A SEARCH FOR MONOCLONAL ANTIBODIES FOR THE IMMUNOLOCALISATION OF COLORECTAL CANCER

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by

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ABSTRACT

For the immunoscintigraphy or antibody targeting of colorectal cancer there is a need for monoclonal antibodies with better specificity and reactivity. The search for such antibodies forms the basis of this thesis. Two approaches were used.

First, new monoclonal antibodies were produced against a crude membrane extract prepared from several colorectal cancers. Ten fusions were performed between immunised spleen cells from Balb C mice and the mouse myeloma cell line NSO, producing a total of 893 hybridomas of which 245 secreted antibodies positive on ELISA. Only 67 of these antibodies stained frozen sections of colorectal cancer. Five hybridomas (UC-ICR 12.1, 20.3, 40.1, 41.2 and 41.6) were cloned to monoclonal stage. UC-ICR 41.2 and 41.6 showed a favourable tissue distribution on immunocytochemistry and may be suitable for immunoscintigraphy.

Second, immunoperoxidase technique was used to test 25 different monoclonal antibodies for cross-reactivity to colorectal cancer. Three monoclonals, M8, 77-1 and 8-30-3, which recognise epitopes of epithelial membrane antigen (EMA), showed a preferential affinity to the cancer when compared to normal colon. One of these, M8 has already been used for the immunoscintigraphy of breast cancer.

The monoclonal antibody 77-1, radiolabelled with iodine-125 and indium-111, was found to retain immunoreactivity on live cell binding assays and xenograft localisation studies.

In a clinical localisation study indium-111 labelled M8 detected 13 out of 21 (62%) tumour sites (13 primary tumours, 5 liver metastases and 3 recurrent

tumours) in 16 patients. The uptake of the radiolabel as measured by the tumour to blood ratio was 5.3 $\pm$ 3.7 and the mean tumour to normal colon ratio was 2.5 $\pm$ 1.20.

Indium-111 labelled 77-1 detected 10 of 17 (59%) tumour sites (12 primary, 3 liver metastases, and 2 recurrent tumours) in 14 patients. The tumour to blood ratio was 3.6 $\pm$ 1.43 and the mean tumour to normal colon ratio was 2.0 $\pm$ 0.81.

These results showed that anti-EMA monoclonal antibodies have a role in the immunoscintigraphy of colorectal cancer. The new monoclonal antibodies UC-ICR 41.2 and 41.6 are worth further evaluation.

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STATEMENT OF ORIGINALITY

In the immunolocalisation of colorectal cancer for the purpose of immunoscintigraphy or therapy, the phenomenon of tumour heterogeneity, the human anti-mouse antibody response and lack of colorectal cancer-specific monoclonal antibodies have defined a need for more monoclonals with better specificity to this cancer. A search for such monoclonal antibodies was therefore undertaken by the author. In this endeavour, the planning of the investigation and the performance of all experiments, tissue culture and the generation of monoclonal antibodies, immunocytochemistry and immunological assays described in the thesis were personally carried out by the investigator. Technical assistance and laboratory facilities have been acknowledged.

1. Five new monoclonal antibodies have been raised to colorectal cancer using a crude membrane extract prepared from several patients. Two antibodies may be suitable for clinical application.

2. Using the phenomenon of the cross-reactivity of monoclonal antibodies, five colorectal cancer-reactive monoclonal antibodies, not previously evaluated for reactivity with this cancer, have been identified by means of immunocytochemistry. Two antibodies with anti-epithelial membrane antigen (EMA) properties were selected for the immunoscintigraphy of colorectal cancer. This finding suggests that other tumour-reactive monoclonal antibodies may be similarly identified.

3. One of the two anti-EMA monoclonal antibodies, not previously radiolabelled or

used for immunoscintigraphy, was radiolabelled with iodine-125 and indium-111 and the retention of *in vitro* and *in vivo* immunoreactivity of the radiolabelled antibody was demonstrated. This enabled the antibody to be used in a radioimmunolocalisation study in colorectal cancer patients.

4. The localisation of the two antibodies to colorectal cancer forms the first detailed clinical study of anti-EMA monoclonal antibodies in the immunoscintigraphy of colorectal cancer, and suggests that such antibodies have a role for the immunodetection of this carcinoma.

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CHAPTER 1

COLORECTAL CANCER AND MONOCLONAL ANTIBODIES

1.1 INTRODUCTION

Colorectal cancer is the second commonest cancer in the Western world, responsible for 67 deaths per 100,000 population in England and Wales in 1978 (Cancer Statistics 1981). Furthermore, its incidence is rising in USA (LeFall, 1981) and in Australia (Dent et al., 1981). Over the past 30 years the survival rate for colorectal cancer following surgery has not improved (Nicholls, 1982; Reasbeck, 1987). The average survival has remained around 20-50% at 5 years (Slaney, 1971; Gill & Morris, 1978; McDermott et al., 1980; Phillips et al., 1984), principally because of late presentation. The result is disappointing considering that this is a potentially curable disease if diagnosed early, as the range of 5 year survival of Dukes A carcinoma is 90%, Dukes B 54-58% and Dukes C 12-27% (Clark et al., 1987). However, less than 6% of patients with diagnosed colorectal cancer have Dukes' A stage (Gill & Morris, 1978). The benefit for early detection was shown in a study by Gilbertsen & Nelms (1978), who performed repeated sigmoidoscopy in 21,150 patients over 45 between 1948-1976. An average of 5.4 examinations were performed in each patient and any polyps present were removed. Initial examinations of these patients detected 27 cancers with another 13 found on follow up. The outcome of surgery was good as these patients had early cancers. The expected incidence of rectal cancers was reduced by 90%.

Patients with diverticular disease may have a co-existing carcinoma or polyp, and colonoscopy in this group of patients detected tumours not revealed by radiology (Boulos et al., 1984). It is an encouragement to observe a rising detection rate (15%) of symptomatic Dukes' A colorectal carcinomas (Vellacott et al.,1987) through the more widely used lower gastrointestinal endoscopy and improved radiology. However, population screening using faecal occult blood test

is probably a more practical way of detecting early colorectal cancer in the asymptomatic subjects (Clark et al., 1987), but this has its difficulties as the compliance rate is generally low, there is still a lack of a test with a high sensitivity and specificity for colorectal cancer and the necessary resources and facility needed to perform such an exercise may not be available. Until a satisfactory means of early colorectal cancer detection is available, the prognosis of this tumour is likely to remain unchanged, particularly as the treatment of metastatic carcinomas with various adjuvant therapies have not made a significant impact.

The current management of colorectal cancer is based on the accurate staging of the disease, appropriate surgery, adjuvant therapy and follow-up to detect early recurrent tumour. More recently efforts have been made to treat liver metastases which are a major influence on the prognosis of the disease. Advances in monoclonal antibodies may contribute to the management of colorectal cancer in their potential role in tumour localisation for the purpose of early diagnosis of recurrent disease and in therapeutic targeting.

1.2 Staging of Colorectal Cancer

Staging of colorectal cancer is important in determining the appropriate treatment. It allows the results of different therapies to be compared and may serve as a prognostic indicator.

Histological staging of rectal cancer was described by Dukes as long ago as 1932 and this is still the most widely adopted method. When the tumour is confined to the bowel wall, it is classified as stage A; stage B when the tumour invades the bowel wall and stage C when there is lymph node involvement. This classification has been modified by others, for example, Astler and Coller (1954)

and Gunderson and Sosin (1974) who subdivide the different stages into the depths of tumour penetration and the level of lymph nodes involvement. However, the newer systems are more complex to record and may cause confusion without significant improvememt in prognostication. The only useful modification is the addition of stage D by Turnbull et al. (1967), for distant metastases. This classification has improved the survival statistics of stage B and C group, but distant metastases may not be obvious to detection and may have already occurred at the time of surgery. This may explain a study by Gilbert et al. (1984) who performed a necropsy and a prospective clinical series examining the extent of tumour metastases. They found that the necropsy series showed a dissemination of recurrent tumour in 73% of patients, and that recurrence in a single site only occurred in 27% of the patients. The clinical series however, revealed a single site recurrence in 55%. The difference between these series is statistically significant and indicates an under-diagnosis of dissemination by clinical investigation. The incidence of local recurrence and liver secondaries was about the same in both series but they usually formed part of a more widespread disease. This study illustrates the deficiency of clinical staging methods and confirms the need for more effective tumour detection techniques.

