.D.0.44) : 1 for intra-operative probing (p=0.000). The mean T/NC ratio found on probing the resected specimen of 4.44 (+/-S.D.1.37) was also higher than that found on weighing and gamma well counting of biopsies of tumour and normal colon from the resected specimen. The resected specimen tumour to normal colon ratios found on probing correlated well with the T/NC ratio calculated by weighing and gamma well counting of biopsies of tumour and normal colon from the resected specimens (Figure 7.4.) ($r = 0.8$, $p<0.001$).

In all patients studied the counts obtained on probing over the liver were significantly greater than those obtained over either the tumours or the normal colon (all $p<0.05$). The mean probe counts over the liver to that of tumour was 2.48 (+/-S.D.1.10) : 1 and to that of normal colon was 3.83 (+/-S.D.0.98) : 1. Of the two patients in the present study with liver metastases one was probed directly over the site of a

Figure 7.3. Intra-operative probing and well counting

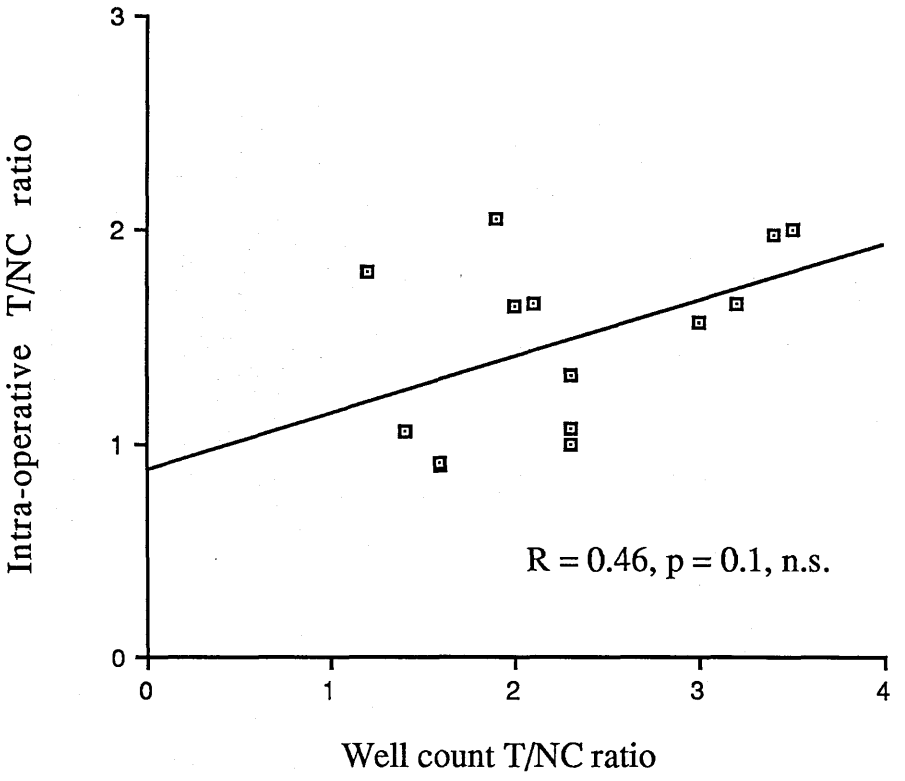
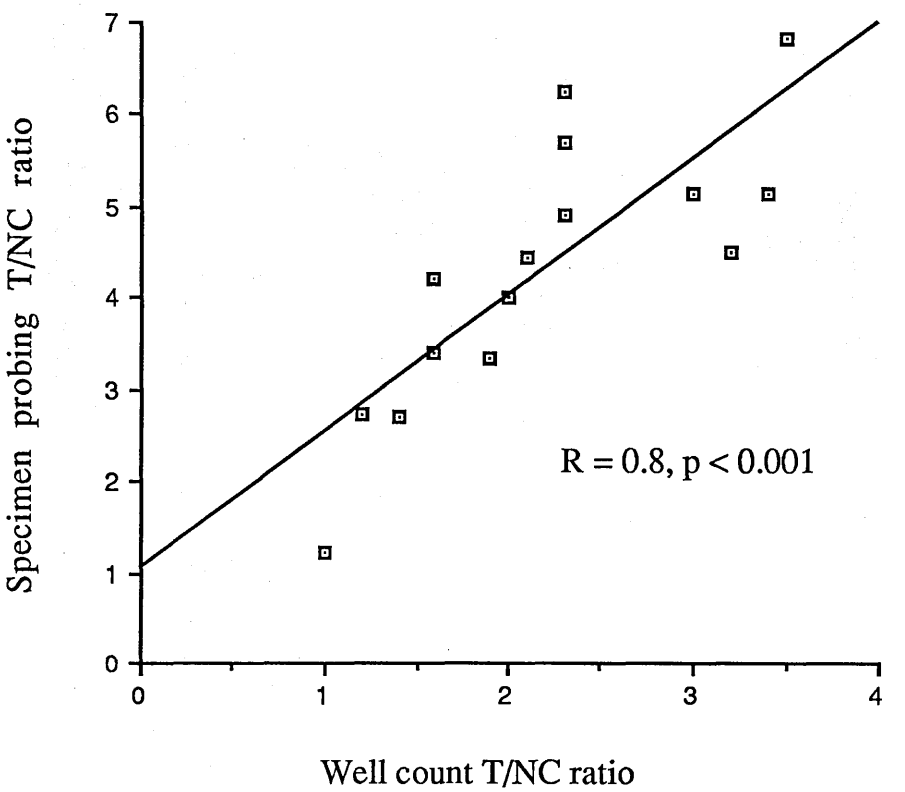


Figure 7.4. Specimen probing and well counting



liver metastasis and in the other the site of the metastasis was not accessible . The mean count over the metastasis was less than that over the normal liver but not significantly so (5100+/-S.D.430 vs 5920+/-S.D.483)

Intra-operative probing of a regional lymph node was carried out in 7 patients who had an easily palpable node in the mesentery. None of the probed nodes had clinically obvious tumour involvement and all were less than 1cm in diameter. Two of the seven nodes probed were subsequently shown on histological examination to contain metastatic tumour. The two lymph nodes containing tumour had a significantly higher uptake of radioactivity relative to normal colon than the five which did not (1.71+/-S.D.0.06:1 against 0.84+/-S.D.0.33:1, $p < 0.05$).

The antigen expression of the resected specimens measured by immunohistochemistry using the indirect immunoperoxidase method demonstrated EMA expression in 12 of the 16 resected tumours and in all regional lymph nodes involved by metastatic deposits. The remaining four resected specimens showed either no EMA expression or a focal pattern of staining alone. The T/NC ratios found on gamma well counting of biopsies were significantly higher with those tumours which expressed the target antigen than those which did not or showed focal staining alone (2.45+/-S.D.0.65 : 1 against 1.30+/-S.D.0.26 : 1, p=0.005). The correlation between antigen expression and T/NC uptake ratios was demonstrated in Table 5.5..

Discussion

In this study 85% of the patients with colorectal cancer had elevated counts obtained over primary cancer compared to that of normal colon as measured by in vivo probing following administration of ^{111}In-ICR2 and in 65% of patients the T/NC ratio was greater than 1.5:1. In a similar study carried out by Sickle-Santanello and colleagues intra-operative probing resulted in a T/NC ratio of greater than 1.5:1 in 83% of the patients studied using the B72.3 MAb raised to the tumour associated antigen Tag-72 and labelled with ^{125}I(150). This similarity in the proportion of cancers showing uptake of the radiolabelled MAb may be explained by the antigen having a similar distribution to that recognised by ICR2 and a similar reactivity with colorectal cancers (J.P. Sloane, unpublished observation, [248, 323, 324]). In the present study, however, false negative results on probing could not be explained by a lack of antigen expression on the cancers as suggested in the study by Sickle-Santanello, as the three tumours showing no preferential uptake expressed EMA (all grade II) on immunohistochemistry.

The time between administration of ^{111}In-ICR2 and intra-operative probing may be important as a tendency was noted for low T/NC ratios to occur more often at 6 rather than three days. From the studies reported in Chapter 5 the time which was optimal for external imaging was found to be 48-72 hrs after administration of ^{111}In-ICR2 at which time satisfactory counts were

obtained and the blood activity had cleared. A wide range has been reported for the optimum time period for colon cancer localisation with ^{111}In labelled MAb (102,105,294) and this may vary with the dose of antibody or radioactivity administered, the nature of the antibody and the particular epitope of the antigen being targeted.

A possible explanation for cancers which express the target antigen on immunohistochemistry not showing specific uptake on probing is that the labelled antibody clears from the tumour tissue due to the turnover of the membrane antigen. This possibility is supported by comparing the results of the external imaging study with that of the intra-operative probing, two of the three patients showing no preferential uptake on probing at six days (T/NC ratio approximately 1) having previously had positive tumour images on external scanning at 24 and 48 hours after administration.

Metastatic tumour deposits were found on histological examination in two of the 7 regional lymph nodes which were probed intra-operatively. The lymph nodes which were involved with metastatic tumour had a significantly higher node to normal colon ratio than those which did not contain metastases. These nodal metastases were less than 1 cm in diameter which is smaller than the tumour deposits reported as being detectable by external immunoscintigraphy (105,315,325,326). Although these metastases were

within the field of surgical resection and hence their detection did not affect the clinical management for that particular patient this finding is of significance since it indicates that tumour deposits which would not be detected by other means can be localised by this technique. As the antigen expression of metastatic and recurrent deposits corresponds to that of the primary cancer (154) the uptake of ^{111}In-ICR2 in tumour tissue demonstrated in the present study is that which would be found in patients undergoing second look laparotomy for suspected recurrent colorectal cancer. It is in this group of patients that intra-operative radioimmunolocalisation has shown greatest clinical benefit (149, 150, 322).

The uptake of ^{111}In-MAb in normal liver tissue was found to be greater than for liver metastases and it may be concluded that ^{111}In labelled MAb's are not likely to have a role in the detection of tumour deposits in the liver. The mechanism of uptake of radioactivity by the liver following administration of ^{111}In-MAb was discussed in Chapter 4 and accumulation in the liver appears to be independent of the MAb used or the antigen targeted. The uptake may be related to instability of the ^{111}In-DTPA-MAb complex as newly developed ^{111}In chelating agents give a reduced uptake of activity by normal liver tissue. Whether this reduction in background will be sufficient to allow the detection of liver metastases by intra-operative

probing remains to be established (286, 291, 327). More favourable results for detecting liver metastases intra-operatively have been obtained following the administration of MAbs labelled with radionuclides of Iodine. Because of the rapid dehalogenation in liver tissue the radioactivity taken up by normal tissue clears rapidly and allows a positive differential in counts to be obtained between tumour and normal liver (150).

In addition to undergoing rapid dehalogenation and clearance from normal liver substance, ^{125}I has the advantage of a long half-life of 60 days thus allowing background activity to clear from the liver and other organs prior to intra-operative probing. This effect may explain the higher T/NC ratios found in studies using ^{125}I than those found in the present study with ^{111}In (149, 150, 321, 322). While higher ratios may be achieved due to clearance of background radioactivity fewer counts will be obtained over both tumour and normal colon due to radioactive decay and dehalogenation so that the statistical significance of the ratio of counts will be reduced. The importance of applying nuclear counting statistics to the interpretation of intra-operative probe counting is discussed and described in part b) of this chapter.

An alternative radionuclide which may be suitable for intra-operative radioimmunolocalisation is ^{99m}Tc. This radionuclide has a short half life of 6 hours which would allow a larger dose to be administered and

higher count rates to be obtained over the tumour tissue as long as adequate tumour uptake of the radiolabelled antibody occurs prior to intraoperative probing and decay of the radionuclide.

The use of ^{99m}Tc has been suggested for labelling MAbs although no studies on intra-operative RIL with this radionuclide have as yet been carried out. A major advantage to its use is that the energy of gamma emissions would allow both pre-operative external imaging and intra-operative probing to be carried out on the same patients with a single administration of ^{99m}Tc labelled MAb. The localisation of the ^{99m}Tc labelled MAb to the tumour deposits would, however, require to be rapid due to the six hour half life of the radionuclide.

Alternative approaches which have been investigated for improving external immunoscintigraphy have been directed at reducing the background uptake of radioactivity and these may also have a role to play in intra-operative probing. For example the radiolabelled MAb may be cleared from the circulation once tumour localisation has occured by administering a liposomally bound antibody directed against the first which forms a circulating complex which is cleared by the reticuloendothelial system (133).

Another technique which has recently been investigated makes use of the exceptionally high affinity of strepavidin for biotin (300-302). In this procedure MAb conjugated to streptavidin is

administered and then the non tumour bound activity is allowed to clear prior to administration of radiolabelled biotin. This technique may result in lower background radioactivities and higher T/NC ratios and would be particularly suitable for radionuclides such as ^{99m}Tc with a good energy of emissions but whose short half life may otherwise prohibit their use as a radiolabel for MAbs.

The NaI probe used in the present study was adapted from other clinical applications . Its large size and the weight of its lead shielding made it difficult to use at operation. It also needs a high voltage for its operation and required to be connected to the mains power supply. A further disadvantage to its clinical use is that the NaI crystal is sensitive to variations in temperature producing difficulty for the cleaning and sterilisation of the probe.

The CdTe probe has advantages in several of these areas. It is small, light and manoeuvrable and therefore easy to us at the time of operation. It also works with a rechargeable 12 volt power supply increasing its safety of use and making it completely portable. The probe does, however, require to be sterilised by ethylene oxide.

In addition to these practical considerations as to which probe may be most easily used at the time of operation the sensitivity of the detectors and the ability of the collimation (the shielding around the aperture) to avoid uptake of scattered radioactivity

are important to the results obtained.

In the direct comparison of the two probes on the same resected specimens the counts obtained with the NaI probe were significantly higher than those with the CdTe probe although the T/NC ratios were not significantly different. The small number of specimens examined, however, did not allow the probes to be adequately compared and the question of which probe is best suited to the detection of small tumour deposits with ^{111}In-MAb was therefore addressed in the following section using a phantom model.

7.2. THE LIMITATIONS OF INTRA-OPERATIVE RADIOIMMUNOLOCALISATION

7.2.a) Introduction

The preceding clinical study on the intra-operative use of the gamma detecting probe showed that the majority of primary tumours and some lymph node metastases less than 1cm diameter could be localised following the injection of ^{111}In-ICR2. As in previous studies on intra-operative RIL no clinical benefit was established for patients with primary colorectal cancers although the results provide an indication to the likely use of the radiolabelled MAb in patients with suspected recurrent disease.(149,150,322).

Some of the possibilities for improving the results of intra-operative probing have been suggested at the end of the preceding section. Although these may lead to improved clinical results no studies have considered the limitations of intra-operative radioimmunolocalisation or considered the necessity for statistical analysis to the probe counting data. To investigate the theoretical limitations of I/O RIL a model has been constructed in which a "tumour" lies within a radioactive "background", the levels of radioactivity in both corresponding to those found in patient studies. This system has been used to evaluate both the NaI and CdTe probes used in the clinical study. By varying the size of the "tumours" and the ratio of radioactivity between "tumour" and "background" the minimum tumour size detectable with

these GDP's could then be established for a given range of tumour to normal tissue ratios and counting times.

7.2.b) Materials and Methods

Biodistribution data

The model was based on data available from studies on patients with cancer to whom ^{111}In labelled monoclonal antibodies have been administered. The percentage uptake of radioactivity by tumour tissue was obtained from the few studies which have provided this data, including the present study, and was taken as being 0.001% I.D./g. (113,130,243,305,). The other parameters chosen for the model are based on those generally used or which have been found in clinical studies namely an injected dose of 100MBq (2.7mCi) of ^{111}In and tumour to background ratios from 2:1 (present study, 101,113) to 8:1 (106). The background activity was calculated from the present clinical study based on the injected activity, uptake in tumour tissue per gram and the tumour to normal colon and tumour to blood ratios calculated in 10 of the patients in the preceding clinical study. (Table 7.5.)

The model of the intra-abdominal tumour.

1) "Tumours"

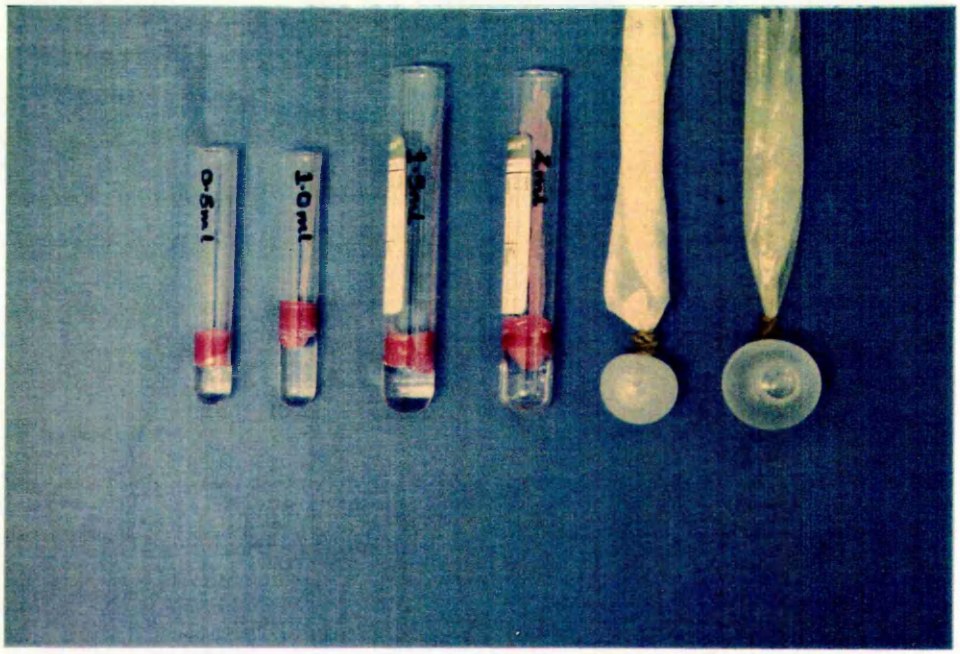
The hollow containers used in this study to represent tumour deposits are shown in Figure 7.5.. The smaller tumour sizes were represented by test tubes in which the liquid was sealed using dental

Table 7.5. Phantom background calculation

No.	Initials	T/Blood	T/N Col
2.	M.B.	2.4	3.4
4.	R.N.	0.8	1.0
5.	D.N.	0.9	1.8
6.	J.L.	1.9	2.0
7.	J.H.	1.4	1.2
8.	B.T.	0.7	2.1
11.	M.J.	3.5	3.5
13.	V.D.	1.4	1.6
14.	F.H.	5.4	2.3
16.	R.D.	1.6	1.6
Mean		2.00	2.05
S.D.		1.46	0.84

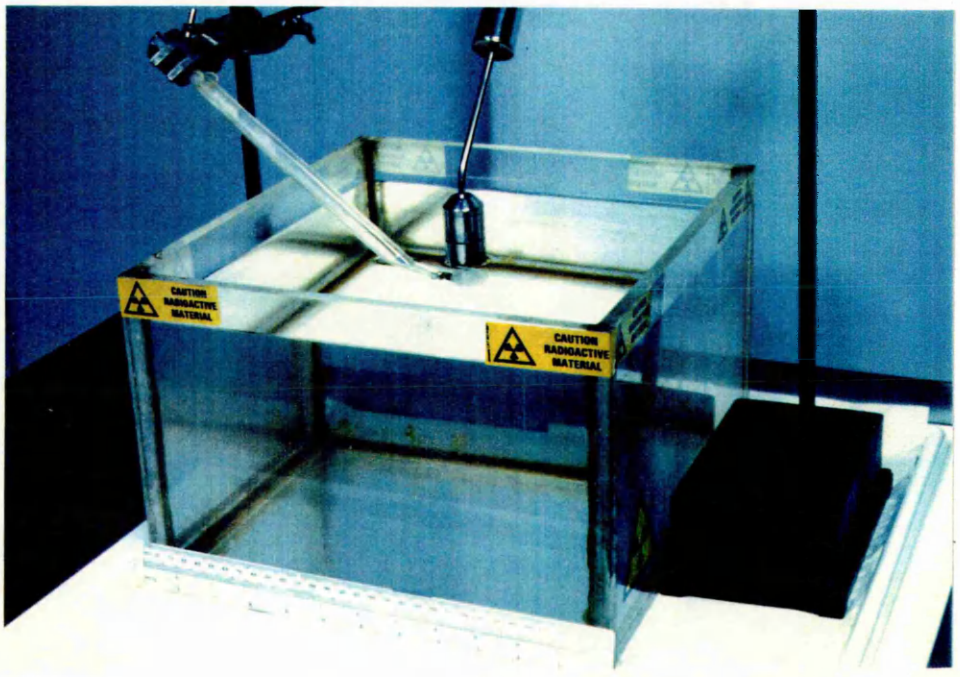
T/Blood and T/N Col : ratio of counts per gram for tumour to blood and tumour to normal colon.

Figure 7.5. Active solutions representing tumour deposits



The sealed solutions of ^{111}In represent tumour volumes of 0.5-10mls as shown.

Figure 7.6. The abdominal phantom



The tank solution represents background activity and contains 15 litres of radioactive water at pH < 2.0.

moulding wax and the larger sizes were formed from latex rubber (Mates Ltd.) These contained a solution of ^{111}In Cl in water, the concentration of radioactivity present being altered for each tumour to background ratio examined. Prior to the containers being filled with the radioactive solution an aliquot was counted in a gamma well counter to ensure that the tumour to background ratio was within +/- 5% of that required.

2) Background activity

The tank used to represent the abdominal cavity is shown in Figure 7.6.. Its dimensions were 30 x 28 x 20 cm and it was filled with radioactive solution to a depth of 17cm to give a volume of 15 litres. The radioactive solution was again comprised of ^{111}InCl in water with the addition of sufficient concentrated HCl to give a final tank pH of less than 2. This pH was required to prevent aggregation of the InCl due to the formation of ^{111}In(OH)n. As with the tumour models the amount of radioactivity present in the tank was checked prior to commencing each probe study by removing a 1ml volume and counting it in a gamma well counter. By comparing this with the 1ml aliquot taken from the "tumours" the accuracy of the tumour to background ratio produced could be measured and altered accordingly if required.

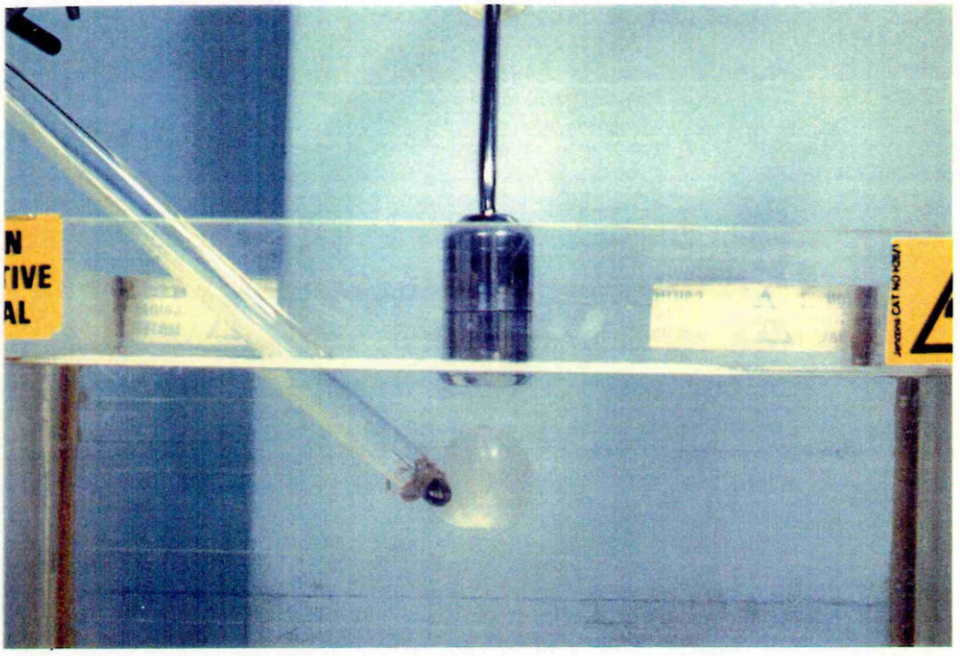
3) **Gamma detecting probes and tumour positioning**

The "tumours" were placed in the centre of the tank representing background activity by attaching them to the end of a perspex rod attached to a retort stand as shown in Figure 7.7.. The upper surface of the tumours was held exactly 1cm below the surface of the active background regardless of the tumour size. The probe under investigation was also supported by a retort stand (Figure 7.7.) and positioned exactly over the centre of the tumour deposit with the tip of the probe collimator touching, and perpendicular to, the surface of the active background.

The collimator is the shielding at the front of the crystal which surrounds the aperture, the shape and size of which influences the radioactivity detected by the crystal. With the NaI probe the collimator was fixed whereas the CdTe detector had two interchangeable collimators. Since this may affect the region from which counts are detected and hence the probes ability to differentiate tumour from background the CdTe probe was analysed with both the wide angle (w.a.) collimator which was virtually a protective cap to the crystal with very little shielding and the parallel hole (p.h.) collimator whose depth of shielding provided a long and narrow aperture.

The energy windows for counting were set at 120-300keV for both probes allowing the two gamma emissions (171 and 245 keV) of ^{111}In to be counted.

Figure 7.7. Positioning of the probe and tumour



The probe was placed perpendicular to the tank solution with the collimator in contact with the surface. Tumours were situated with their upper surface 1cm below the liquid surface.

4) Counting and statistical analysis

The CdTe probe (RMD) is supplied with a counter/timer device and can be preset to count for a fixed period. The NaI scintillation detector was used with a multichannel analyser also set to count for a fixed period (Figure 7.1.). For all tumour sizes and all uptake ratios counting with both probes was carried out for both 20 and 100 seconds and repeated three times. Counting over the tumour was carried out with the probe positioned as previously described whereas counts over background were taken at a site remote from the tumour which was chosen to be 10cm. The shorter count time was selected as being a practical time period for counting over one site at the time of operation and the longer counting period was included to allow the importance of counting time to be analysed. The threshold of counts that can be taken as a reliable measure of tumour detection was based on simple statistical principles outlined in Appendix 7.1.. Differences between the counts obtained over the tumour to that of the background activity were measured by the number of standard deviations between the mean counts obtained over the tumour to those obtained over background. Three standard deviations of the difference in mean counts was taken as a level of detectability for the tumour (Appendix 7.1.). Because the time involved in carrying out each part of this study was significant in relation to the half life of ^{111}In(2.8 days) all counts recorded were corrected for

radioactive decay to the time at which the first measurements were taken.

7.2.c) Results

The results of the statistical analysis is shown in Tables 7.6.-7.12. and the data on which they were calculated in Appendices 7.2.-7.12.. The mean counts for both tumour and background have been calculated for each tumour size (marked on the left of the table) at each tumour to background ratio (top of tables) and the number of standard deviations of the difference in counts calculated (data in the centre of the tables).