Staging may be performed clinically by means of a physical examination, including different imaging techniques such as radioisotope scanning, ultrasound and computer tomographic (CT) scanning. In a comparison of these three imaging techniques for hepatic secondaries, CT scan was the best, followed by ultrasound and radioisotope scan (Finlay et al., 1982). Ultrasound and CT scan detected an incidence of 24% occult liver metastases (Finlay & McArdle, 1986) which were not found at laparotomy. The presence or absence of these metastases at the time of apparently curative resection for colorectal

carcinoma predicts the majority of deaths from disseminated disease. More recently, intra-operative ultrasound has been used to detect liver metastases with tumour as small as 0.4 cm being identified (Machi et al., 1986). CT scan is also useful in the assessment of tumour bulk and the extent of tumour spread in rectal carcinomas (Dixon et al., 1981). It is the best available method for the detection of recurrent rectal cancer following an abdomino-perineal excision of the rectum (Williams & Husband, 1987). However, rectal cancer may be staged accurately by a rectal examination performed by an experienced clinician (Nicholls et al., 1982) who can assess the mobility of the tumour and the presence of lymph nodes in the mesorectum. The interpretation of tumour fixity poses difficulty as this may be due to an inflammatory process rather than tumour dissemination. An ultrasound probe placed inside the rectum appears to be a reliable indicator of local tumour invasion. When compared with CT scan and digital rectal examination, the ultrasound technique was best giving an accuracy of 91%, sensitivity of 94%, specificity of 87%, positive predictive value of 97% and negative predictive value of 78% (Beynon et al., 1986). Neither CT scan nor endoluminal ultrasound can reliably identify lymph node metastases in rectal cancer and therefore it cannot be used to select patients for local excision of tumour (Holdsworth et al., 1988).

Carcinoembryonic antigen (CEA) is a marker described by Gold and Freedman (1965 a,b) and may contribute in staging of colorectal cancer. It was originally thought to be a specific marker for colorectal cancer but it was later found in normal colon mucosa (Martin & Martin, 1970; Fritsche & Mach, 1977; Shively et al., 1978), pancreatic cancer, breast cancer, small cell carcinoma of the lung (O'Brien et al., 1980), other non-gastrointestinal carcinomas (Pusztaszeri & Mach, 1973) as well as different liver disorders such as hepatitis, cirrhosis, and cholestasis due to benign or malignant diseases (Zamcheck,1983). CEA also

shares antigenic determinants with cross-reacting substances present in large amount in normal adult tissues (Mach & Pusztaszeri, 1972; Von Kleist et al., 1972; Turberville et al., 1973). Abnormal liver function tests, particularly a raised alkaline phosphatase, may indicate liver metastases. These biochemical tests are not entirely specific and confirmation with other investigations are usually necessary.

The various staging methods available are essential in the management of colorectal cancer, and they are often complementary to each other. A detailed staging technique for rectal cancer called extended staging (ES) has been used by Williams et al. (1985). This consisted of CT scans of the liver and pelvis, serum CEA measurement, acute phase reactant proteins, multiple superficial and deep biopsies of the tumour for the determination of histological grade and DNA cellular content as measured by flow cytometry. A combination of these tests gave a better assessment of true tumour bulk and the extent of tumour spread, enabling a decision to be made about the type of operation, such as anal sphincter sparing and pre-operative radiotherapy or the administration of intra-operative chemotherapy as appropriate. However, despite recent advances, better detection methods for more accurate tumour staging, particularly in recurrent cancer, are still needed.

1.3 Surgery for Colorectal Cancer

Surgery is the only treatment that offers the prospect of a cure in patients with colorectal cancer. In recent years operative mortality and the necessity for colostomies in colorectal cancer surgery have been reduced by better antibiotics, bowel preparation for elective and emergency procedures (Christensen & Kronborg, 1981; Goligher et al. , 1970; Dudley et al. 1980) and the development

of stapling techniques (Heald, 1980). However, the overall 5-year survival rate following curative operations remains unchanged at 20-50%.

As early as 1908, Miles pointed out the importance of en bloc excision of lymph nodes and tumour, while Moynihan (1908) emphasised the need to remove proximal lymph nodes in rectal cancer by flush ligation of the inferior mesenteric artery at its origin. Pezim and Nicholls (1984) reported the experience of St Mark's Hospital when the 5-year survival of 1370 patients who underwent resection for sigmoid and rectal cancers was examined. "High ligation" i.e. ligation of the inferior mesenteric artery above the origin of the left colic artery was found not to confer any benefit, compared to those who had "low ligation" when the inferior mesenteric artery was ligated below the left colic artery. Glass et al. (1985) again reported the experience of St Mark's Hospital between 1960 and 1981 when the technique of extended abdomino-iliac lymphadenectomy was developed to try to improve the prognosis in patients thought to have unfavourable rectal tumours, including Dukes' C tumours. The five-year survival rate has not, however, shown any improvement over conventional techniques. A technique has been described by Turnbull et al. (1967) in which the tumour is not touched until the vascular pedicle is isolated to prevent dissemination of the tumour through handling, as during resection tumour cells can be found in the peripheral and portal veins (Griffiths et al., 1973). An apparently higher 5 years survival rate was found in Dukes' C patients compared with a historical control, but the extent of resection and randomisation were different in the two groups, particularly in the exclusion of locally advanced cases, making definitive conclusions difficult. In another study applying this technique, Wiggers et al. (1988) reported a tendency for reduction in the number of and in the time to develop liver metastases in the no-touch group particularly when the tumour was located in the sigmoid colon

with angio-invasive growth, but there was no difference in survival in the two groups. Recently, Heald and Ryall (1986) pointed out the importance of careful dissection of the mesorectum in colorectal cancers. This procedure has reduced the local recurrence rate to 4% with a long-term survival of over 80%. The absence of concurrent controls in this study may, however, limit the true significance of this report.

1.4 Adjuvant Therapies for Colorectal Cancer

The apparent limitation of surgery in improving the prognosis of colorectal cancer has led to an increasing interest in adjuvant therapies.

Interest in radiotherapy for rectal cancer has been prompted by reports from the Memorial Hospital and the Sloan-Kettering Institute that preoperative radiotherapy (1000-2000 rad or 10-20 Gy in 10 daily fractions) given to patients suffering from rectal cancer resulted in a better survival at 5 years (55% compared with 45% in the control group) which was sustained to 10 years (Stearns et al., 1959; 1974). This study, however, was not randomised. More promising results were reported by the Veterans' Administration Surgical Adjuvant Group (VASAG) in a randomised trial in which low dose preoperative radiotherapy of 2000 rad (20 Gy) was given over 10 days to patients with rectal cancer (Roswit et al., 1975). The incidence of Dukes' C stage cancer was reduced in the irradiated group compared to the control (27.8% v 41.2%). Autopsies showed that recurrent tumours in patients following abdominoperineal excision were also significantly reduced at the 5% level in the study group compared to the control (49% v 68%). The MRC Working Party (1982;1984) organised a randomised, multicentre study of 824 patients with operable rectal cancers to examine the effect of preoperative

low dose radiotherapy. Patients were randomised to receive surgery alone, surgery plus 500 rad as a single fraction or surgery plus 2000 rad in 10 daily fractions. The patients with the 2000 rad group had fewer Dukes' C cases and had tumours which were significantly smaller than the other groups. However, an analysis at 5 years has shown no benefit in survival, recurrence or metastatis rates, although the complication rates were similar in all 3 groups. In another study into pre-operative radiotherapy by the Stockholm Rectal Cancer Study Group (1987), 694 patients were randomised into a radiotherapy (25 Gy over 5-7 days) plus surgery group and another group with surgery alone. Although the incidence of local recurrence was significantly reduced, no difference was observed in the pattern of distant metastases or any improvement in survival. A detailed review of adjuvant radiotherapy for rectal carcinoma was made by Cummings (1986) who suggested that future trials with higher radiation doses were needed to determine the effect of such therapy.

In a randomised trial adjuvant cytotoxic perfusion of liver was performed by Taylor et al. (1979a) in colorectal cancer patients who underwent surgical resection and in whom there was no evidence of liver metastases, as judged by liver isotope scans and ultrasound. One gram of 5-fluorouracil (5-FU) was infused daily in 5% dextrose for seven days post-operatively into the portal vein cannulated via the 'obliterated' umbilical vein at the time of cancer resection. A total of 244 patients was randomised into a treatment and a control group. In the control group 38 patients had died and of these 17 developed liver metastases. In the perfusion group 19 patients had died with recurrent disease and liver metastases were present in 5. A median follow-up at 34 months suggested that the incidence of liver metastases might be reduced by this technique (Taylor, 1981). Later it was found that increased survival and fewer liver secondaries were only

evident in patients with Dukes B cancers subjected to this treatment (Taylor et al., 1984; Taylor et al., 1985).

The effect of immediate postoperative intraportal infusion of 5FUand mitomycin C on the outcome of colorectal cancer was reported by Metzer et al. (1987) in a randomised control study. The patients suitable for evaluation included 187 in the control group and 231 in the study group. There was no difference in the post-operative mortality or morbidity in the two groups. During a median follow-up of 24 months, death from recurrent disease was found in 25 patients in the control group and 18 in the study group, indicating that such therapy may be of value.