By defining a difference between tumour and background of 3 S.D's. as a threshold of detectability (outlined in bold in Tables 7.6.-7.11), with a 2:1 tumour /background ratio and a 20 second count none of the tumours were detectable with the CdTe probe whereas only the 10ml was detectable with the NaI probe. With a counting period of 100 seconds at this ratio the 10 ml tumour could be detected with the CdTe probe (p.h.) (Table 7.11.) and those of 5 and 10mls with the NaI probe. (Table 7.7.).

When the tumour to background ratio was increased to 4:1 then a counting period of 20 seconds allowed the detection of tumours of 1.5mls and greater with the NaI probe (Table 7.6.) and only those of 10mls with the CdTe probe using either collimator (Table 7.8. and 7.10.). Extending the counting period to 100 seconds at 4:1 ratio improved the tumour detection with both

probes, allowing all tumours to be detected with the NaI probe (Table 7.6.) , those above 5mls with the CdTe probe fitted with the wide angle (w.a.) collimator (Table 7.9.) and those above 1ml with the parallel hole (p.h.) collimator. A similar improvement with extended counting time was seen with a 6:1 ratio of uptake, tumours of 1.5mls and above and 5mls and above being detected with the NaI and CdTe (both collimators) probes at 20 second counts (Tables 7.6., 7.8., 7.10) whereas all sizes could be detected at 100 seconds with the NaI probe (Table 7.7.) and those of 1ml and above with the CdTe(either collimator)(Table 7.9. and 7.11). At an 8:1 tumour to background ratio both probes could detect tumours of 1ml and above when counting for twenty seconds (Table 7.6., 7.8. and 7.10) and all tumours (0.5cm and above) with a 100 second count (Tables 7.7., 7.9. and 7.11).

A summary of the tumour sizes detectable, based on three standard deviations as a threshold of detectability, is shown for the different probes, uptake ratios and counting times in Table 7.12.

Table 7.6. NaI probe : 20 second count time

Tumour size(mls)	Tumour to background ratio			
	2:1	4:1	6:1	8:1
0.5	-0.72	-0.17	2.42	1.38
1.0	-0.94	2.88	2.29	**5.42**
1.5	0.16	**3.27**	**3.68**	**7.92**
2.0	0.37	0.38	**5.15**	**10.82**
5.0	2.37	**8.00**	**12.19**	**14.78**
10.0	**3.81**	**9.09**	**16.78**	**18.71**

Table 7.7. Na I probe : 100 second count time

Tumour size (mls)	Tumour to background ratio			
	2:1	4:1	6:1	8:1
0.5	-1.12	**5.44**	**4.47**	**6.26**
1.0	2.63	**4.39**	**5.75**	**11.16**
1.5	1.30	**6.80**	**11.53**	**15.24**
2.0	-0.45	**4.37**	**13.16**	**23.49**
5.0	**3.32**	**15.44**	**24.66**	**31.28**
10.0	**8.53**	**20.79**	**35.76**	**47.17**

Table 7.8. CdTe probe (w.a.) : 20 second counts

Tumour size (mls)	Tumour to background ratio			
	2:1	4:1	6:1	8:1
0.5	0.17	0.39	-0.31	2.86
1.0	-0.80	1.13	-1.62	**3.00**
1.5	-2.33	-1.98	1.82	**4.09**
2.0	-0.89	0.15	1.72	**5.70**
5.0	1.37	2.82	**6.49**	**6.20**
10.0	2.13	**6.19**	**9.56**	**15.55**

Table 7.9. Cd Te probe (w.a.) : 100 second counts

Tumour size (mls)	Tumour to background ratio			
	2:1	4:1	6:1	8:1
0.5	-1.11	-0.93	-2.15	**5.24**
1.0	-1.66	1.82	**3.24**	**4.35**
1.5	-2.84	1.15	**4.33**	**7.25**
2.0	-0.04	1.51	**3.19**	**9.71**
5.0	2.16	**7.86**	**13.10**	**17.77**
10.0	2.50	**10.54**	**21.59**	**33.13**

Table 7.10. CdTe probe (p.c.) : 20 second count

Tumour size (mls)	Tumour to background ratio			
	2:1	4:1	6:1	8:1
0.5	0.51	-1.57	1.15	1.19
1.0	1.91	1.67	0.58	**3.95**
1.5	2.03	2.24	0.57	1.94
2.0	1.23	1.81	2.58	**4.80**
5.0	2.50	2.67	**4.72**	**4.41**
10.0	1.42	**4.29**	**5.42**	**8.57**

Table 7.11. CdTe probe (p.c.) : 100 second count

Tumour size (mls)	Tumour to background ratio			
	2:1	4:1	6:1	8:1
0.5	-1.10	1.29	2.31	**4.04**
1.0	1.52	**4.01**	**4.52**	**3.28**
1.5	1.36	**4.04**	1.86	**6.29**
2.0	-0.55	**4.27**	**6.18**	**8.74**
5.0	1.83	**6.28**	**11.53**	**11.35**
10.0	**3.17**	**7.64**	**13.42**	**19.08**

Table 7.12. Minimum tumour sizes detectable

Probe		NaI		CdTe (w.a.)		CdTe (p.h.)	
Count time (secs)		20	100	20	100	20	100
	2:1	10.0	5.0	none		none	10
Ratio	4:1	1.5	0.5	10.0	5.0	10.0	1.0
	6:1	1.5	0.5	5.0	1.0	5.0	1.0
	8:1	1.0	0.5	1.0	0.5	1.0	0.5

The minimum tumour sizes detectable are shown(mls) based on a level of detectability of 3 S.D.'s.. Figures are for 20 second and 100 second counting periods. w.a. : wide angle collimator, p.h. : parallel hole collimator.

7.2.d) Discussion

This is the first study to be carried out to investigate the potential and limitations of intra-operative gamma detecting probes for the localisation of tumour deposits following the administration of ^{111}In labelled monoclonal antibodies.

The results suggest that intra-operative probing may be able to detect tumour deposits as small as 1.5ml at a tumour to background ratio of 4:1 (NaI probe) and one ml at an 8:1 ratio (both probes) with a counting period of 20 seconds. This size of tumour deposit could easily be overlooked by the surgeon at the time of laparotomy and its detection may significantly alter the prognosis for the patient involved.

The accuracy of the present model is dependent on the premises on which it is based and in particular on the %I.D. taken up in the tumour tissue. This figure is based on the findings of the present study with ^{111}In labelled ICR2 in patients with colorectal cancer and is in agreement with other studies using ^{111}In labelled MAb's (113,130,243,305). Many studies on MAb's used in colorectal cancer patients have not, however, quoted the tumour uptake as %I.D./g or the differential ratio. These labelled antibodies may, therefore, provide better or worse results than those determined from the present study.

Another factor of prime importance in evaluating the clinical correlation of the present model is that

in patients the primary, metastatic or recurrent tumours do not lie within an even background of activity but may occur in relation to non tumour sites of high uptake of labelled antibody, such as the liver, spleen and bone marrow.

The liver may be responsible for uptake of 30-40% (125) of the injected dose of ^{111}In labelled antibody and this would therefore produce considerably higher background activity when probing in its vicinity. Similarly the pelvis and retroperitoneal tissues are common sites of recurrent colorectal cancer and intra-operative probing in this area will be influenced by the high uptake of activity in the bony pelvis and lumbar vertebrae. Consequently the results obtained in vivo may be considerably worse than those calculated from the present model.

Of the two probe types analysed the NaI scintillation detector was able to detect smaller tumours at lower tumour to background ratios than the CdTe probe at either of the counting times investigated. This is due to the higher number of counts being recorded over both the tumour and the background with the NaI probe resulting in improved counting statistics and a greater statistical significance to the differences between the mean counts. The number of counts detected for any individual probe will depend on the intrinsic properties of the detecting material (NaI or CdTe), the

size and shape of the crystal used and the collimation of the probe itself. Although both of the probes used in the present study were suitable for detecting ^{111}In some improvement might be achieved by increasing the crystal thickness and hence the sensitivity of the CdTe probe, thereby improving the detectability of small tumours at low tumour to background ratios.

A novel approach to the problem of background activity has been investigated by Hickernell and colleagues using a probe with two concentric detectors. By this means the area of suspected tumour may be counted simultaneously with an adjacent area of normal tissue and the results subsequently compared. By using a computer simulation of the surgical staging of metastases this dual detecting probe was shown to perform better than a single detector probe [328]. A clinical evaluation of the probe is awaited.

The statistical analysis of the tumour and background counts used in the present study points to the way ahead for future clinical studies on intra-operative probing. The system presently used is based on an arbitrary cut off level of 1.5 for suspected tumour to background counts [149,150]. Although this system is easily applied in a clinical situation the significance of this ratio will depend on the actual number of counts on which it is based. Hence a ratio of 1.5 based on counts of 1500 and 1000 has an entirely different statistical significance to a ratio

of 1.5 based on counts of 15 and 10. The system developed for the interpretation of tumour detectability in the phantom model could be applied in the clinical situation. This would require the number of standard deviations to be calculated between the mean tumour and background counts and would allow the significance of this difference in counts to be calculated. Taking a confidence limit of two standard deviations between the counts would result in a 95% certainty that the difference in counts is significant and with three standard deviations there would be less than a 1% chance that the difference in counts is due to random error ([329-331], Appendix 7.1.).

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Appendix 3.1. : Serum EMA levels

	EMA levels by patient group (ng/ml)			
No.	Controls	Breast Ca.	Colon Ca.	Other Cancers
1	105	760	990	580
2	430	500	270	580
3	560	1030	240	185
4	240	1550	280	220
5	110	1230	210	67400
6	100	760	390	9720
7	610	1240	1100	120
8	700	620	310	1720
9	350	3000	845	5360
10	400	1010	455	
11	830	560	640	
12	550	550	1600	
13	650	790	140	
14	570	250	440	
15	720	250	380	
16	470	880	750	
17	210	460	470	
18	220	1120	470	
19	1830	1560	710	
20	365	290	480	
21	630	740	9920	
22	780	440	650	
23	635	300	680	
24	152	1000	1150	
25	510	1110	670	
26	1000	1000	58	
27	1950	890	750	
28	1820	650	495	
29	108	960	1350	
30	980	470	420	
31	280	650	410	
32	920	800		
33	1750	1320		
34	400	830		
35	1000	260		
36	540	700		
37	720	550		
38	470	600		
39	630	2010		
40	440	320		
41	930	200		
42	540	337		
43	560	990		
44	3680	16430		
45		1500		
46		410		
47		470		
48		630		
49		210		
50		540		
51		750		
52		2390		
53		1150		

Appendix 4.1. Folin-Lowry protein assay

The cells from the uptake experiments were kept at -20^{o}C until the protein assay was carried out. After thawing at room temperature each samples was sonicated for 30 seconds (Soniprep 150, Fisons) to produce an even cell suspension and 0.5mls of each sample mixed with an equal volume of 0.5M sodium hydroxide. After a mixing period of one hour, to each sample was added 5mls of 2% sodium carbonate solution containing (1ml for every 50mls) sodium and pottasium tartrate (2% solution) and copper sulphate(1% solution). This was mixed thoroughly and after exactly 10 minutes 250ul of Folin and Ciocalteau's reagent (2.2M)(diluted 5mls in 8mls of water) was added to each tube.

As a standard solutions of bovine serum albumin(BSA) containing protein concentrations of 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30mg /ml were prepared and treated in the same manner.

After leaving for one hour to allow colour development all samples were read on a u.v., spectrophotometer (Unicam SP1800) at 500nm. A standard curve was then produced from the dilutions of BSA and the protein concentrations of the other samples read from the standard curve.

Appendix 4.2. Baseline uptake of ^{111}In-ICR2

1) MCF7 tumour cells

Cpm/plate	Protein conc. (mg/ml)	cpm/mg	Applied (cpm x 10^5)	Uptake (% applied/mg)
244,632	0.23	1,063,617	31.0	34.3
292,810	0.29	1,009,698	31.4	32.2
255,377	0.24	1,064,070	28.2	37.7

2) Hepatocytes

Cpm/plate	Protein conc. (mg/ml)	cpm/mg	Applied (cpm x10^5)	Uptake (% applied/mg)
23,589	0.20	117,945	26.4	4.47
21,387	0.20	106,935	31.8	3.36
19,242	0.20	96,210	29.6	3.25

Appendix 4.3. Temperature and cell uptake of ^{111}In-ICR2

1) MCF7 cells at 37^{o}C

Applied cpm	Bound cpm	Uptake as % applied cpm
528,497	50,585	9.57
495,359	54,546	11.00
582,752	56,550	9.70

2) Hepatocytes at 37^{o}C

Applied cpm	Bound cpm	Uptake as % applied cpm
561,149	3048	0.54
573,149	1880	0.33
552,876	2429	0.44

3) MCF7 cells at 4^{o}C

Applied cpm	Bound cpm	Uptake as % applied cpm
635,032	38,360	6.04
679,836	25,904	3.81
655,709	30,608	4.60

4) Hepatocytes at 4^{o}C

Applied cpm	Bound cpm	Uptake as % applied cpm
622,927	1673	0.27
618,020	1912	0.31
699,811	1894	0.27

Appendix 4.4. Preparation of Transferrin (Tf)

Iron precipitates without a chelator at neutral pH so the iron saturated transferrin was prepared at acidic pH. A 90% iron saturated solution of Transferrin was prepared by the reaction of 20mg of Transferrin in 0.5mls of molar Tris / HCl pH 8.5 with 0.4uM of ferrous ammonium sulphate in 0.1M HCl (previously bubbled with N_2). A few grains of sodium bicarbonate were then added to ensure an alkaline pH. After 30 minutes the solution was then made up to 1ml with PBS and dialysed against RPMI 1640 medium (Gibco,U.K.) for two hours. A final concentration of 2 g/l was achieved by the application of the transferrin solution to the culture medium of the cells.

Appendix 4.5. Tf and the hepatocyte uptake of ^{111}In-ICR2

Cell group	Applied (cpm x 10^5)	Cell uptake (cpm/plate)	% applied counts
Control	3.81	300	0.078
Control	3.09	331	0.107
Control	3.77	445	0.118
Tf	3.34	362	0.108
Tf	3.84	392	0.102
Tf	3.89	297	0.076
ApoTf	3.95	438	0.110
ApoTf	3.65	223	0.061
ApoTf	3.77	429	0.114

Appendix 4.6. Effect of apoTf on cell uptake of radioactivity

1) MCF7

Cell group	Counts applied (cpm x 10^6)	Uptake per plate (cpm x 10^3)	% applied counts
Control	2.45	285	11.7
Control	2.47	277	11.2
Control	2.45	336	13.7
ApoTf	2.36	243	10.3
ApoTf	2.32	271	11.6
ApoTf	2.46	313	12.7

2) Hepatocytes

Cell group	Counts applied (cpm x 10^6)	Uptake per plate (cpm x 10^3)	% applied counts
Control	2.37	5.63	0.24
Control	2.43	5.46	0.23
Control	2.44	6.65	0.27
ApoTf	2.37	5.76	0.24
ApoTf	2.39	6.27	0.26
ApoTf	2.46	4.48	0.18

Appendix 4.7. The effect of DTPA co-administration

1) MCF7

Cell group	Applied (cpm x 10^5)	Uptake/ plate (cpm x 10^3)	(%) applied	Protein (mg/ml)	Uptake/mg (cpm x 10^3)
Control	5.11	15.3	3.0	3.7	4.12
Control	5.53	17.7	3.2	3.7	4.78
Control	5.60	16.6	3.0	3.7	4.47
DTPA	5.57	12.6	2.9	3.7	4.47
DTPA	5.61	20.0	3.6	3.7	5.41
DTPA	5.61	10.0	1.8	3.5	2.94

2) Hepatocytes

Cell group	Applied (cpm x 10^5)	Uptake/ plate (cpm x 10^3)	(%) applied	Protein conc. (mg/ml)	Uptake/mg (cpm x 10^3)
Control	4.07	6.02	1.48	3.7	1.63
Control	5.37	7.96	1.48	3.7	2.15
Control	5.68	7.47	1.31	3.7	2.02
DTPA	5.72	4.48	0.78	3.7	1.21
DTPA	5.56	4.41	0.79	3.7	1.19
DTPA	5.85	3.24	0.55	3.5	0.92

Appendix 4.8. Cell incubation with DTPA

1) MCF7's

Cell group	Applied (cpm x 10^5)	Uptake/plate (cpm x 10^3)	Uptake (%)	Protein (mg/ml)	Uptake cpm/mg
control	5.11	15.23	3.0	3.7	4116
control	5.53	17.71	3.2	3.7	4787
control	5.60	16.56	2.9	3.7	4475
DTPA	5.20	17.10	3.3	3.7	4622
DTPA	5.50	12.21	2.2	3.7	3301
DTPA	5.40	14.02	2.6	3.7	3788

2) Hepatocytes

Cell group	Applied (cpm x 10^5)	Uptake/plate (cpm x 10^3)	Uptake (%)	Protein (mg/ml)	Uptake (cpm/mg)
control	4.07	6.02	1.48	3.7	1627
control	5.37	7.96	1.48	3.7	2152
control	5.68	7.47	1.31	3.5	2020
DTPA	5.64	4.52	0.80	3.7	1223
DTPA	5.64	5.32	0.94	3.7	1439
DTPA	5.72	4.89	0.85	3.7	1322

Appendix 4.9. : Dose response of DTPA

1) MCF7's

[DTPA]	Applied (cpm x 10^6)	Uptake (%)	Uptake/plate (cpm x 10^3)	Prot. (mg/ml)	cpm x 10^3 per mg
0	3.10	7.89	244.63	0.23	1063.62
0	3.14	9.32	292.81	0.29	1009.69
0	2.82	9.05	255.37	0.24	1064.07
100uM	3.04	9.07	275.95	0.26	1061.36
100uM	3.01	6.22	187.33	0.21	892.05
100uM	2.86	8.16	233.57	0.26	898.34
1mM	3.24	9.94	322.08	0.26	1238.76
1mM	3.04	10.92	332.12	0.34	976.83
1mM	2.12	13.35	283.13	0.26	1088.97
10mM	2.88	7.80	229.79	0.24	957.45
10mM	3.14	8.63	270.87	0.28	967.41
10mM	3.16	9.32	294.63	0.28	1052.27

2) Hepatocytes

[DTPA]	Applied (cpm x 10^6)	Uptake (%)	Uptake/plate (cpm x 10^3)	Protein (mg/ml)	cpm x 10^3 per mg
0	3.16	0.75	23.59	0.20	117.94
0	3.18	0.67	21.39	0.20	106.93
0	2.96	0.65	19.24	0.20	96.21
100uM	2.92	0.73	21.21	0.21	100.99
100uM	2.49	0.74	18.56	0.22	84.37
100uM	2.54	0.68	17.04	0.20	85.18
1mM	3.22	0.13	4.33	0.20	21.66
1mM	2.40	0.13	3.27	0.16	20.45
1mM	3.23	0.13	4.34	0.20	21.69
10mM	3.19	0.14	4.46	0.22	20.25
10mM	2.93	0.13	3.89	0.22	17.69
10mM	3.10	0.11	3.48	0.21	16.56

Appendix 4.10. DTPA incubation period and cell uptake

1) MCF7's

Cell group	Applied (cpm x 10^5)	Uptake (%)	Uptake/plate (cpm x 10^3)	Prot. conc. (mg/ml)	cpm x 10^3/mg
control	5.30	4.8	25.29	0.36	70.26
control	4.62	7.8	36.25	0.43	81.99
control	4.96	6.0	29.59	0.34	87.04
5 mins	4.77	5.4	26.05	0.28	93.03
5 mins	4.80	4.5	21.87	0.28	78.11
5 mins	4.49	7.4	33.53	0.36	93.13
15 mins	4.55	5.7	25.76	0.32	86.50
15 mins	4.57	5.1	23.51	0.32	73.47
15 mins	4.47	5.0	22.89	0.20	81.78
30 mins	4.89	6.1	29.89	0.31	96.44
30 mins	4.93	6.6	32.52	0.36	90.33
30 mins	4.74	5.1	24.43	0.28	87.24
60 mins	5.22	4.0	20.82	0.28	74.36
60 mins	4.62	4.5	20.94	0.29	72.21
60 mins	3.20	7.2	23.16	0.28	81.25

2) Hepatocytes

Cell group	Applied (cpm x 10^5)	Uptake (%)	Uptake/plate (cpm x 10^3)	Prot. conc. (mg/ml)	cpm x 10^3/mg
control	5.30	1.13	6.02	0.92	6.54
control	5.09	1.27	6.48	1.03	6.29
control	5.35	1.57	8.43	0.94	8.97
5 mins	4.69	1.02	4.32	0.94	4.60
5 mins	4.66	1.02	4.76	0.91	5.23
5 mins	4.92	0.88	4.36	0.88	4.96
15 mins	4.51	0.83	3.73	0.78	4.78
15 mins	4.30	0.78	3.36	0.96	3.50
15 mins	4.73	0.83	3.92	0.96	4.08
30 mins	4.87	0.98	4.76	1.03	4.62
30 mins	4.67	0.80	3.75	0.91	4.12
30 mins	4.71	0.57	2.68	0.93	2.88
60 mins	4.79	1.16	5.55	0.99	5.60
60 mins	4.55	0.61	2.80	0.81	3.45
60 mins	4.53	0.73	3.32	1.03	3.22

Appendix 4.11. Cell uptake of ^{111}In and ^{111}In-DTPA

1) MCF7's : Free ^{111}In

Applied (cpm x 10^5)	Uptake cpm/plate	Uptake (%)	Protein mg/ml	Uptake/mg (cpm)
1.84	1258	0.68	0.40	3140
1.85	622	0.34	0.21	2962
1.87	1102	0.59	0.42	2624

2) MCF7 : Free ^{111}In / co-admin DTPA

Applied (cpm x 10^5)	Uptake cpm/plate	Uptake (%)	Protein mg/ml	Uptake/mg (cpm)
1.84	780	0.42	0.37	2108
1.84	480	0.27	0.35	1371
1.88	580	0.31	0.35	1657

3) Hepatocytes : Free ^{111}In

Applied (cpm x 10^5)	Uptake cpm/plate	Uptake (%)	Protein mg/ml	Uptake/mg (cpm)
1.82	1522	0.83	0.75	2029
2.03	1568	0.77	0.74	2119
1.87	938	0.51	0.45	2084

4) Hepatocytes : Free ^{111}In / co-admin DTPA

Applied (cpm x 10^5)	Uptake cpm/plate	Uptake (%)	Protein mg/ml	Uptake/mg (cpm)
1.84	800	0.43	0.56	1428
1.83	718	0.39	0.75	957
1.86	874	0.47	0.82	1065

5) MCF7's : ^{111}In-DTPA

Applied (cpm x 10^5)	Uptake cpm/plate	Uptake (%)	Protein mg/ml	Uptake/mg (cpm)
1.90	498	0.26	0.46	1083
1.95	432	0.22	0.43	1005
1.99	638	0.32	0.38	1679

6) MCF 7 : ^{111}In-DTPA / co-admin DTPA

Applied (cpm x 10^5)	**Uptake** cpm/plate	**Uptake** (%)	**Protein** mg/ml	**Uptake/mg** (cpm)
1.87	548	0.29	0.38	1442
1.91	630	0.33	0.43	1465
1.86	660	0.35	0.38	2750

7) Hepatocytes : ^{111}In-DTPA

Applied (cpm x 10^5)	Uptake cpm/plate	Uptake (%)	Protein mg/ml	Uptake/mg (cpm)
1.80	290	0.16	0.74	392
1.83	494	0.27	0.72	686
1.86	382	0.20	0.48	796

8) Hepatocytes : ^{111}In-DTPA / co-admin DTPA

Applied (cpm x 10^5)	Uptake cpm/plate	Uptake (%)	Protein mg/ml	Uptake/mg (cpm)
1.92	478	0.25	0.62	771
1.81	502	0.27	0.69	727
1.91	464	0.24	0.66	642

Appendix 4.12. A comparison of chelators

Cell group	Applied (cpm x 10^6)	Uptake/plate (cpm x 10^3)	Uptake (%)	Protein (mg/ml)	cpm/mg (x 10^3)
MCF7 ctr	1.32	73.88	5.6	0.175	211.09
MCF7 ctr	1.36	42.47	3.1	0.115	184.66
MCF7 ctr	1.44	50.94	3.5	0.135	188.65
MCF7 ctr	1.39	31.11	2.2	0.115	135.26
MCF7 EDTA	1.44	67.95	4.7	0.180	188.74
MCF7 EDTA	1.28	53.36	4.2	0.120	222.35
MCF7 EDTA	1.30	32.43	2.5	0.110	147.40
MCF7 EDTA	1.27	14.95	1.2	0.065	114.97
MCF7 DTPA	1.28	61.38	4.8	0.085	361.06
MCF7 DTPA	1.29	30.39	2.4	0.100	151.96
MCF7 DTPA	1.30	68.14	5.3	0.140	243.35
MCF7 DTPA	1.36	45.34	3.3	0.075	302.28
MCF7 ccDTPA	1.34	66.62	5.0	0.145	229.72
MCF7 ccDTPA	1.38	28.67	2.1	0.120	119.47
MCF7 ccDTPA	1.50	89.09	5.9	0.195	228.43
MCF7 ccDTPA	1.33	26.93	2.0	0.085	158.42
MCF7 CP040	1.27	34.51	2.7	0.085	203.00
MCF7 CP040	1.23	23.16	1.9	0.060	192.97
MCF7 CP040	1.26	82.30	6.5	0.085	484.13
MCF7 CP040	1.15	37.17	3.2	0.075	247.83
Heps ctr	1.38	66.40	4.8	0.320	103.74
Heps ctr	1.34	52.78	3.9	0.375	70.37
Heps ctr	1.37	73.24	5.3	0.310	118.13
Heps ctr	1.32	58.10	4.4	0.355	81.82
Heps EDTA	1.28	61.76	4.6	0.375	84.40
Heps EDTA	1.36	63.30	4.6	0.375	84.40
Heps EDTA	1.35	59.24	4.4	0.475	62.35
Heps EDTA	1.44	57.53	4.0	0.505	56.96
Heps DTPA	1.33	53.60	4.0	0.445	60.23
Heps DTPA	1.41	46.88	3.3	0.475	49.35
Heps DTPA	1.37	43.04	3.1	0.445	48.36
Heps DTPA	1.27	45.99	3.6	0.385	59.73
Heps ccDTPA	1.40	50.71	3.6	0.385	65.86
Heps ccDTPA	1.52	58.68	3.9	0.330	88.91
Heps ccDTPA	1.20	37.84	3.1	0.390	48.51
Heps ccDTPA	1.50	59.84	4.0	0.335	89.31
Heps CP040	1.53	129.15	8.4	0.400	161.43
Heps CP040	1.48	143.66	9.7	0.400	179.58
Heps CP 040	1.61	139.62	8.7	0.415	168.22
Heps CP040	1.66	152.82	9.2	0.415	184.11