In another study (Windle et al., 1987), 141 patients were randomised into 3 groups following curative surgery for colorectal cancer. One group received a 6 month course of 5-FU, another group received levamisole in addition to 5FUand the third group supportive treatment only. Follow-up at 5 years showed that 52% of the control group, 44% of the 5FUgroup and 32% of the 5-FU/levamisole group had died of recurrent tumour. Levamisole thus appeared to confer a significant survival advantage to these patients.

A study from the National Surgical Adjuvant Breast and Bowel Project of USA was reported by Wolmark et al. (1988) in which 1166 patients with Dukes' B or C colorectal cancer were randomised into three groups, consisting of surgery alone, postoperative systemic chemotherapy (5FU, semustine and vincristine) or postoperative BCG. A study period extending over an average of 77.3 months showed that the chemotherapy group had a significantly better survival and the incidence of recurrence than the other groups.

An antimitotic drug razoxane (ICRF 159) which was developed from a chelating agent ethylenediamine tetra-acetic acid (EDTA, Sequestrene), which

blocks cell division in the premitotic and early mitotic phase, has been used in the treatment of lymphoproliferative disorders. It has shown activity in advanced colorectal cancer and a randomised trial (Gilbert et al., 1986) has been performed in 272 postoperative patients (133 control, 139 treatment) with a median follow-up of 5 years. Treatment was given continuously on a long term basis. The result shows a prolongation of time to recurrence in Dukes' C patients alone, but acute leukaemia was found in three patients (2.45%) after prolonged exposure, so that further study has to be limited to patients who are at high risk of developing recurrent disease.

1.5 Treatment of Liver Metastases

A major influence in the prognosis of colorectal cancer is the presence of liver metastases. The number of patients with liver metastases at diagnosis has been estimated to be about 20-25% (Bengmark & Hafstrom, 1969; Wood et al., 1976), and their mean survival rate is about 6 months. A prospective study showed that 70% of patients with liver metastases had involvement of both lobes of the liver, and localised involvement of one lobe or segment of the liver occurred in only 13% of the patients, while solitary metastases were present in 17% of the patients. Patients with localised or solitary metastasis had a similar prognosis in the first 3 years with a survival probability of 26% and 21% respectively. At 5 years however, those with a solitary metastasis had a survival probability of 16%, while all other groups of patients were dead (Wood, 1988). It is against this background that intense interest has been generated in tackling liver metastases using various techniques.

Recently, several groups have reported an increase in survival following

resections of colorectal cancer hepatic secondaries, with survival times varying from 2 to 10 years (Wilson & Adson, 1976; Foster et al., 1978; Adson & Van Heerden, 1980; Morrow et al., 1982). However, Adson and Van Heerden (1980) pointed out that although about 20% of patients with colorectal cancer have hepatic metastases, only 5% or less of these patients with liver secondaries would have lesions suitable for surgery. The total number of patients that might benefit from such surgery would, therefore, be relatively small. A review of the current status of hepatic resection for colorectal cancer metastases by Greenway (1988) shows that survival following liver resection is not related to the extent of the resection, but to the number and size of the metastases. The results of surgery indicates that efforts should be concentrated on those patients with less than 4 hepatic metastases. However, 75% of patients die within 5 years of recurrences (the majority within 3 years) and half of the recurrences are at extra-hepatic sites only, demonstrating the limitation of staging by current methods. This finding argues for the necessity of postoperative chemotherapy. August et al. (1985) reported a trend towards increased survival in patients who received intraperitoneal 5-fluorouracil.

Liver metastases derive their blood supply mainly from the hepatic artery (Taylor et al., 1979b). Drugs such as 5-fluorouracil (5-FU) or 5-fluorodeoxyuridine (5-FUDR), have a high extraction rate during the first pass through the liver. They can be delivered by continous infusion into the hepatic artery to the tumour, resulting in less systemic drug exposure. Thus Watkins et al. (1970) reported a response rate of up to 73% when 5FUwas infused by this means. Oberfield et al., (1979) also found good responses when 5FU and 5-FUDR were infused into the hepatic arteries continuously over a prolonged period. The median survival was 6.9 to 8.5 months in those who responded

compared with 3.6 months for non-responders. Ariel and Padula (1982) observed an average survival of 31 months when a bolus of combined chemotherapy consisting of Platinol (TM), methotrexate and 5FU was given intra-arterially in asymptomatic patients.

Recently, an implantable infusion pump for the continuous delivery of drug into the hepatic artery has been introduced. Intra-arterial 5-FUDR has been reported to give a mean survival rate of 26 months compared with 8 months for historical control patients (Balch et al., 1983). In a comparison of intra-arterial infusion with intravenous administration of 5-FUDR for liver metastases, 64 patients were randomised into two groups (Chang et al., 1987). The response was measured by CT scans of the liver, chest and CEA levels. A significantly improved response rate was found in the arterial infusion group (62%) as against the intravenous group (17%). The 2-year actuarial survival rates for the two groups were 22% and 25% respectively, which were not statistically significant. If analysis was performed in a subgroup with negative lymph node involvement, a significant increase in survival was found in the intra-arterial infusion group. Considerable toxicity was caused by the hepatic infusion, and side-effects included chemical hepatitis (79%), biliary sclerosis (21%), peptic ulcers (17%) and gastritis/duodenitis (21%). The small gain in survival in the subgroup of patients with negative hepatic nodes appeared to be offset by the toxicity of intra-arterial infusion. Toxic side-effects such as chemical hepatitis and sclerosing cholangitis have also been reported by others (Kemeny et al., 1986; Hohn et al., 1987) in similar randomised trials comparing the effect of intra-arterial verses intravenous infusion of 5-FUDR for liver metastases. The objective response rates for intra-arterial groups were 46% (Kemeny et al., 1986) and 37% (Hohn et al., 1987) while the intravenous groups were 23% and 10% respectively. Although the

reponses were significantly higher for the intra-arterial infusion groups, there was no increase in survival. The application of such therapy has to be balanced against the quality of survival, as no patients can expect a cure (Clark, 1986).

As only 5% (Adson & Van Heerden, 1980) to 20% (Bengmark, 1988) of patients with hepatic metastases are suitable for resection, other therapies such as arterial ligation or embolization which cause tumour necrosis by ischaemia have been tried. Relatively little data are available on these treatments. Allison et al. (1985) reported that the benefit of hepatic embolisation in patients with non-endocrine metastases was miminal. This technique appear to afford pain relief but no evidence to suggest any prolongation in survival (Taylor, 1985). An interesting study in which an implantable occluding device is placed around the hepatic artery and connected to a subcutaneous port, so that the patients can occlude the artery for up to 60 minutes a day, has been reported by Bengmark (1988). Calcification of the metastases, suggestive of tumour regression, has been observed after more than a year of therapy.

Prasad et al. (1977) reported that hepatic irradiation was effective in symptomatic patients with liver metastases. Seventy per cent of patients showed relief of pain and some experienced reduction in liver size. However, survival was not improved. Ariel and Padula (1982) infused Yttrium microspheres and 5FU into the hepatic artery. These patients survived an average of 26 months, but unfortunately the effect of radiation alone was not determined.

1.6 Second Look Laparotomy

The rationale for this procedure is that recurrence often cannot be detected by clinical investigation. Second-look laparotomy performed at intervals

can thus offer better chance of detecting a recurrent cancer and improve the prospect of a further curative operation. This concept was first proposed by Wangensteen et al. (1951,1954), and they reported that 17% of these patients could be returned to a cancer-free state. However, this practice did not gain wide acceptance because some patients did not have recurrences and others had tumours beyond curative surgery. The postoperative morbidity and mortality following resections were also unacceptable (Minton et al., 1978).

To obviate unneccessary surgery, rising serum CEA levels has been used as an indicator of recurrence and for second-look laparotomy. Minton et al. (1978) performed 22 second-look laparotomies using serum CEA as a guide and found recurrent tumours in 19 patients of whom only 6 had resectable tumours. A steep rise in CEA usually signifies liver metastases, while a slow rise is suggestive of local recurrence (Wood, 1980). However, such CEA-guided second-look laparotomy saves only a few lives as the plasma CEA levels do not reflect early localised tumour (Zamcheck, 1983). An explanation of normal plasma CEA levels in patients with early tumour recurrences is the regulation of CEA by the liver (Zamcheck, 1983) which prevents a rise of CEA from becoming apparent. A controlled trial designed to evaluate the effectiveness of serial CEA measurement for second-look laparotomy is currently underway in the UK. Recently, imaging with radiolabelled antibodies has been used to supplement serum CEA level in selecting patients with localised disease for second look laparotomy.This technique was more effective than conventional radiology in discriminating between localised from disseminated disease, and appeared to be a useful investigation to improve the success of a second look laparotomy (Begent et al., 1986).

1.7 General Properties of Monoclonal Antibodies

In 1975 Kohler and Milstein described a technique for the production of monoclonal antibodies of predefined specificity, introducing the possibility of another modality in the diagnosis and treatment of colorectal cancer, that of immunoscintigraphy and immunotherapy.