Appendix 4.13. The effect of pooled rat IgG

Cell group	Applied (cpm x 10^6)	Uptake/plate (cpm x 10^3)	Uptake (%)	Protein (mg/ml)	cpm/mg (x 10^3)
MCF7 ctr	9.7	70.56	7.3	0.42	168.00
MCF7 ctr	10.0	73.32	7.3	0.37	198.17
MCF7 ctr	9.8	75.32	7.7	0.47	160.26
MCF7 x 10	9.7	71.27	7.3	0.40	285.08
MCF7 x 10	10.1	74.51	7.4	0.50	149.00
MCF7 x 10	10.3	80.97	7.9	0.43	188.30
MCF7 x 50	10.9	84.09	7.7	0.37	227.26
MCF7 x 50	9.6	66.58	6.6	0.42	158.53
MCF7 x 50	10.1	76.95	7.6	0.45	171.00
MCF7 x 100	10.2	77.78	7.6	0.42	185.20
MCF7 x 100	10.4	85.14	8.2	0.45	189.20
MCF7 x 100	10.0	86.12	8.6	0.42	205.04
MCF7 x 1000	9.4	57.68	6.1	0.40	144.20
MCF7 x 1000	9.4	63.75	6.5	0.50	127.50
MCF7 x 1000	9.8	63.56	6.5	0.43	147.82
Heps ctr	9.1	2.93	0.32	0.53	5.52
Heps ctr	9.0	3.59	0.40	0.64	5.61
Heps ctr	9.2	4.98	0.54	0.57	8.74
Heps x 10	9.7	6.33	0.64	0.51	12.41
Heps x 10	9.8	6.36	0.65	0.57	11.15
Heps x 10	9.6	6.31	0.66	0.64	9.85
Heps x 50	10.2	5.27	0.52	0.72	7.32
Heps x 50	10.3	4.52	0.44	0.69	6.55
Heps x 50	9.9	5.31	0.54	0.66	8.04
Heps x 100	10.4	4.77	0.46	0.72	6.63
Heps x 100	10.2	6.55	0.64	0.72	9.10
Heps x 100	14.0	6.71	0.48	0.58	11.58
Heps x 1000	8.0	4.00	0.50	0.61	6.56
Heps x 1000	6.1	3.39	0.56	0.61	5.56
Heps x 1000	10.2	4.41	0.43	0.61	7.22

Appendix 5.1. Method of conjugation of ICR2

Antibody was stored in the preservative sodium azide (0.01% w/v). This was removed prior to conjugation by dialysing (Dialysis membrane PJC/400/050/L, Medicell International Ltd.) against Hepes buffer (0.05M, pH 7.5) for 48 hours with changes of dialysate 12 hourly. The antibody was then concentrated to 10-20mg/ml using a protein concentrator No. A25(Amicon Corp. Lexington. Mass., U.S.A.). The amount of DTPA required for reaction with the antibody was then calculated using the following formula,

$$\text{DTPA (mg)} = \frac{\text{MWt DTPA}}{\text{MWt MAb}} \times \text{C.R.} \times \text{mg MAb}$$

where C.R. is the conjugation ratio required. A solution of DTPA was prepared in the inorganic solvent dimethylsulfoxide (DMSO)(Sigma) at 1 mg DTPA per ml of DMSO, the procedure requiring vigorous mixing. The concentrated antibody was then added to the amount of ccDTPA/DMSO as calculated from the above formula and vortexed for one minute. Unwanted DTPA in the reaction mixture was then removed by dialysis against Hepes buffer pH 7.5 0.05M for 48 hours. Following dialysis the conjugated antibody was further purified by gel chromatography using a 50 x 2 cm column of G25 Sephadex pretreated with 2mls of human serum albumin in Hepes buffer at pH 7.5 to block non specific protein binding on the Sephadex. The conjugated antibody was applied and eluted with Hepes buffer pH 7.5. Fractions of 2ml were collected using an LKB ultrorak and the protein content of each fraction was determined from the U.V. absorption at 282nm using a Cecil CE272 linear U.V. spectrophotometer.

Appendix 5.2. Labelling and in vitro stability of ^{111}In-ICR2

A wide range of labelling efficiencies and specific activities have been reported for ^{111}In labelled MAb's which may be related to the antibodies themselves or their methods of conjugation or labelling. The stability of the radiopharmaceutical is likely to have an important bearing on the results of its clinical use. Prior to clinical use some preliminary studies were carried out on the effect of labelling pH and storage on the stability of ^{111}In-ICR2 and on the effect of DTPA conjugation on its immunoreactivity.

Indium chloride is supplied in 0.04M HCl because at neutral pH it hydrolyses in water to form insoluble complexes of In(OH)n. There are two possible approaches to prevent hydrolysis occurring during the labelling procedure. Firstly the reaction with MAb-DTPA can be carried out at acid pH possibly leading to damage to the antibody or alternatively the InCl can be reacted with sodium acetate at pH 6-7 to produce the weakly bound indium acetate. The effect of pH on labelling efficiency was assessed for ICR2 at pH 3.5, 7.5 and 8.95.

Effect of pH on labelling efficiency

1) **Labelling at pH 3.5**

200ug of ICR2-DTPA was mixed with 15MBq of InCl in 0.04 M HCl for 20mins. A BioRad 20 x 1 cm chromatography column was filled with Sephadex G 25 which had been swollen in 0.01 M glycine buffer overnight at pH 3.5. The column was eluted with 10mls glycine, 2mls of 1% HSA to block any protein binding sites then washed with a further
10mls of glycine buffer pH 3.5. Labelled antibody was applied to the column, eluted with 10mls glycine and 1 ml fractions collected and counted in a gamma well counter.

2) **Labelling at pH 7.5**

The same amount of antibody was labelled with the same activity as in the previous experiment (200ug ICR2 with 15MBq InCl). The indium chloride in 0.04M HCl was reacted with an equal volume of molar sodium acetate prior to adding the antibody to this mixture for twenty minutes. The sephadex G25 was swollen overnight in Hepes buffer (0.05M, pH 7.5) and the column prepared as before but with Hepes buffer at pH 7.5 replacing glycine. 1ml fractions were collected and counted.

3) Labelling at pH 8.95

The procedure as outlined above was repeated but using a carbonate/bicarbonate buffer at pH 8.95. The activity of column fractions within the protein peak were summated to give the labelled activity. Eluting non protein bound activity and that remaining on the column was assessed by gamma well counting. The labelling efficiency was expressed as a ratio of antibody bound activity to total applied activity.

Results

Buffer	pH	Activity in protein peak	Activity in column	Labelling efficiency
Glycine	3.5	10.6	4.3	71%
Hepes	7.5	13.6	1.3	91%
Bicarb	8.95	13.8	1.2	92%

Figures are mean of three determinations

The preliminary experiments on labelling pH suggested that a satisfactory labelling efficiency may be achieved at a physiological pH. The stability of the labelled antibody with alterations in the pH at which it is stored was then investigated.

Procedure

100ug of ICR2 was labelled with ^{111}In via ccDTPA as previously described using a glycine buffer at pH 3.5 or Hepes buffer at pH 7.5. Aliquots were taken 1, 2 and 7 days from the time of labelling, stored at this pH and then subjected to gel chromatography using a PD 10 column (bed volume 9.1ml, bed height 5cm). Sephadex G 25 was swollen in normal saline containing methiolate as preservative (0.1g/l) and the non ionic detergent Berol 048. Aliquots of ^{111}In-ICR2 were eluted with 0.5ml volumes of TBS (0.15M, pH7.4) and 0.5ml fractions collected and counted. The activity associated with the protein peak was calculated as a percentage of total activity eluting.

Results : % protein bound activity

Buffer	Glycine pH 3.5	Hepes pH 7.5
Days after labelling		
Day 1	98%	93%
Day 2	92%	95%
Day 7	87%	66%

These results suggested that ^{111}In-ICR2 labelled at either pH was stable over a 48 hour period. Due to the short half life of ^{111}In (2.8 days) antibody labelled with this isotope is used shortly after the labelling procedure and the pH of storage is therefore not likely to be a major factor. On injection of the labelled antibody into a patient, however, a rapid change in pH will occur with antibody labelled at pH 3.5. The stability of ^{111}In-ICR2 with changes in pH of storage were therefore investigated.

Procedure

The protein bound activity was compared in samples labelled and stored at pH 3.5,. those labelled and stored at pH 7.5 and those labelled at pH 3.5 and stored at pH 7.5. Two samples of 200ug of ICR2 were labelled at pH 3.5 or pH 7.5 as outlined above. After labelling, an aliquot of the sample labelled at pH 3.5 was passed through a PD 10 column of Sephadex G 25 which had been equilibrated with TBS at pH 7.5. Fractions of 0.5ml were collected from the protein peak. The three samples were then compared for protein bound activity by repeating the column chromatography after storage for 1 hour and for 1 week.

Results

All three samples had > 90% protein bound activity when re-run after 1 hour whereas the sample labelled at pH 3.5 but stored at pH 7.5 showed only 13% of initial radioactivity was protein bound after one week compared with 88% in the control samples kept in Glycine buffer at pH 3.5 or Hepes buffer at pH 7.5.

Overall conclusions

For patient studies ICR2 would be labelled with ^{111}In at a physiological pH of 7.5 in Hepes buffer. The problem of ^{111}In forming insoluble indium hydroxide was overcome by the addition of 1M sodium acetate to the InCl/HCl solution prior to labelling. Storage and pH changes after labelling were avoided.

Appendix 5.3. The immunoreactivity of ICR2

To preserve the immunoreactivity of the antibody it is necessary to determine the effect of the conjugation with DTPA on antibody immunoreactivity. To assess this a competition radioimmunoassay was carried out using 96 well PVC plates (Dynatech Ltd.) coated with MID/2, a monoclonal anti-idiotypic antibody directed against the combining site of ICR2 (Dean et al, unpublished data.) Serial dilutions of the DTPA conjugate were assessed for the ability to inhibit the binding of ^{125}I labelled ICR2 to the MID/2 coated plates. The results showed that while the immunoreactivity of the 1:1 conjugate was 98% of unconjugated antibody , the 2:1 conjugate was reduced to 65% of the unconjugated ICR2. (C. Dean et al, Institute of Cancer Research, Sutton, Surrey, personal communication) A 1:1 conjugation ratio of ICR2 to ccDTPA was therefore selected for patient studies.

Appendix 5.4. Antibody labelling for patient administration

For patient studies all procedures were carried out using aseptic technique in a laminar flow hood. Antibody for administration was assessed for sterility , pyrogenicity and freedom from viruses. All materials and reagents were autoclaved and tested for sterility.

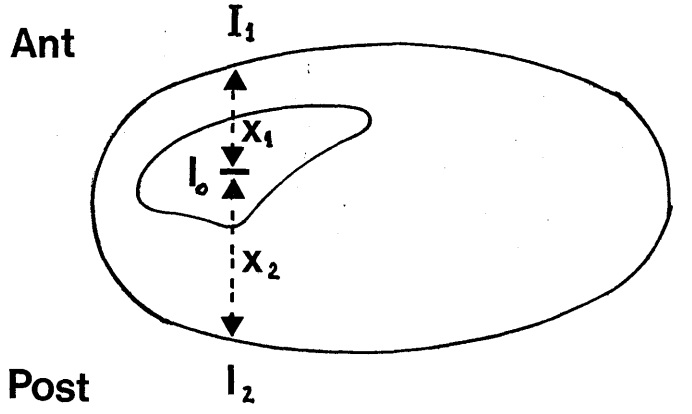
Procedure

Sealed vials containing at reference date 2mCi of ^{111}InCl in 0.2mls of 0.04M HCl were obtained from Amersham U.K.. The vials were assayed for their radioactivity on the day of labelling in a gamma well counter (Vintner U.K.). The vials were then opened in the sterile hood and 0.2mls of 1M sodium acetate added to achieve a pH of 6-7. One mg of antibody was then added which had been conjugated to DTPA at a ratio of 1:1 and the mixture left to stand for 20 minutes with occassional agitation. A 20 x 1cm column (Bio-Rad) was filled to a bed height of 17cm with G25 Sephadex which had been swollen overnight in Hepes buffer and sterilised in a liquids autoclave. The column was then eluted with 10mls of Hepes buffer pH 7.5 (0.05M) followed by 2mls of 1% human serum albumin to block protein binding . Further elution with 20mls of Hepes buffer removed any excess unbound human albumin from the column. Labelled antibody was added to the column after the 20 minute incubation period and eluted through the column with exactly 10mls of Hepes buffer. One ml fractions were collected in sterile tubes (Sterilin U.K.) and assayed individually in a well counter. Using this method the protein peak eluted from the column in fractions 4-6 which were pooled and assayed for their protein bound activity by thin layer chromatography as described by Carrasquillo and colleagues[243]. The pooled fractions of labelled antibody were then filtered through a 0.22um filter (Millipore , France) into a sterile universal container (Sterilin, U.K.) to which 20 mls of normal saline were added. The labelled antibody was then once again assayed for radioactivity in a gamma well counter. Following preparation of the injectate aliquots were removed for the preparation of a liver phantom and a standard for each patient (Chapter 6).

The labelling procedure was the same for patients in the DTPA group except that after the antibody had been incubated for 20 minutes with indium acetate an equal volume of 500uM DTPA was added to the mixture for 15 minutes prior to the gel chromatography.

Appendix 6.1. Liver attenuation correction for patient size

An attenuation correction factor was derived to allow the counts detected over the patients liver to be correlated with those over the liver phantom (Temex trunk). It is based on the following mathematical model.



Where

I_1 = anterior detected liver counts per second decay corrected to time of injection.

I_2 = posterior detected liver counts per second decay corrected to injection.

Io = actual liver counts per second decay corrected to injection time.

u = linear attenuation coefficient of tissue equivalent for ^{111}In cm-1

Geometric mean (GM) $= \sqrt{I_1 \times I_2} = \sqrt{I_o^2 e^{-u(X_1 + X_2)}} = I_o e^{-1/2(X_1 + X_2)}$ where

$X_1 + X_2$ = Antero-posterior diameter of the patient.

To obtain the attenuation correction factor (f) by comparing the counts from the standard and the patient liver.

$$\frac{IT}{IP} = \frac{I_o e^{-1/2uYT}}{I_o e^{-1/2uYP}}$$

Where

IT = geometric mean of activity detected from the standard

IP = geometric mean of activity detected from the patient

YT = AP diameter of the phantom

YP = AP diameter of the patient at the level of the xiphisterum.

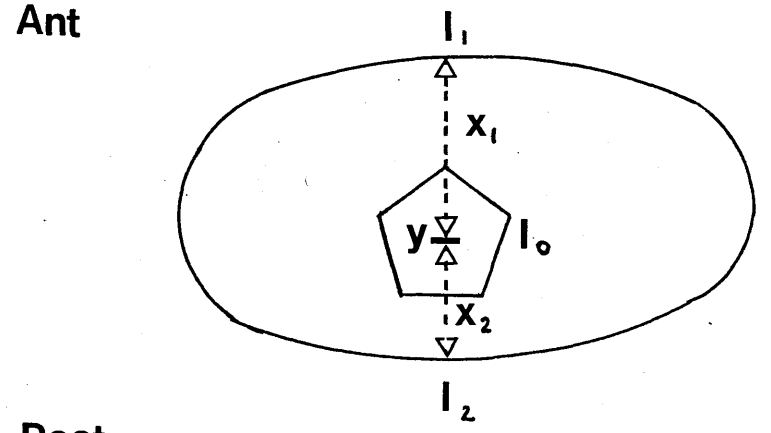
$$\frac{IT}{IP} = e^{-1/2u(YT-YP)} = f$$

The correction factor was then computed for different patient AP diameters using the log of this formula in the form $-1/2u(YT-YP) = \ln f$
The measured value of u for Temex (tissue equivalent) = 0.102
AP diameter of the Temex trunk = 23 cms in midline at liver level. The patients AP diameter was measured at the xiphisternum.

Patient diameter(cms)	Correction factor f
15	0.665
16	0.700
17	0.736
18	0.775
19	0.815
20	0.858
21	0.903
22	0.950
23	0.0
24	1.052
25	1.107
26	1.165
27	1.226
28	1.291
29	1.358
30	1.429

Appendix 6.2. Attenuation correction for other organs

The attenuation correction for the spleen, kidney and bone marrow uptake of radioactivity was based on the following mathematical model.



Transverse section of the patients body where

I_0 = organ cps at point y

I_1 = anterior detected organ cps at point y

I_2 = posterior detected organ cps at point y

u = linear attenuation coefficient (0.102 cm^{-1})

$I_1 = I_0e^{-u(X_1)}$ $\quad I_2 = I_0e^{-u(X_2)}$

The geometric mean of the counts $= \sqrt{I_1 \times I_2} = \sqrt{I_0{}^2e^{-u(X_1 + X_2)}}$

$= I_0e^{-1/2u(X_1 + X_2)}$

This can be applied to all other points within the organ.

Geometric mean of organ cps(Ip) $= I_A e^{-1/2u(X_1 + X_2)}$ where

I_A = unattenuated organ cps

$X_1 + X_2$ = patient antero-posterior diameter at the level of the xiphisternum

$f = e^{-1/2u(X_1 + X_2)}$

Dividing I_p by f corrects for attenuation of I_A

$$I_A = \frac{I_p}{e^{-1/2u(X_1 + X_2)}} = \frac{Ip}{f}$$

Errors in f may occur due to

1) The approximate nature of the measurement of u

2) Attenuation over the full patient width is corrected for using u.

3) The patients antero-posterior diameter is measured at the level of the xiphisternum and may differ over the organ in question.

Appendix 6.3. Urinary excretion and blood clearance data

		Urinary excretion (% I.D.)			Blood activity (% 10 min)				
No.	Initial	First 12hrs	Next 24hr	Next 24hrs	10min	30min	24hrs	48hrs	72hrs
1.	M.D.	20.1	3.3	3.7	100	92.4	56	44	10.3
2.	M.B.	NA	NA	NA	NA	NA	NA	NA	NA
3.	R.S.	21.3	3.4	1.9	100	105	67	43	NA
4.	R.N.	7.5	3.0	2.0	100	91.4	57.1	45	23
5.	D.N.	8.3	2.6	2.0	100	91	76	47	33
6.	J.L.	NA	NA	NA	100	106	56	42	25
7.	J.H.	15.5	4.7	NA	100	96	60	46	30
8.	B.T.	24	2.0	0.8	100	93	63	46	22
9.	J.P.	NA	NA	NA	100	91	56	NA	NA
10.	K.H.	NA	NA	NA	100	91	56	38	26
11.	M.J.	1.9	2.5	2.9	100	93	65	42	30
12.	B.B.	1.1	2.2	2.4	100	95	57	34	25
13.	V.D.	NA	NA	NA	100	97	70	33	29
14.	F.H.	NA	NA	NA	NA	NA	NA	NA	NA
15.	B.S.	3.6	1.9	NA	100	97	51	26	20
16.	R.D.	5.2	1.5	2.0	100	91	47	34	NA
17.	J.P.	5.2	3.0	2.3	100	96	53	40	NA
18.	E.B.	1.3	0.7	0.9	100	95	61	33	21
19.	D.O.N.	3.74	1.9	NA	100	91	52	38	NA
20.	D.F.	3.9	1.4	3.1	100	91	52	38	NA
21.	G.X.	4.5	4.4	1.5	100	80	54	NA	NA
22.	J.B.	6.1	2.7	2.4	100	94	53	28	17.5

Appendix 6.4. Dynamic liver uptake (first 30 mins)

Counts calculated from ROI analysis and expressed as cpm/ MBq injected dose.

Patient group	Time after administration(mins)					
Control (n=10)	5	10	15	20	25	30
	692	706	738	729	725	746
	308	325	309	NA	NA	NA
	470	462	459	456	463	459
	597	586	585	578	579	572
	695	673	665	663	663	644
	861	845	838	823	821	830
	664	648	638	644	638	647
	493	424	413	NA	NA	NA
	627	653	635	NA	NA	NA
	560	583	645	674	643	732
DTPA alone (n=3)	432	464	470	485	501	511
	615	632	660	676	690	685
	770	761	812	816	844	851
Cold MAb (n=5)	539	508	531	544	546	551
	421	426	413	410	403	407
	706	697	692	699	NA	NA
	463	439	436	434	430	432
	1022	1040	1001	1004	1005	1020

Appendix 6.5. Liver uptake of ^{111}In-ICR2 (% I.D.)

No.	Init.	Time after injection 45min	24hr	48hr	Patient group
1.	M.D.	22	25	30	Ctr
2.	M.B.	20	NA	NA	Ctr
3.	R.S.	20	23	23	Ctr
4.	R.N.	17	19	21	Ctr
5.	D.N.	18	18	19	Ctr
6.	J.L.	22	20	20	Ctr
7.	J.H.	15	17	19	Ctr
8.	B.T.	14	16	16	Ctr
9.	J.P.	13	22	NA	Ctr
10.	K.H.	20	26	27	Ctr
11.	M.J.	23	29	33	DTPA alone
12.	B.B.	20	23	30	DTPA alone
13.	V.D.	NA	25	27	DTPA alone
14.	F.H.	24	25	31	DTPA alone
15.	B.S.	27	34	36	DTPA alone
16.	R.D.	19	25	27	Cold MAb
17.	J.P.	17	20	NA	DTPA alone
18.	E.B.	12	19	24	Cold MAb
19.	D.O.N.	25	26	29	Cold MAb
20.	D.F.	18	27	30	Cold MAb
21.	G.X.	12	16	19	Cold MAb
22.	J.B.	26	24	26	Cold MAb

Appendix 6.6. Spleen uptake of ^{111}In-ICR2(% I.D.)

No.	Init.	Time after injection 45mins	24hours	48hours	Patient group
1.	M.D.	NA	NA	NA	Ctr
2.	M.B.	NA	NA	NA	Ctr
3.	R.S.	NA	NA	NA	Ctr
4.	R.N.	NA	2.0	1.7	Ctr
5.	D.N.	4.6	3.7	3.1	Ctr
6.	J.L.	NA	1.9	1.7	Ctr
7.	J.H.	1.9	1.6	1.7	Ctr
8.	B.T.	1.3	1.1	1.0	Ctr
9.	J.P.	1.4	1.5	NA	Ctr
10.	K.H.	3.0	3.2	2.4	Ctr
11.	M.J.	NA	2.9	2.9	DTPA alone
12.	B.B.	2.3	2.6	2.5	DTPA alone
13.	V.D.	NA	2.8	2.8	DTPA alone
14.	F.H.	4.0	3.8	3.2	DTPA alone
15.	B.S.	2.9	2.3	2.3	DTPA alone
16.	R.D.	2.7	2.6	2.4	Cold MAB
17.	J.P.	NA	1.2	NA	DTPA alone
18.	E.B.	3.3	3.3	2.5	Cold MAb
19.	D.O.N.	2.4	2.2	2.8	Cold MAb
20.	D.F.	2.5	2.4	2.1	Cold MAb
21.	G.X.	2.8	3.8	3.9	Cold MAb
22.	J.B.	4.3	3.5	3.3	Cold MAb

Appendix 6.7. Spleen uptake (cts/5 min/MBq)

No.	Init.	Time after injection 45mins	24hours	48hours	Patient group
1.	M.D.	340	389	396	Contol
2.	M.B.	335	NA	NA	Control
3.	R.S.	376	428	383	Control
4.	R.N.	385	486	455	Control
5.	D.N.	764	685	676	Control
6.	J.L.	442	320	298	Control
7.	J.H.	432	378	431	Control
8.	B.T.	322	302	290	Control
9.	J.P.	287	467	NA	Control
10.	K.H.	645	768	671	Control
11.	M.J.	456	453	436	DTPA
12.	B.B.	497	538	479	DTPA
13.	V.D.	NA	510	516	DTPA
14.	F.H.	690	659	630	DTPA
15.	B.S.	625	700	570	DTPA
16.	R.D.	671	676	610	Cold
17.	J.P.	747	830	NA	DTPA
18.	E.B.	706	782	752	Cold
19.	D.O.N.	648	888	730	Cold
20.	D.F.	939	965	823	Cold
21.	G.X.	922	950	1037	Cold
22.	J.B.	1004	895	851	Cold

Appendix 6.8. Renal uptake of ^{111}In-ICR2(% I.D.)

No.	Init.	Time after injection 45mins	24hours	48hours	Patient group
1.	M.D.	NA	NA	NA	Control
2.	M.B.	NA	NA	NA	Control
3.	R.S.	NA	NA	NA	Control
4.	R.N.	2.5	3.2	2.8	Control
5.	D.N.	1.5	1.7	1.9	Control
6.	J.L.	2.2	2.2	1.8	Control
7.	J.H.	1.6	1.3	1.6	Control
8.	B.T.	2.4	2.0	1.5	Control
9.	J.P.	2.5	2.7	NA	Control
10.	K.H.	1.5	1.6	1.3	Control
11.	M.J.	1.4	1.9	1.4	DTPA
12.	B.B.	1.7	2.5	2.3	DTPA
13.	V.D.	NA	1.9	1.9	DTPA
14.	F.H.	3.2	3.9	2.9	DTPA
15.	B.S.	2.5	2.6	2.4	DTPA
16.	R.D.	2.1	2.8	2.8	Cold
17.	J.P.	1.4	1.5	NA	DTPA
18.	E.B.	1.8	2.3	2.6	Cold
19.	D.O.N.	2.4	2.9	2.1	Cold
20.	D.F.	2.5	2.4	2.1	Cold
21.	G.X.	1.6	2.1	2.0	Cold
22.	J.B.	2.8	2.5	1.6	Cold

Appendix 6.9. Renal uptake of ^{111}In-ICR2 (cts / 5 min / MBq)

No.	Init.	Time after injection 45mins	24hours	48hours	Patient group
1.	M.D.	532	534	516	Control
2.	M.B.	328	NA	NA	Control
3.	R.S.	270	317	267	Control
4.	R.N.	537	655	588	Control
5.	D.N.	334	356	366	Control
6.	J.L.	450	448	394	Control
7.	J.H.	490	449	475	Control
8.	B.T.	553	505	440	Control
9.	J.P.	510	627	NA	Control
10.	K.H.	329	375	348	Control
11.	M.J.	249	252	230	DTPA
12.	B.B.	453	448	484	DTPA
13.	V.D.	NA	346	329	DTPA
14.	F.H.	611	717	687	DTPA
15.	B.S.	512	567	585	DTPA
16.	R.D.	507	526	604	Cold
17.	J.P.	357	481	NA	DTPA
18.	E.B.	376	494	524	Cold
19.	D.O.N.	610	731	659	Cold
20.	D.F.	655	699	647	Cold
21.	G.X.	381	474	446	Cold
22.	J.B.	614	684	640	Cold

Appendix 6.10. Marrow uptake of ^{111}In-ICR2 (Cts/5min/MBq)

No.	Init.	Time after injection 45mins	24hours	48hours	Patient group
1.	M.D.	133	143	150	Control
2.	M.B.	NA	NA	NA	Control
3.	R.S.	101	115	163	Control
4.	R.N.	121	135	136	Control
5.	D.N.	129	142	141	Control
6.	J.L.	214	189	186	Control
7.	J.H.	229	219	220	Control
8.	B.T.	187	165	169	Control
9.	J.P.	222	165	169	Control
10.	K.H.	135	136	150	Control
11.	M.J.	111	96	108	DTPA
12.	B.B.	141	135	157	DTPA
13.	V.D.	NA	165	169	DTPA
14.	F.H.	161	167	160	DTPA
15.	B.S.	178	217	191	DTPA
16.	R.D.	114	128	118	Cold
17.	J.P.	NA	NA	NA	DTPA
18.	E.B.	126	124	135	Cold
19.	D.O.N.	223	225	253	Cold
20.	D.F.	178	207	222	Cold
21.	G.X.	109	127	125	Cold
22.	J.B.	152	137	166	Cold

Appendix 6.11. Cold MAb and immune complex

No cold MAb

No.	Initial	Pre ^{111}In-ICR2	Post ^{111}In-ICR2 10min	30min	24hrs	48hrs
7.	J.H.	NA	40	36	36	NA
9.	J.P.	33	33	24	NA	NA
11.	M.J.	NA	28	33	26	NA
12.	B.B.	30	39	35	34	28
13.	V.D.	33	NA	NA	NA	NA
14.	F.H.	28	NA	NA	NA	NA
15.	B.S.	30	28	25	18	NA
17.	J.P.	33	NA	NA	NA	NA

Cold MAb

No.	Initial	Pre ^{111}In-ICR2	Post cold	Post ^{111}In-ICR2 10min	30min	24hr	48hr
16.	R.D.	27	NA	20	18	13	NA
18.	E.B.	34	28	35	35	28	28
19.	D.O.N.	27	29	30	29	22	17
20.	D.F.	35	31	32	29	23	NA
21.	G.X.	40	33	NA	NA	NA	NA
22.	J.B.	35	30	32	28	28	NA

The results shown are the % of the circulating activity present in high molecular weight form.