Antibodies are proteins that are produced by higher organisms in response to foreign materials, defined as antigens. They act as part of a protective mechanism to provide a defence against harmful agents such as toxins, bacteria or viruses. They are also known as immunoglobulins as they belong to a general class of plasma proteins known as globulins. They are secreted by B lymphocytes, so-called because they were originally recognised in the Bursa of Fabricius in birds, and which are found in bone marrow in man. These cells are distinct from the T lymphocytes (processed in the thymus), which are responsible for cellular immune responses, and which do not secrete immunoglobulins. The production of specific antibodies is a complex process involving macrophages, B and T lymphocytes and the immunising antigen. According to the clonal theory, one lymphocyte is responsible for the secretion of any one specific antibody following immune stimulation (Burnet, 1957). This clone of cells will secrete the same antibody which is by definition a monoclonal antibody. In response to an immunological challenge large amounts of specific antibodies are produced by the clonal expansion of such reactive cells. In the body many similar but not identical antibodies are produced in response to an antigen in what is generally a polyclonal response, due to the presence of many clones of lymphocytes with appropriate specificities. The only situation when a monoclonal antibody is produced *in vivo* is in cases of malignant clonal proliferation of antibody secreting cells (e.g. multiple myeloma, Waldenstrom's macroglobulinaemia etc.). In this case the specificity of

such antibodies is determined on a random basis and is of no practical use.

Kohler and Milstein discovered a way of preparing monoclonal antibodies of predetermined specificity in vitro, using rodent myeloma (plasmacytoma) cells as the cellular factory, and cell fusion to generate specificities from B lymphocytes retrieved from immunised animals. This method was made possible by the development of tissue culture technique in three areas. First the establishment of mouse myeloma cell lines, induced in Balb/c mice with mineral oil (Horibata & Harris, 1970), which unlike lymphocytes can be grown indefinitely in tissue culture. Secondly, advances in somatic cell fusion using fusogens such as Sendai virus (Harris and Watkins, 1965), and later polyethylene glycol (PEG) (Ringertz and Savage, 1976), which enables the fusion of a lymphocyte with a myeloma cell to form an immortal hybridoma cell. Thirdly, the development of mutant myeloma cell strains lacking specific nucleotide metabolising enzymes such as hypoxanthine-guanine phosphoribosyl transferase (HGPRT), which allows their selective destruction with anti-metabolites such as aminopterin, without damage to the fused hybridoma cells.

The experiment which established the production of predefined monoclonal antibodies was described by Kohler and Milstein. A Balb/c mouse was immunised against sheep red blood cells (sRBC), and the spleen cells of the immunized mouse were removed and fused with Balb/c mouse myeloma cells lacking HGPRT, thus forming hybrid cells called hybridomas. These cells were grown in selective tissue culture medium containing aminopterin. Normal lymphocytes did not survive long-term in tissue culture and the myeloma cell line was killed by the anti-metabolite. The hybridomas, however, contained the enzyme HGPRT inherited from the fused lymphocytes and therefore continued to grow in the aminopterin-containing medium, provided that it was supplemented with

hypoxanthine and thymidine. The hybridomas secreting antibodies to the sRBC were isolated by repeated cloning and screening the supernatant for reactive antibody. In this way positive clones were identified. This basic technique has now become a standard method for the production of pure antibodies of desired specificity, and includes several improvements. Thus the potential contamination of the monoclonal antibodies secreted by the myeloma cell has been eliminated by the use of non-secreting myeloma cell-lines. This *in vitro* monoclonal antibody technique can therefore produce specific antibodies in pure form, in large quantities and indefinitely.

The essential feature of monoclonal antibody technique is to enable the immortalisation of a lymphocyte secreting a particular antibody, so that a large amount of this pure antibody can be prepared. This technique enables the 'capture' of an antibody response to a particular stimulus, whether a response to a cancer, an autoimmune process, a bacteria, a virus or a parasite or any other substance. By injecting human cancer into a mouse and generating monoclonal antibodies, the existence of tumour associated antigens can be analysed. By generating human monoclonal antibodies using lymphocytes from patients suffering from cancer, the patients' own antibody response to a particular cancer can be determined. In a wider context, the limitless availability of monoclonal antibodies has permitted standardisation of a wide range of immunoassays and other methods using antibodies as reagents, as well as facilitating their improvement.

1.8 Tumour Associated Antigens

Ehrlich was the first to suggest the possible existence of tumour antigens and the treatment of tumours using antibodies (Carroll et al., 1984).

Experiments in the last century showed that when tumours from one species were transplanted to animals of the same species the tumours were rejected. It was concluded that the tumour cells expressed potent antigenic determinants that caused their rejection by the host. In fact this mechanism of rejection was not related to tumour antigens, but as it is now known, to the difference in histocompatibility. Nevertheless, passive immunotherapy was used to treat human cancer. For example, beneficial effects were claimed by Hericourt and Richet (1895) who treated 50 patients with anti-tumour antisera raised in dogs and donkeys.

i Evidence for the existence of tumour antigens

Later transplant experiments did provide, however, some more specific evidence for the existence of tumor antigens. Clowes and Baeslack (1905) demonstrated that in animals in which tumour regressed spontaneously, implantation of the same tumour did not grow. This resistance could not be transferred by serum or inheritance. However, Woglom (1929) pointed out that all tumour transplant experiments using animals which lacked genetic homogeneity could not provide conclusive evidence of specific immunological responses to tumours.

In the 1940's in-bred (syngeneic) mice became available. Gross (1943) repeated the experiments similar to those performed by Clowes and Baeslack (1905) using such mice. Sarcomas were induced in these animals by means of a chemical carcinogen, methylcholanthrene, and transplanted. Spontaneous regression of the sarcoma was observed in about 20% of the animals, and further implantation of the tumour in these sarcoma-resistant animals resulted in rejection. This rejection showed tumour specificity as these mice were not protected from the spontaneous development of mammary tumours, the incidence of which was the

same as in other mice of the same strain.

Prehn and Main (1957) later showed that tumour-specific resistance to rechallenge by the tumour was found in animals which had been exposed to the tumour previously, although the tumour was later removed by amputation of a tumour-bearing limb or by excision of the tumour. The same phenomenon was observed if the animal was treated with irradiated tumour cells. Antigens detected by these type of experiments are known as tumour-specific transplantation antigens (TSTA).

ii Tumour specific transplantation antigens (TSTA)

Antigens of this type have been found in chemically-induced tumours (Reiner & Southam, 1967), virus-induced tumours (Heppner & Pierce, 1969), plastic film-induced tumours (Klein et. al., 1963), and ultraviolet light-induced tumours (Daynes et al., 1977). A feature of these transplantation antigens is their polymorphism, with each tumour having a transplantation antigen unique to itself despite common derivation from mice of the same inbred strain. Attempts to isolate and biochemically characterize TSTA on surfaces of chemically induced tumours have only been partially successful (DeLeo et. al., 1977). A major difficulty in the study of these antigens is the poor humoral response despite prolonged immunisation and a resulting high degree of resistance to challenge with tumour grafts. So far two antigens, the Meth A and CMS4, have been detected to methylcholanthrene-induced sarcomas in Balb/c mice. The nature and significance of these antigens remain to be defined (Old, 1981).

Tumours induced by virus may share the same viral antigens as well as antigens specific to a tumour (Currie, 1974). Chemically induced tumours also show cross-reactivity, but interpretation of the results is complicated by the fact

that chemically-induced mouse sarcomas often express antigens of the endogenous murine leukemia virus (MuLV) on the cell surface (Grant et. al., 1974). Normal mouse serum also often contains antibody to the MuLV antigens (Nowinski & Kaehler, 1974). The expression of MuLV viral gene in mouse leukaemia is poorly understood (Old, 1981). In the analysis by Old (1981) of mouse leukaemias of thymic origin, antigens that are leukaemia specific i.e. those that are not present in normal cells have not been found. Antigens such as the Thymus Leukaemia (TL) antigens coding genes are present universally in the mouse, but in some strains of mice these genes and those of MuLV are never activited or de-repressed. However, during malignant transformation these silent genes are activated directly or indirectly. The products of these genes have the characteristics of transformation-specific products, as their appearance is restricted to the tumour cells in these strains (Old, 1981).

iii Tumour-associated antigens (TAA)

In most, if not all, spontaneous (as distinct from induced) tumours in animals and man, there is no evidence of TSTA's equivalent to those described above (Wiseman & Rao, 1986). However, there is evidence of antigens which are associated with (although not specific to) tumours, which can evoke an immune response. These tumour-associated antigens (TAA) have been reported in man (Currie, 1974) using several different methods to examine the interactions of tumour cells with the various effector limbs of the immune response.

These have included the production of xenogeneic antisera by innoculating human tumours in other species. The resulting antisera are absorbed with corresponding normal tissue in order to obtain the tumour specific components. Antisera to human leukaemic cells have been prepared by this

technique by immunising monkeys with leukaemic blast cells (Metzgar et al., 1972) and similarly to malignant melanoma (Ghose et al., 1972; Carrel & Theilkaes, 1973).

Another approach has been to screen cancer patients for antibody reponse to cell lines of the same tumour type derived from other patients by means of the immunofluorescence technique (1.10.i). This consists of exposing tumour cell line to the patient's serum and the reaction between the antibody and the tumour cells is demonstrated by an antibody labelled with a fluorescent dye. Antibodies to the Burkitt's lymphoma have been found in patients with Burkitt's lymphoma, nasopharyngeal carcinoma and infectious monoclonucleosis. Epstein-Barr virus may be associated with these conditions (Currie, 1974). The immunofluorescent technique has also demonstrated antibodies in malignant melanoma to both cytoplasmic and cell surface antigens (Morton et al., 1968; Lewis et al., 1969). Antibodies have been found in patients with sarcomas by means of immunofluorescence (Morton & Malmgren, 1968), and this has been confirmed by complement fixation and complement-dependent serum cytotoxicity tests (Currie, 1974). There is a high incidence (70%) of autoantibodies to normal tissues in the serum of cancer patients (Whitehouse & Holborow, 1971), for examples, anti-nuclear factor, gastric parietal cell autoantibodies, small muscle antibody and cytoplasmic microtubules. The presence of these antibodies is not related to tumour bulk and is probably not a response to tumour necrosis.

A further method has been to examine the activity of the patient's lymphoid cells when they are exposed to tumour cells or tumour cell extracts in tissue culture. The interpretation of the results of this kind of experiments is difficult as the tests are not entirely specific. Lymphocytes previously stimulated by a tumour may secrete chemical mediator substances called 'lymphokines' when

confronted by the specific antigen. One of the substances inhibits the migration of macrophages and leucocytes and is known as migration inhibitory factor (MIF). Crude tumour extract has been shown to inhibit the migration of autologous leucocytes in 58% of 57 cancer patients (Segall et al., 1972).

A more commonly used assay, is the examination of the cytotoxic activity of peripheral lymphocytes from cancer patients, including those with colonic cancer, on tumour cells in culture. Cytotoxic lymphocytes to the patient's tumour or to tumour of a similar histological type have been demonstrated (Hellstrom et al., 1971). In a small group of patients with malignant melanoma, it was found that the cytotoxic effect of lymphocytes was blocked when there was an extensive tumour load. A reduction of tumour bulk by surgery resulted in a diminution of serum blocking activity and was sometimes accompanied by a rise in lymphocyte cytotoxicity (Hellstrom & Hellstrom, 1973). Similar serum blocking activity was reported by Currie (1973) in patients with malignant melanoma and hypernephroma and the inhititory activity disappeared following the removal of the tumours. The molecular weight, affinity and specificity of these serum inhibitors suggested that they are shed tumour antigen. Similar activity has been found in the serum, tumour, tumour cells and the urine of patients with hypernephroma or tumour cell culture supernatant (Currie, 1974).

Finally, the reactions of cancer patients' sera (primarily those with malignant melanoma, astrocytoma, and renal cancer) with surface antigens of their own and other patients' cultured cancer cells has been studied, notably by the group of Old at the Sloane-Kettering Memorial Hospital. With this method 3 classes of surface antigens with different extents of normal tissue and tumour distribution have been defined. Class 1 antigens are restricted to the autologous tumour and cannot be found on autologous normal cells or any other normal or

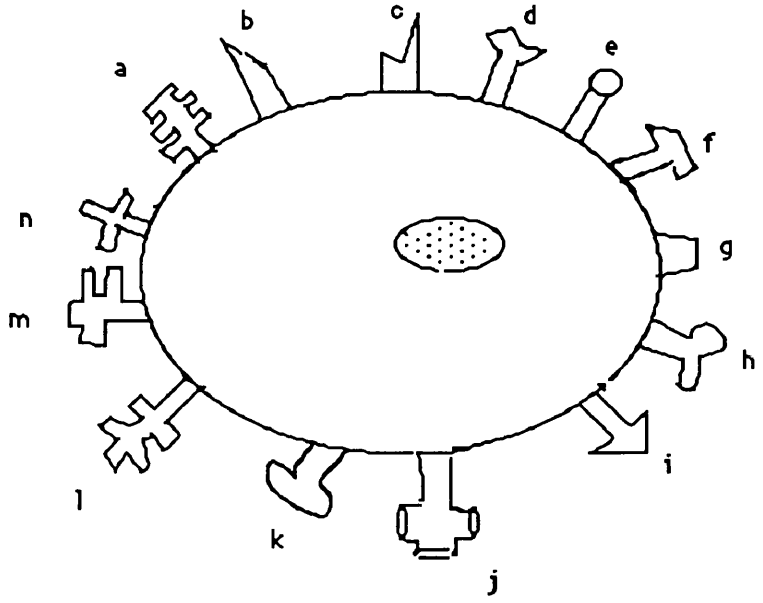
malignant cell type. Class 2 antigens are shared tumour antigens, found on autologous as well as on allogeneic tumours of similar and in some cases dissimilar origins. They are not detected on a wide variety of other tumour types or on normal B-cells, kidney cells, or fibroblasts. Class 3 antigens are widely distributed on normal and malignant cells, autologous, allogeneic, and xenogeneic (Old, 1981). Most autologous antibodies detected in cancer patients react with Class 3 antigens. Antibodies to Class 3 antigens can give the impression of tumour specificity in direct tests as tumour cells may express higher levels of these antigens. However, in all these approaches problems remain in distinguishing histocompatibility antigens from the tumour associated antigens, particularly in the case of rare allotypes. Figure 1 illustrates some of the antigens that may be present on a tumour cell.

1.9 Monoclonal Antibodies and Colorectal Cancer Antigens

In general no convincing evidence of the equivalent of tumour-specific transplantation antigens has been obtained from the several studies in man; although clearly there are responses to tumour-associated antigens including those of colonic origin.

Monoclonal antibodies are a better probe for tumour associated antigens because they can detect unique antigenic determinants rather than a mixture of antigens which react with polyvalent antisera. The purification of polyvalent sera depends on a process of absorption of common antigens using normal tissue. A weakly immunogenic antigen which may be an important tumour associated antigen may be overlooked or missed in the process of purification because of its lower concentration and / or immunogenicity. However, in monoclonal antibody

Figure 1 Some antigens present on a cancer cell



a Tumour specific transplantation antigen
b General transformation antigen
c Histocompatibility antigen
d Blood group antigen
e Differentiation antigen
f Ir (DR) antigen
g Forssman (heterophile) antigen
h Oncofetal antigen
i Tissue-specific antigen
j Organ-specific antigen
k Private antigen
l Unknown antigen
m Virus-induced antigen
n Tumour-associated antigen

(from Wiseman and Rao, 1986)

production the desirable antibodies are searched for at an early stage by an active proccess of selection by means of various screening methods. Once identified, the antibodies can be produced in its pure form indefinitely. Polyvalent antibodies have the disadvantages of inconsistency due to batch to batch variations in quantity and quality and other impurities that cannot be removed. A summary of the differences between monoclonal and polyvalent antibodies is provided by Edwards (1981) and is shown in Table 1.

Monoclonal antibody technique has generated intense interest in the search for tumour-associated antigens because of the manner in which it facilitates identification of specific antigens and the epitopes which are the subunits of an antigen to which individual antibodies bind. The monoclonal nature of individual hybridomas means that it is not neccessary to use a pure antigen as the immunogen in order to raise highly selective reagents. In cases in which relatively pure antigen is available (eg. CEA), the monoclonal technique enables specific domains or epitopes on the molecule to be detected individually.

i Carcinoembryonic antigen (CEA)

CEA epitomises a human tumour-associated antigen (TAA) and was one of the first to be isolated and chemically characterised. Gold and Freedman (1965) induced immune tolerance of new born rats to normal human colon. The animals were then immunised with colorectal cancer. Using this strategy it was hoped that the antibody response would be directed primarily against the cancer. The antigen was called carcinoembryonic antigen (CEA) as it was originally thought to be confined to fetal colon and colorectal cancer but belief in its property as a specific marker for colorectal cancer was unfounded (see 1.1).

Table 1 Comparison of the properties of monoclonal and polyclonal antibodies (Edwards, 1981)

Property	Polyclonal antisera	Monoclonal antibodies	
		Culture supernatant	Ascites fluid
Useful antibody content:	0.1 - 1.0mg/ml	5 -25ug/ml	0.5 -5mg/ml
Irrelevant immunoglobulin:	10mg/ml	None	0.5-1mg/ml
Other serum proteins:	present	10% (v/v)foetal calf serum	Some present
Binds to:	All antigenic determinants of all components of immunizing material	One antigenic determinant (epitope) of one component of immunizing material	
Reproducibility of specificity and affinity:	Varies between batches	Invariant	
Cross-reactions with other antigens:	Partial with antigens bearing common antigenic determinants	Usually absent, but complete if binds to a common determinant.	
Applicability of conventional immunological procedures, eg.precipitation of antigen, class and subclass of immunoglobulin:	Applicable.	May not work.	
	Mixture of all.	One only; may be any	
Physical properties of immunoglobulin:	Typical spectrum	Individual property of antibody	
Kinetics of binding:	Typical spectrum, though may vary	Wide variation expected; selected by screening method	

Carcinoembryonic antigen (CEA) has, nevertheless, been an obvious choice as an immunogen for the production of antibodies reactive with colorectal cancer. CEA is a complex glycoprotein molecule of 180,000 daltons and the production of monoclonal antibodies from different laboratories has defined many different epitopes on this antigen.