Appendix 7.1. : Statistical significance of data

Radioactive decay is a process of random disintegration of nuclei and as such is described mathematically by the statistics of Poisson processes. Random variations in disintegration rate give rise to a range of recorded values on successive measurement with a distribution about the mean value described by the Poisson distribution. An important feature of this distribution is that the variance of the distribution, σ^2 is equal to the mean, μ of the distribution $\sigma^2 = \mu$
and therefore that the standard deviation, σ , is given by $\sigma = \sqrt{\mu}$
Now, if a large number of readings are taken, then the mean value $\bar{x}$ is a good approximation to μ and so $\sigma^2 \approx \bar{x}$, $\sigma(x) \approx \sqrt{\bar{x}}$
Thus the standard deviation of a number x is approximately equal to the square root of the mean. For readings of large values, typical in radiation counting measurements, where the discrete distribution of numbers approximates to a distribution of a continuous variable then the Poisson distribution is virtually indistinguishable from the more general Gaussian (or normal) distribution for the case where $\sigma^2 = \mu$
For a Gaussian distribution it may be shown(329) that the propogation of errors for a result computed from a number of readings is such that the standard deviation of the sum or difference of two readings N1 and N2 , $\sigma(N_1 \pm N_2)$ is given by $\sigma(N_1 \pm N_2) = \sqrt{N_1 + N_2}$
and the standard deviation $\sigma(\bar{N})$ of a mean $\bar{N}$ of a number of readings n is given by $\sigma(\bar{N}) = \sqrt{\bar{N}/n}$
Thus , for the study described, the standard deviation of the mean tumour and background counts are respectively

$$\sigma(\bar{T}) = \sqrt{\bar{T}/3} \quad , \quad \sigma(\bar{B}) = \sqrt{\bar{B}/3}$$

and the standard deviation $\sigma(\bar{T}-\bar{B})$ of the difference between mean tumour and mean background counts is such that

$$\sigma(\bar{T}-\bar{B}) = \sqrt{\frac{\bar{T}}{3} + \frac{\bar{B}}{3}}$$

It can also be shown that the percentage error in the reading represented by the standard deviation is given by

$$\frac{\sqrt{N}}{N} \times 100\,\% = \frac{100\,\%}{\sqrt{N}}$$

This indicates a very important factor in radiation measurement techniques ; that as the counts obtained for a result are allowed to increase (by increasing counting time or use of a more sensitive detector) so the percentage uncertainty decreases and the statistical reliability of the result increases.

The variance σ^2 is a parameter such that 68.3% of results fall within +/-σ of the true mean. Additionally 90% of results lie within +/-1.64 σ , 95% within +/- 2 σ and 99.7% within +/- 3 σ . These are termed confidence levels and from this it can be stated that if a result lies beyond +/-3 σ from the mean, there is a chance of less than 1% that this is due to random error alone, and the difference can be considered to be statistically significant.

Thus if 3 s.d. (3 σ) is used as a threshold of detectability then the difference in mean tumour and background counts must exceed this for the tumour to be deemed detectable

$$\bar{T} - \bar{B} \geqslant 3\sqrt{\frac{\bar{T}}{3} + \frac{\bar{B}}{3}}$$

Appendix 7.2. : CdTe probe , 2:1 ratio, w.a.collimator,

Size (mls)	Decay factor[1]	Tumour mean[2]	S.D.[3]	Corrected mean[4]	Background mean[2]	S.D.[3]	Corrected mean[4]	Signif.[5]
20 second counts								
0.5	1.023	2487	32.0	2544	2480	75.5	2537	0.17
1.0	1.029	2449	18.5	2520	2481	37.0	2553	-0.80
1.5	1.031	2382	25.9	2456	2474	53.6	2551	-2.33
2.0	1.037	2457	60.7	2548	2493	60.9	2585	-0.89
5.0	1.044	2503	11.4	2613	2448	118.3	2556	1.37
10.0	1.047	2489	32.9	2606	2405	65.3	2518	2.13
100 second counts								
0.5	1.023	12381	119.6	12666	12481	171.2	12768	-1.11
1.0	1.029	12281	161.9	12637	12430	212.6	12790	-1.66
1.5	1.031	12135	62.2	12511	12388	92.3	12772	-2.84
2.0	1.037	12265	84.4	12719	12269	113.7	12723	-0.04
5.0	1.044	12268	74.8	12808	12078	93.9	12609	2.16
10.0	1.047	12409	123.8	12992	12188	74.5	12760	2.50

1 Correction for radioactive decay occuring during the period of the experiment
2 Mean counts corrected for room background activity
3 Standard deviation of the raw counts
4 Mean counts corrected for radioactive decay
5 Number of standard deviations between mean tumour and mean background counts (by method outlined in Appendix 7.1.).

Appendix 7.3. : CdTe probe , 2:1 ratio, p.h. collimator

Size (mls)	Decay factor[1]	Tumour mean[2]	S.D.[3]	Corrected mean[4]	Background mean[2]	S.D.[3]	Corrected mean[4]	Signif.[5]
20 second counts								
0.5	1.053	84.7	12.7	89.2	81.0	3.4	85.3	0.51
1.0	1.056	88.0	8.0	92.9	74.3	6.6	78.5	1.91
1.5	1.058	79.0	4.6	83.6	65.3	5.5	69.1	2.03
2.0	1.062	83.7	7.4	88.9	75.0	4.4	79.7	1.23
5.0	1.066	83.3	9.9	94.1	70.7	10.8	75.3	2.50
10.0	1.069	89.3	23.3	95.5	79.0	9.0	84.5	1.42
100 second counts								
0.5	1.053	390.3	19.5	410.9	391.7	28.4	412.5	-0.10
1.0	1.056	416.3	8.1	439.6	392.0	41.6	413.9	1.52
1.5	1.058	401.7	4.6	425.0	380.3	27.3	402.4	1.36
2.0	1.062	394.0	23.1	418.4	402.7	27.3	427.7	-0.55
5.0	1.066	417.3	19.5	444.8	388.3	8.6	413.9	1.83
10.0	1.069	440.0	13.4	470.4	389.0	3.5	415.8	3.17

1-5 as in Appendix 7.2.

Apppendix 7.4. : NaI probe, 2:1 ratio

Size (mls)	Decay factor[1]	Tumour mean[2]	S.D.[3]	Corrected mean[4]	Background mean[2]	S.D.[3]	Corrected mean[4]	Signif.[5]
20 second counts								
0.5	1.100	684.3	31.3	752.7	699.0	13.5	768.9	-0.72
1.0	1.095	681.7	31.8	746.5	701.0	10.6	767.6	-0.94
1.5	1.092	703.0	19.7	767.7	699.7	35.9	764.0	0.16
2.0	1.089	683.3	18.5	744.1	675.7	7.8	735.8	0.37
5.0	1.083	743.3	32.5	804.9	693.3	50.5	750.9	2.37
10.0	1.080	772.3	33.3	834.1	691.3	37.8	746.6	3.81
100 second counts								
0.5	1.100	3420	58.1	3762.7	3472.0	38.0	3819.2	-1.12
1.0	1.095	3500	28.7	3832.9	3380.0	46.0	3701.1	2.63
1.5	1.092	3497	71.6	3819.1	3437.6	22.9	3753.9	1.30
2.0	1.089	3492	47.1	3802.4	3512.3	21.0	3824.9	-0.45
5.0	1.083	3707	19.8	4014.3	3549.7	19.4	3844.3	3.32
10.0	1.080	3803	113.5	4107.2	3400.7	37.5	3672.7	8.53

1-5 as in Appendix 7.2.

Appendix 7.5. : CdTe 4 : 1 ratio, w.a. collimator

Size (mls)	Decay factor[1]	Tumour mean[2]	S.D.[3]	Corrected mean[4]	Background mean[2]	S.D.[3]	Corrected mean[4]	Signif.[5]
20 second counts								
0.5	1.046	2661	65.0	2783.8	2645	17.4	2766.7	0.39
1.0	1.048	2690	16.8	2819.1	2643	43.1	2770.2	1.13
1.5	1.053	2618	53.5	2757.5	2700	21.9	2843.1	-1.98
2.0	1.056	2672	151.9	2821.9	2656	71.7	2815.3	0.15
5.0	1.058	2745	93.7	2904.9	2629	45.4	2782.2	2.82
10.0	1.061	2838	49.1	3010.8	2582	38.2	2739.9	6.19
100 second counts								
0.5	1.046	13219	290.0	13827	13304	139.4	13916	-0.93
1.0	1.048	13416	120.4	14050	13239	71.5	13874	1.82
1.5	1.053	13393	154.4	14103	13288	120.9	13992	1.15
2.0	1.056	13243	105.3	13985	13106	175.3	13840	1.51
5.0	1.058	13864	26.0	14668	13139	166.3	13901	7.86
10.0	1.061	14075	135.7	14934	13102	66.6	13901	10.54

1-5 as in Appendix 7.2.

Appendix 7.6. : CdTe probe, 4:1 ratio, p.h. collimator

Size (mls)	Decay factor[1]	Tumour mean[2]	S.D.[3]	Corrected mean[4]	Background mean[2]	S.D.[3]	Corrected mean[4]	Signif.[5]
20 second counts								
0.5	1.023	84.7	6.3	85.59	95.7	12.9	97.87	1.57
1.0	1.026	99.3	12.7	101.92	86.3	12.2	88.58	1.67
1.5	1.029	101.0	4.0	103.93	83.7	17.0	86.09	2.24
2.0	1.033	109.0	14.0	112.60	94.3	12.8	97.45	1.81
5.0	1.037	103.3	8.1	107.16	82.7	6.1	85.73	2.67
10.0	1.039	119.0	13.2	123.64	84.3	8.4	87.62	4.29
100 second counts								
0.5	1.023	468.3	11.0	479.11	447.0	16.4	456.26	1.29
1.0	1.026	503.0	11.5	516.08	433.0	35.4	444.26	4.01
1.5	1.029	513.0	9.0	527.88	443.0	11.8	454.82	4.04
2.0	1.033	520.7	14.0	537.85	445.3	24.6	460.03	4.27
5.0	1.037	573.0	46.1	594.20	458.7	43.4	475.64	6.28
10.0	1.039	595.3	16.3	618.55	455.0	9.5	472.75	7.64

1-5 as in Appendix 7.2.

Appendix 7.7. : Na I probe, 4:1 ratio

Size (mls)	Decay factor[1]	Tumour mean[2]	S.D.[3]	Corrected mean[4]	Background mean[2]	S.D.[3]	Corrected mean[4]	Signif.[5]
20 second counts								
0.5	1.242	658	20.3	790.3	639.3	8.1	794.1	-0.17
1.0	1.239	693	20.3	831.4	617.3	41.1	764.9	2.88
1.5	1.235	705	18.1	843.1	621.3	29.0	767.9	3.27
2.0	1.229	681	22.3	836.9	673.7	20.2	827.9	0.38
5.0	1.228	810	43.4	995.1	651.0	23.8	799.4	8.00
10.0	1.217	834	25.9	1015.4	673.0	12.1	792.3	9.09
100 second counts								
0.5	1.242	3367	85.1	4182.2	3140	58.4	3900.0	5.44
1.0	1.239	3387	30.9	4196.1	3202	26.0	3967.3	4.39
1.5	1.235	3431	118.9	4237.3	3144	81.3	3883.3	6.80
2.0	1.229	3447	87.9	4237.2	3261	64.1	4008.2	4.37
5.0	1.228	3926	60.7	4820.7	3244	91.7	3984.5	15.44
10.0	1.217	4170	33.2	5074.9	3203	93.2	3935.4	20.79

1-5 as in Appendix 7.2.

Appendix 7.8. : CdTe probe , 6:1 ratio , w.a. collimator

Size (mls)	Decay factor[1]	Tumour mean[2]	S.D.[3]	Corrected mean[4]	Background mean[2]	S.D.[3]	Corrected mean[4]	Signif.[5]
20 second counts								
0.5	1.058	2757	76.9	2916.9	2775	52.6	2930.3	-0.31
1.0	1.056	2828	107.4	2986.4	2887	36.1	3058.9	-1.62
1.5	1.052	2908	48.4	3059.6	2831	47.6	2977.9	1.82
2.0	1.049	2894	28.8	3035.8	2820	44.3	2958.9	1.72
5.0	1.046	3081	60.6	3222.7	2803	47.6	2928.8	6.49
10.0	1.040	3299	22.0	3431.3	2874	87.8	2988.9	9.56
100 second counts								
0.5	1.058	13741	77.8	14538	13942	128.8	14751	-2.15
1.0	1.056	14283	190.3	15083	13977	17.3	14760	3.24
1.5	1.052	14324	40.1	15069	13914	68.5	14638	4.33
2.0	1.049	14241	64.3	15967	13966	205.0	14650	3.19
5.0	1.046	15240	71.6	15941	14006	60.8	14619	13.10
10.0	1.040	16526	179.3	17187	14376	91.7	14619	21.59

1-5 as in Appendix 7.2.