Accolla et al. (1980) were the first to produce monoclonal antibodies to CEA, using purified CEA as immunogen. Two hybrids VII-23 and VII-37 were obtained and they react with different epitopes on the CEA molecule. In another series of monoclonal antibodies raised against highly purified CEA, Hedin et al. (1982) produced 8 different monoclonals which recognised at least six different epitopes in the peptide moiety of CEA and two of which cross-reacted strongly with nonspecific cross-reacting antigen (NCA). Rogers et al. (1981) reported an anti-CEA monoclonal which binds weakly to CEA in perchloric acid extracts of tumour but strongly to CEA similarly extracted from serum. There is evidence to suggest that CEA present in different tumours may contain specific epitopes for that organ or tumour. This is shown by the anti-CEA monoclonals, produced against a breast cancer liver secondary, which showed different quantitive binding to breast and colon cancer (Reynoso et al., 1985). Reynoso et al., (1985) postulate that monoclonals to CEA may be divided into 3 classes: class I shows identical or near identical affinities for CEA, NCA and MA (meconium antigen). Class II reacts with CEA and MA. Class III reacts with CEA alone. The epitopes defined by anti-CEA monoclonal antibodies are different to those shown by polyclonal anti-CEA antibodies which cross-react with a larger number of other tissue antigens. More recently, monoclonals reactive with different epitopes of CEA have been reported by Price (1988), some of which as expected cross-reacted with NCA.

ii Monoclonal antibodies to other colorectal antigens

There are several reports of monoclonal antibodies raised against colorectal cancer using immunogens other than CEA. These have usually been cell lines or cells from freshly resected colon cancer specimens. These antibodies have varying reactivity to colorectal cancer and normal colon as well as other tissues.

The first monoclonal antibodies to colorectal cancer were produced by Koprowski's group at the Wistar Institute (Herlyn et al., 1979; Koprowski et al., 1979) using colorectal cancer cell lines as the immunogen. Further antibodies were produced using cells obtained from freshly resected carcinomas (Koprowski & Steplewski, 1981). The antigens recognised by these antibodies have been investigated and both glycolipid and protein antigens have been detected. Four glycolipid reactive antibodies (1116NS-10, 1116NS-33a, 1116NS-38a and 1116NS-43a) were found to be specific for the Lewis^b saccharide structure (Brockhaus et al., 1981); another three (ZWG13, ZWG14 and ZWG11) were specific for 3 fucosyl-N-acetyllactosamine (Brockhaus et al., 1982) and a further two (1116NS-52a and 1116NS-19-9) were directed at a sialic acid substituted Lewis^a structure present on a monosialoganglioside (Magnani et al., 1981). The monoclonal antibodies 9-1 and 9-2 reacted with protein antigens both of which comprised a 28,000 mol. wt. alpha chain and a 22,000 mol. wt. beta chain (Koprowski & Steplewski, 1981), and antibodies 116NS-3d and 116NS-20 reacted with CEA (Koprowski et al., 1979).

The reactivity of one of these antibodies, 1116NS-19, has been studied using an indirect immunoperoxidase method on paraffin sections (Atkinson et al., 1982; Arrends et al., 1983a). It stained 40 out of 68 colorectal cancers (59%). It also reacted with columnar cells in the stomach mucosa, bronchial glands and the ducts of the breast, prostate, salivary glands and liver. It did not react with normal

colon, small intestine, oesophagus, skin, heart muscle, the genitourinary system, endocrine system and skeletomuscular system. Monoclonal antibody 1083-17-1A (IgG2a) was produced using SW 1083 cell line as immunogen. It reacts with eight out of nine colorectal carcinomas maintained in culture but did not bind to other human tumours of different origin or to normal human fibroblast. It has cytotoxic effects on colorectal cancer cell lines through mediating the antibody-dependent cell-mediated cytotoxicity (ADCC) as measured by the chromium release assay (Herlyn et al., 1979a; Koprowski et al., 1979). It also inhibits growth of colorectal cells in nude mice (Herlyn et al., 1980). This antibody has been used for immunoscintigraphy and therapy of colorectal cancer (Mach et al., 1983; Sears et al., 1982).

Colorectal cancer cell line HT29 has been used by two groups to produce antibodies (Voak et al., 1980; Thompson et al., 1983). Those produced by Voak et al. react with blood group A substance. On immunoperoxidase staining antibody 1/3.12 reacted with blood vessels and smooth muscle; antibodies 2/38.9 and 2/44.3 reacted with both normal and cancer sections; antibodies 1/1.1, 2/9.12 and 2/13.4 reacted with normal colon but little with colorectal cancer; antibody 2/12.1 reacted preferentially with cancer compared with normal colon (Finan et al., 1982). Antibody 2/12.1 also reacted with a crude extract of CEA (Berry et al., 1981). Out of 102 HT29 reactive clones produced by Thompson et al. (1983) one antibody, 250-30.6 showed the greatest colorectal cancer specificity on screening with cell lines. However, on immunocytochemistry the reactivity of this antibody was found to be more widespread, including normal, benign and malignant colonic epithelium and other cells of the gut, respiratory system and genitourinary system.

Monoclonal antibodies AUA1, D20L and C15L were raised against a cell line LoVo (Arklie and Bodmer, 1981). These antibodies were epithelium

specific when screened against a panel of carcinoma, fibroblast and lymphoid cell lines using a radioimmunoassay. Antibody C15L recognises a protein with a mol. wt. of 60,000 while antibody D20L a protein of 40,000. Immunoperoxidase showed that D20L reacted with the small intestine while AUA1 reacted with both the large and small bowel.

The monoclonal antibody C14/1/46/10 (IgM) was raised against extra-nuclear membrane of colorectal cancers and it recognises the Y hapten carbohydrate antigen (Brown et al., 1983). It reacts with 96% of colorectal cancer and 100% of colorectal adenomas (Brown et al., 1984).

The monoclonal antibody CAM.5 (Makin, 1986) was raised against colorectal cancer cell line, cell membrane extract and meconium. It reacts with all epithelial cells by reacting with 3 low molecular weight cytokeratin proteins of 50,000, 43,000 and 39,000 daltons. Although CAM.5 is not a tumour marker it can be used to detect tumour metastases in the lymph nodes or bone marrow where epithelial cells are not normally present.

As the above results illustrate in no case has the monoclonal antibody approach yet led to the identification of specific colorectal tumour antigens, although several examples of preferential reactivity have been described.

A summary of monoclonal antibodies reactive with colorectal cancer is shown in Table 2.

There is substantial evidence that man can produce a humoral response to cancer (Old, 1981) - see 1.7.iii - although its clinical significance is debatable. In colorectal cancer a direct way of determining this response is to examine antibody secretion from human lymphocytes with respect to their reaction to colorectal cancer. Immortalization of these lymphocytes by the monoclonal antibody technique enables such studies to be performed. In practice however, the

Table 2 Monoclonal antibodies raised to colorectal cancer

Antibody	Immunogen	Class	Reference
1083-17-1A	SW1083 cell	IgG2a	Koprowski et al. 1979
1116NS-19	SW1116 cell	IgG1	Koprowski et al. 1979
AUA1	LoVo cell	NA	Arklie & Bodmer 1981
D20L	LoVo cell	NA	Arklie & Bodmer 1981
C15L	LoVo cell	NA	Arklie & Bodmer 1981
H58	HT29 cell	IgG1	Rogers et al. 1984
YPC2/12.1	HT29 cell	IgG2a	Finan et al. 1982
250-30.6	HT29 cell	IgG2b	Thompson et al. 1983
CCOL1	COLO205 cell	IgG3k	Kaszubowski et al. 1984
C14/1/46/10	Cell membrane	IgM	Brown et al. 1983
CAM5.2	Cells & membrane	IgG2a	Makin 1986
CAM17.1	and meconium	IgG1	Makin 1986
LoVoF4/2E10	LoVo cell	IgG1	Drewinko et al. 1986
Mab 23	CEA	IgG1	Accolla et al. 1980
MA-1	CEA	NA	Rogers et al. 1981
c-1; c-3; c-5; c-19;	CEA	NA	Stahli et al. 1981
c-24		NA	
mab9	CEA	IgG1	Hedin et al. 1982
38S1	CEA	IgG1	Hedin et al. 1982
mab 48	CEA	IgG1	Hedin et al. 1982
Mab 35	CEA	IgG1	Haskell et al. 1983
Mab 202	CEA	IgG1	Haskell et al. 1983
NP1,2,3&4	CEA	IgG1	Primus et al. 1983
Mab 1-15	CEA	various	Price 1988

NA = not available

production of human antibodies has been fraught with technical difficulties, and only a very limited number of human monoclonal antibodies have so far been raised (O'Hare & Yiu, 1987).