Appendix 7.9. : CdTe probe , 6:1 ratio, p.h. collimator

Size (mls)	Decay factor[1]	Tumour mean[2]	S.D.[3]	Corrected mean[4]	Background mean[2]	S.D.[3]	Corrected mean[4]	Signif.[5]
20 second counts								
0.5	1.021	105.0	13.1	107.21	95.7	11.5	97.68	1.15
1.0	1.026	103.0	8.5	105.68	98.3	7.4	100.89	0.58
1.5	1.028	105.7	16.2	108.63	101.0	6.2	103.83	0.57
2.0	1.031	113.0	7.2	116.50	92.0	7.9	94.85	2.58
5.0	1.034	135.6	19.6	140.28	95.0	12.1	98.23	4.72
10.0	1.037	140.3	5.0	145.53	93.3	8.1	96.79	5.42
100 second counts								
0.5	1.021	528.7	24.3	539.77	486.7	18.8	496.89	2.31
1.0	1.026	544.3	8.7	558.49	462.7	46.1	474.69	4.52
1.5	1.028	541.0	30.8	556.15	506.7	16.2	520.85	1.86
2.0	1.031	585.6	35.0	603.82	471.3	18.6	485.95	6.18
5.0	1.034	662.0	10.5	684.51	444.3	6.8	459.44	11.53
10.0	1.037	738.6	33.6	765.99	473.6	6.4	491.19	13.42

1-5 as in Appendix 7.2.

Appendix 7.10. : Na I probe, 6:1 ratio

Size (mls)	Decay factor[1]	Tumour mean[2]	S.D.[3]	Corrected mean[4]	Background mean[2]	S.D.[3]	Corrected mean[4]	Signif.[5]
20 second counts								
0.5	1.073	808	34.3	866.6	754	22.6	809.4	2.42
1.0	1.075	815	24.8	876.5	739	43.3	822.0	2.29
1.5	1.080	826	21.2	892.4	745	22.3	804.9	3.68
2.0	1.083	862	25.4	933.9	748	24.4	809.7	5.15
5.0	1.086	1010	48.3	1097.6	729	41.6	791.7	12.19
10.0	1.089	1102	36.7	1200.4	707	24.8	770.3	16.78
100 seconds counts								
0.5	1.073	3999	93.5	4291.6	3780	33.6	4055.9	4.47
1.0	1.075	4138	34.7	4448.3	3851	54.0	4140.5	5.75
1.5	1.080	4283	77.1	4626.4	3710	20.2	4007.5	11.53
2.0	1.080	4345	152.6	4705.9	3690	45.6	3996.9	13.16
5.0	1.086	5022	26.9	5453.9	3743	57.0	4064.9	24.66
10.0	1.089	5502	32.0	5991.3	3718	66.2	3934.6	35.76

1-5 as in Appendix 7.2.

Appendix 7.11. : CdTe probe, 8 : 1 ratio, w.a. collimator

Size (mls)	Decay factor[1]	Tumour mean[2]	S.D.[3]	Corrected mean[4]	Background mean[2]	S.D.[3]	Corrected mean[4]	Signif.[5]
20 second counts								
0.5	1.048	2961	36.2	3102.4	2837	60.8	2973.9	2.86
1.0	1.050	2971	13.1	3118.5	2841	55.5	2983.8	2.99
1.5	1.053	2933	141.7	3088.8	2759	34.0	2905.9	4.09
2.0	1.058	2962	11.1	3134.1	2721	53.8	2878.9	5.70
5.0	1.060	3070	54.3	3254.6	2804	49.4	2972.2	6.20
10.0	1.064	3303	74.4	3514.7	2633	20.3	2801.1	15.55
100 second counts								
0.5	1.048	14692	58.9	15397	14190	179.4	14871	5.24
1.0	1.050	14697	111.7	15432	14280	64.1	14994	4.35
1.5	1.053	14745	58.3	15526	14052	5.0	14797	7.25
2.0	1.058	14673	130.3	15524	13754	64.6	14552	9.71
5.0	1.060	15650	112.1	16589	13936	51.2	14772	17.77
10.0	1.064	16709	29.0	17779	13487	17.0	14350	33.13

1-5 as in Appendix 7.2.

Appendix 7.12. : CdTe probe, 8:1 ratio, p.h. collimator

Size (mls)	Decay factor[1]	Tumour mean[2]	S.D.[3]	Corrected mean[4]	Background mean[2]	S.D.[3]	Corrected mean[4]	Signif.[5]
20 second counts								
0.5	1.080	97	7.0	104.76	88.0	7.2	95.04	1.19
1.0	1.079	115	6.1	124.44	84.3	12.0	90.99	3.95
1.5	1.075	113	5.0	121.48	97.3	7.5	104.63	1.94
2.0	1.071	127	6.8	136.37	88.0	6.2	94.25	4.80
5.0	1.069	131	15.0	140.39	94.3	8.5	100.84	4.41
10.0	1.066	165	10.4	175.89	88.7	8.1	94.52	8.57
100 second counts								
0.5	1.080	516	30.0	557.64	446	6.4	482.40	4.04
1.0	1.079	531	24.0	571.15	471	25.5	508.93	3.28
1.5	1.075	573	16.2	515.15	460	17.3	494.50	6.29
2.0	1.071	599	28.6	641.53	441	16.2	473.03	8.74
5.0	1.069	673	48.4	719.08	459	8.0	491.03	11.35
10.0	1.066	840	36.5	899.35	459	38.6	488.94	19.08

1-5 as in Appendix 7.2.

Appendix 7.13. : NaI probe , 8:1 ratio

Size (mls)	Decay factor[1]	Tumour mean[2]	S.D.[3]	Corrected mean[4]	Background mean[2]	S.D.[3]	Corrected mean[4]	Signif.[5]
20 second counts								
0.5	1.217	726	14.2	883.5	698	5.0	850.28	1.38
1.0	1.221	782	21.5	954.42	673	13.6	822.55	5.42
1.5	1.255	811	37.4	1017.81	654	3.8	821.61	7.92
2.0	1.264	845	14.4	1068.08	631	18.3	798.00	10.82
5.0	1.260	963	34.5	1212.9	657	23.1	827.40	14.78
10.0	1.270	1085	17.9	1378.8	682	13.4	866.78	18.71
100 second counts								
0.5	1.217	3708	26.0	4512.6	3431	65.1	4175.5	6.26
1.0	1.221	3943	133.1	4814.4	3442	44.0	4202.7	11.16
1.5	1.255	4061	57.5	5096.9	3384	28.5	4246.5	15.24
2.0	1.264	4286	71.6	5417.9	3239	87.5	4094.9	23.49
5.0	1.260	4812	170.0	6063.1	3357	72.9	4230.7	31.28
10.0	1.270	5492	149.2	6974.8	3236	52.9	4109.7	47.14

1-5 as in Appendix 7.2.

Publications and presentations on this work

Papers

1) **Davidson B.R.**, Yiu C.Y., Styles J., Ormerod M.G., Clark C.G., Dean C.,
A comparison of carcinoembryonic antigen (CEA) and epithelial membrane antigen (EMA) in human colorectal cancer.
Int J Cancer 1988 ; Supp 3 : 56-60

2) **Davidson B.R.**, Sams V., Styles J., Dean C., Boulos P.B.
A comparative study of carcinoembryonic antigen and epithelial membrane antigen expression in normal colon, adenomas and adenocarcinomas of the colon and rectum.
Gut 1989 (in press)

3) Yiu C.Y., Baker L., **Davidson B.R.**, et al
Imaging of colorectal tumours with an antibody to epithelial membrane antigen (EMA).
Dis Col Rectum 1989 (in press)

4) **Davidson B.R.**, Sams V., Styles J., Dean C., Boulos P.B.,
The detection of occult nodal metastases in patients with colorectal cancer.
Cancer 1989 (in press)

5) **Davidson B.R.**, Boulos P.B., Porter J.,
The role of chelating agents in inhibiting the non specific liver uptake of In111 labelled monoclonal antibodies.
J Nucl Med 1989 (submitted for publication)

Abstracts

1) **Davidson B.R.**, Sams V.R., Styles J., Clark C.G.,
Carcinoembryonic antigen (CEA) and epithelial membrane antigen (EMA) in colorectal cancers.
Gut 1988 ; 29 : A1484

2) Yiu-Yiu C., Baker L., **Davidson B.R.** et al,
Immunoscintigraphy of colorectal cancers with an antibody to epithelial membrane antigen(EMA).
Proceedings of Advances in the Applications of Monoclonal Antibodies in Clinical Oncology. Royal Postgraduate Medical school, London, 25-27th May 1988.

3) **Davidson B.R.**, Yiu-Yiu C., Styles J., Ormerod M., Clark C.G., Dean C., A comparison of carcinoembryonic antigen and epithelial membrane antigen in human colorectal cancer. Proceedings of Advances in the Applications of Monoclonal Antibodies in Clinical Oncology, Royal Postgraduate Medical School, London, 25-27th May 1988.

4) Yiu-Yiu C, Baker L., **Davidson B.R.**, et al. Immunoscintigraphy of colorectal cancers using Indium labelled monoclonal antibody 77.1. Proceedings of Advances in the Applications of Monoclonal Antibodies in Clinical Oncology, Royal Postgraduate Medical school, London, 25-27th May 1988.

5) **Davidson B.R.**, Waddington W.A., Short M.D., Boulos P.B., Clinical use of a radiation detecting probe for the intra-operative localisation of colorectal cancers and metastases. Nucl Med Commun 1989 ; 10 : 222

6) **Davidson B.R.**, Boulos P.B., Porter J.P., Gastrointestinal cancer localisation with ^{111}In labelled monoclonal antibodies (MAb's) : can ^{111}In chelating agents improve the results obtained ? Nucl Med Commun 1989 ; 10 : 246

7) **Davidson B.R.**, Waddington W.A., Short M.D., Boulos P.B., Intra-operative localisation of colorectal cancers and their metastases with a radiation detecting probe. Gut 1989 ; 30 (5) : A 713.

8) **Davidson B.R.**, Young H., Waddington W., Clarke G., Short M., Boulos P., Styles J., Dean C., Ell P. Epithelial membrane antigen (EMA) : A target antigen for radiolabelled monoclonal antibodies (MAb's) in colorectal cancer imaging ? J Nucl Med 1989 ; 30 : 808.

9) Babich J., **Davidson B.**, Young H., Waddington W., Clarke G., Short M., Boulos P., Styles J., Dean C., Cold antibody administration and the biodistribution of In111 labelled monoclonal antibody (MAb) in colorectal cancer patients. J Nucl Med 1989 ; 30 : 809

10) Waddington W.A., **Davidson B.R.**, Short M.D.,
An evaluation of two probe systems for the intra-operative detection of 111In-labelled monoclonal antibodies (MAb's) against gastrointestinal cancer.
J Nucl Med 1989 ; 30 : 891

11) **Davidson B.**, Waddington W., Short M., Boulos P.,
Intra-operative localisation of colorectal cancers and their metastases with a radiation detecting probe.
J Nucl Med 1989 ; 30 : 908

12) **Davidson B.**, Boulos P., Porter P.,
Radiolabelled antibody imaging of gastro-intestinal cancers : can chelating agents improve the results obtained?
J Nucl Med 1989 ; 30 : 909

Presentations

1) **Davidson B.R.**, Yiu-Yiu C., Styles J., Ormerod M., Clark C.G., Dean C.,
A comparison of carcinoembryonic antigen (CEA) and epithelial membrane antigen (EMA) in human colorectal cancer.
5th International meeting on the applications of monoclonal antibodies in cancer detection and therapy, Royal Postgraduate Medical School, 27-29 th May 1988

2) **Davidson B.R.**, Yiu-Yiu C., Styles J., Ormerod M., Clark C.G., Dean C.,
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International Society of Colon and Rectal Surgeons, Glasgow, 10-14th July 1988.

3) **Davidson B.R.**, Sams V., Styles J., Clark C.G.
The expression of CEA and EMA in colorectal cancer
British Society of Gastroenterology, Sheffield, 14-17th September 1988

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Clinical use of a radiation detecting probe for the intra-operative localisation of colorectal cancers and metastases.
British Nuclear Medicine Society, London, 10th April 1989.

5) **Davidson B.R.**, Boulos P.B., Porter J.,
Gastro-intestinal cancer localisation with 111In labelled monoclonal antibodies (MAb) : can 111-In chelating agents improve the results obtained ?
British Nuclear Medicine Society, London, 11th April 1989.

6) **Davidson B.R.**, Waddington W.A., Short M.D., Boulos P.B.,
Intra-operative localisation of colorectal cancers and their metastases with a radiation detecting probe.
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7) **Davidson B.R.**, Young H., Waddington W., Clarke G., Short M., Boulos P.B., Styles J., Dean C., Ell P.J.,
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Cold antibody administration and the biodistribution of ^{111}In labelled monoclonal antibody (MAb) in colorectal cancer patients.
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12) **Davidson B.R.**, Waddington W.A., Short M.D., Boulos P.B.,
Intra-operative detection of cancer using radiolabelled monoclonal antibodies : clinical use and experimental evaluation.
Surgical Research Society, Newcastle, 6 - 7th July 1989.