1.10 Monoclonal Antibody Localisation to Colorectal Cancer

An initial step in the application of a monoclonal antibody to colorectal cancer is the demonstration of its localisation to the tumour, a requirement first pointed out by Pressman some 40 years ago. The first studies with radiolabelled antibodies were carried out to establish the extent of such localisation. Pressman and Keighley (1948) and Pressman (1949a,b) studied the binding of antisera to normal tissue and tumours in animals. Antisera against rat kidney were labelled with iodine-131 and injected into rats. Subsequent autoradiography showed that the antibody localised to the glomerular tufts of the rat kidneys (Pressman, 1948). By using iodine-labelled antibodies raised in rabbit against Wagner's osteogenic sarcoma of mice (Pressman & Korngold, 1953) and Murphy lymphosarcoma of rats (Korngold & Pressman, 1954), localisation of antibodies to these tumours was also demonstrated. In these experiments, the specific localisation of the radiolabelled antisera to the tumours was determined in one group of tumour-bearing animals and the non-specific localisation of radiolabelled immunoglobulins was determined in another comparable group of animals. Specific localisation of the radiolabelled antisera to the tumours is confirmed by its higher uptake to the tumours compared to that of the radiolabelled immunoglobulins. However, such localisation study is difficult to justify in the human. Pressman et al. (1957) devised a paired-labelled method to circumvent

these difficulties. The specific antisera and non-specific immunoglobulins were radiolabelled with two different isotopes (I-131 and I-133 were used in their animal studies) and administered simultaneously. The relative uptake of the specific antiserum and the control immunoglobulins by the tumours could then be compared. Thus, the experimental subject serve as its own control. The experimental work by Pressman and his colleague formed the basis of immunoscintigraphy of tumours using radiolabelled antibodies.

The radiolabelled antibody localisation of tumours was soon applied to patients. Day et al. (1959) and Spar et al. (1959) used antibodies raised against fibrin for tumour localisation on the basis that fibrin was present in the tumour due to an associated inflammatory process. However, clinical application met with limited success as fibrin was present in many other organs (Dewey et al., 1963). Another early study used an antibody to human chorionic gonadotrophin for localisation of a human choriocarcinoma xenograft transplanted to the hamster cheek pouch (Day et al., 1959) but was unsuccessful. It showed, however, that the access of an antibody to tumour was important for its localisation. Failure of antibody localisation can also occur if the antibody cross reacts with other antigens. An antibody raised against a mouse myeloma protein failed to localise to a mouse plasma cell tumour for this reason (Reif et al., 1971).

Antibody localisation studies were extended to colorectal cancer in the early 1970's. Mach et al. (1974) used goat anti-CEA antibodies labelled with ^{131}I for localisation of human colorectal cancer xenografts transplanted to nude mice. They showed that 9 times as much antibody was bound in the tumour as compared with the liver. A non-specific antibody gave a tumour localisation of no more than 2.3 times that of the liver. There was, however, a variation in the antibody uptake by different colorectal cancer xenografts, confirming the histological differences

between them. Goldenberg et al. (1974) and Hoffer et al. (1974) used human colorectal cancer transplanted to the hamster cheek pouches for localisation studies with anti-CEA antibodies. Positive external images were reported by these authors, forming the basis for immunoscintigraphy of colorectal cancer, using initially polyclonal antisera and subsequently monoclonal antibodies.

Initial clinical studies of iodinated anti-CEA polyclonal antibodies failed to show localisation to colorectal cancer in man (Reif et al., 1974; Mach et al., 1978). It was suggested that CEA present in the other organs such as the liver (Kupchik & Zamchek, 1972), lung (Pusztaszeri & Mach, 1973) or colon (Martin & Martin, 1970) could prevent antibody localisation to the tumour by sequestration of circulating antibody. Another explanation offered was the binding of antibodies to cross-reacting antigens in the normal lung and spleen (Mach & Pusztaszeri, 1972). However, Goldenberg et al. (1978) were able to demonstrate tumour localisation with ^{131}I labelled anti-CEA antibodies by using a blood pool subtraction method to remove background activity. This technique involved the use of $^{99m}TcO_4$ and ^{99m}Tc-albumin, in order to outline both the extra- and intra-vascular compartments, which were then subtracted from the body image to reduce the non-tumour background activity. They claimed that most of the CEA producing tumours from 18 patients were positive. Specific localisation of anti-CEA antibodies to colorectal cancer was also demonstrated by Mach et al. (1979). When resected tissue was examined for increased radioactivity, uptake was detected in 16 patients. However, only 6 of these patients showed positive images. Nevertheless, Goldenberg et al. (1980) using goat anti-CEA antibodies reported further encouraging results in a study involving 142 patients with various CEA expressing tumours, with 85% of colorectal cancer being positive.

At present, several monoclonal antibodies have been used in the

immunoscintigraphy of colorectal cancer. For example, an anti-CEA monclonal antibody C46 which detected 83% of primary and metastatic cancers (Armitage et al., 1986); another anti-CEA monoclonal Mab 23 detected 50% of colorectal and pancreatic cancers (Mach et al., 1981); a monoclonal raised against colorectal cancer-associated antigen localised to 31 of 52 (60%) of patients with primary and secondary colorectal cancer (Mach et al., 1983) and another monoclonal YPC2/12.1 to 13 of 16 (81%) patients (Smedley et al., 1983). A monoclonal antibody 791T/36T produced against an osteogenic carcinoma cell line detected 13 out of 24 primary colorectal cancer or 54% and 21 out of 26 metastatic sites or 85% (Armitage et al., 1985). Another antibody raised against a breast cancer metastasis (B72.3) detected about 75% of colorectal cancers (Colcher et al., 1987). In general the tumour detection rate of monoclonal antibodies is in the region of 50-80%.

Although immunoscintigraphy is still under development, technical advances have been made in the preparation of suitable antibodies and their fragments, the labelling of antibodies with appropriate isotopes, and in new imaging techniques, such as emission computerised tomography (ECT) which employs the computerised technique used in CT scanning. The monoclonal antibodies generated with the use of colorectal tumour-associated antigens have the potential to be applied for the immunoscintigraphy or therapy of colorectal cancer. The extent to which these antibodies are likely to be successful in this context depends on a number of factors, of which the specificity of the antibodies is probably the most important one. The use of monoclonals for imaging and targeting therapy is, however, potentially compromised by tumour heterogeneity; a problem which has recently received significant attention (Edwards, 1985).

The appearance and morphology of cancer cells on histological sections

may be similar; however, they may not necessarily express the same antigens. Thus it is commonly found that on immunocytochemical staining of a cancer that only a proportion of the cells will react with the antibody, particularly when cell surface antigens are being stained, although this may be a quantitative rather than absolute qualitative difference. This phenomenon reflects tumour heterogeneity and it may occur between tumours or between cells within the same tumour.

This heterogeneity has significant clinical implication when monoclonal antibodies are used in immunoscintigraphy or targeting. For maximum efficacy, as many cells as possible should react with the monoclonals. One possible solution to tumour heterogeneity is the use of a "cocktail" of monoclonal antibodies, each recognising different antigenic determinants with different levels of expression to achieve maximal reaction between tumours and antibodies. In a clinical study, a mixture of $F(ab)_2$ fragments of monoclonal antibodies administered to a limited number of patients has shown a tendency towards improved tumour detection sensitivity (Chatal et al., 1984). In a xenograft antibody localisation study, two $F(ab')_2$ fragments of monoclonal antibodies GA 73-3 and CO 29.11 were compared when given alone or in combination. The tumour contrast was enhanced when a mixture of the two antibodies was given. Furthermore, increasing the protein doses of either antibody alone above the total dose used in the mixture did not produce a better image than the antibody mixture. The improved tumour contrast is likely to be due to the binding of the antibodies to distinct antigenic determinants, resulting in an increased total antibody binding. The radioactivity bound per cell is likely to be increased rather than the percentage of cells labelled, as monoclonal GA 73-3 binds to 100% and CO 29.11 binds to 60% of the cells in the xenograft. The results also showed that the binding of the one antibody to an individual cell did not interfere with binding from another antibody. It was also

found that the biological half-life of the mixture was longer than with either antibody fragment alone, although the mechanism was not clear (Munz et al. 1986).

A potential obstacle to the use of monoclonal antibody is the human anti-mouse antibody response (HAMA) (Chatenoud et al., 1989), some of which is directed at the anti-idiotypic region i.e. the antibody-antigen binding site, and this may prevent a repeated use of a monoclonal antibody. For these reasons, more monoclonal antibodies with a high affinity for colorectal cancer are required for the immunoscintigraphy of this tumour. Human monoclonal antibodies may be more suitable in this respect.

1.11 Monoclonal antibodies for targeting

Monoclonal antibodies can be used as carriers for cytotoxic agents such as toxins, radioisotopes or drugs when more effective ones become available. Thus radiolabelled monoclonal antibodies have already been used to treat ovarian, breast and gastrointestinal cancers (Epenetos et al., 1982b; 1986). In a phase I/II trial up to 30 mg of monoclonal antibodies (HMFG1, HMFG2, AUA1, and H17E2) have been given to patients with drug resistant ovarian tumours (Hnatowich et al., 1988). Response was obtained in two patients with tumours less than two cm in size. A pilot study has been performed using ^{131}I monoclonal antibodies in the therapy of leptomeningeal tumours (Lashford et al., 1988). Five patients who had not responded to conventional therapy were given between 11 mCi and 40 mCi radiolabelled antibody intrathecally. The choice of antibodies (UJ181.4, HMFG2, F8-11-13, Mel 14, 4C6 and FD32) was determined by prior immunoreactivity study between the antibody and the patient's tumour. There was no acute toxicity and the therapy was well tolerated. Four of the five patients

showed objective response to treatment which has been sustained from seven months to two years. There was no evidence of chronic toxicity in patients up to two years after therapy. The monoclonal antibody 791T/36 has been conjugated to methrotrexate either directly or via a human serum albumin bridging agent (Baldwin et al., 1987). The retention of antibody immunoreactivity with target cells was demonstrated by flow cytometry, and the retention of drug cytotoxicity was assessed by *in vitro* cytotoxicity and colony inhibition assays. The *in vivo* activity of the antibody-drug conjugates was tested in human tumour xenografts, and has shown a better toxic effect on the tumour than methrotrexate alone.

With regards to cytotoxic antibodies, mouse monoclonal antibodies of the IgG2a and IgG3 isotypes can mediate antibody-dependent cellular cytotoxicity (ADCC) in the presence of human effector cells and/or activate human compliment (Hellstrom et al., 1986). The mouse monoclonal antibody L6, an IgG2a identifies a ganglioside that is strongly expressed in most human carcinomas. It had been shown to kill antigen-positive cells in conjunction with either human lymphocytes (as effector cells) or human serum (as complement), and to inhibit the outgrowth of L6 antigen-positive human tumour in nude mice (Hellstrom et al., 1986). Sears et al. (1982) infused the monoclonal antibody 17.1, an IgG2a, intravenously or via the hepatic artery to 4 patients with hepatic secondaries from gastrointestinal tumours. The patient who received antibody incubated with his own monoclonal nuclear cells injected via the hepatic artery showed reduction in tumour size. Resected specimens showed heavier monocytic infiltration of the liver.

For clinical application, Abrams & Oldham (1985) listed the following characteristics of an ideal antibody and that of the antigen which the antibody recognises:

1. Antibody binds to an antigen on the cell surface;

2. Antibody binds with high affinity;
3. Antigen highly expressed on cell surface and found on most or, preferably, all cells in a tumour;
4. Antigen expressed at very low levels on a very limited number of normal tissues and/or found only on occasional cells in normal tissues;
5. Antigen-antibody complexes internalise;
6. Antibody mediates antibody-dependent cellular cytotoxicity (ADCC);
7. Biodistribution studies reveal negligible uptake of antibody by reticuloendothelial system.

The binding of antibodies to the cell surface antigens is necessary for radioimmunolocalisation to occur as intra-cellular antigens, being enclosed within the living cell by the cell membrane, cannot be detected by antibodies. Such intra-cellular antigens, however, may be demonstrated by immunocytochemistry as the cell membrane is removed during tissue processing and the antigens are thereby exposed. If immunocytochemistry is used to evaluate the potential of a monoclonal antibody for immunoscintigraphy, it is essential to examine for surface membrane reactivity between the antibody and the cell. The retention of immunoreactivity of the antibodies following radiolabelling is also important for radiolocalisation, as the radiolabelling process may damage the binding sites of the antibodies and prevent the binding of the radiolabelled antibodies to the target antigens. The size of a monoclonal antibody can influence its ability to localise to a tumour, as a larger molecule cannot cross tissue compartments as easily as a smaller molecule (Larson and Carrasquillo, 1987). Antibodies of the IgG subclasses would be better

than the larger IgM immunoglobulin in crossing tissue spaces to reach the tumour, and the smaller Fab fragments of an immunoglobulin may have a better penetration of the tumour than the intact IgG molecule (Schlom, 1986).

1.12 MONOCLONAL ANTIBODY - ANTIGEN REACTIONS : DETECTION TECHNIQUES

i Immunocytochemistry

Immunocytochemistry is the identification of tissue constituents in situ by means of antibodies in conjunction with suitable colour-generating reagents. Although it began in the 1930's it's use has been greatly extended, both in scope and in quantity by the development of monoclonal reagents. The different methods in use relate principally to the type of visualization system used. In early studies antibodies were generally coupled directly to coloured reagents, but in recent years the use of second antibodies and indirect techniques have predominated, as suitable species and class specific antibodies have been developed.

a Immunofluorescence

Reiner (1930) first demonstrated that the properties of pneumococcal antisera were not damaged by their coupling to chemical such as diazotised atoxyl. Later, Marrack (1934) labelled anti-typhoid and anti-cholera antibodies with R-salt-azo-benzidine. This preparation reacted with the organisms and produced a pink colour, although Coons found that the colour was not sufficiently strong for diagnostic use. About the same time Creech and Jones (1941) developed a fluorescent labelling method. They coupled the isocyanates of several aromatic hydrocarbons to various proteins and produced highly fluorescent substances. A

blue fluorescence was obtained when a fluorescent B-anthryl carbamido derivative of rabbit anti-pneumococcus III serum was agglutinated with the bacteria. Unfortunately, this product was similar to the normal colour of tissue. Another compound, fluorescein-4-isocyanate was used instead. This produces a high intensity greenish-yellow fluorescence of fluorescein even in high dilution.

Coons and his colleagues (1941) applied this method to label antibodies and thus initiated the immunofluorescence technique. Subsequently it was shown that the fluorescent dye fluorescein isothiocyanate (FITC) can be even more readily conjugated to antibodies, and to-date FITC has remained the fluorescent label of choice. There are however some disadvantages in using fluorescent dyes. They require a special microscope and are quenched by conventional mounting reagents, and bleached by repeated examination. Furthermore, their sensitivity is limited by the number of fluorescent molecules that can be successfully bound to the antibody.

b Enzyme-immunocytochemical techniques

These shortcomings of fluorescence stimulated the development of covalent coupling of enzymes to antibodies. Nakane and colleagues (Nakane & Pierce, 1966, 1967; Ram et al., 1966) were principally responsible for this development with significant contributions made by Avrameas (1968, 1969) and Sternberger et al., (1970). The standard technique of enzyme cytochemistry with development of an insoluble coloured product at the site of enzyme binding, is used to demonstrate the localisation of antibody-enzyme conjugates on tissue sections. Unlike immunofluorescence, the end results of these reactions are more permanent, can be observed using an ordinary microscope, and are potentially more sensitive. The enzymes commonly used for this purpose are peroxidase,

alkaline phosphatase and glucose oxidase. They may be coupled to the antibody in the direct method, or more commonly are conjugated to a second antibody, for example, a rabbit anti-mouse Ig antibody, which reacts with the mouse first antibody. This is known as the indirect method. The advantage of the indirect technique is that the second antibody can react with many different monoclonals which form the first antibodies. Furthermore, the indirect method has an amplification effect as several enzyme coupled second antibody molecules may bind to the first antibody, thus increases the sensitivity of the reaction. A precaution to be taken is the presence of endogenous enzymes in the tissues, for example, alkaline phosphatase in the colon. A false positive results may occur if the substrates react directly with such endogenous enzymes. A blocking agent is commonly used to suppress these endogenous enzyme activity with good effect. Hydrogen peroxide in methanol 0.5% v/v is used to block peroxidase activity, while glacial acetic acid in distilled water 15% v/v is used to block phosphatase activity. The enzyme blocking procedure is not possible with frozen sections, as this will damage the tissue. Endogenous peroxidase in macrophages is therefore commonly seen in frozen sections. The use of suitable controls are thus important in the interpretation of results. In the case of the colon, it would be appropriate to compare the alkaline phosphatase and the immunoperoxidase techniques. A diagramatic representation of an indirect immunocytochemical reaction is illustrated in Figure 2.

ii Enzyme-linked immunsorbent assay (ELISA)

This assay was developed independently by Engvall and Perlmann (1971) and van Weemen and Schuurs (1971), and was based on the same basic principle as the enzyme immunocytochemical technique. It is an assay system in

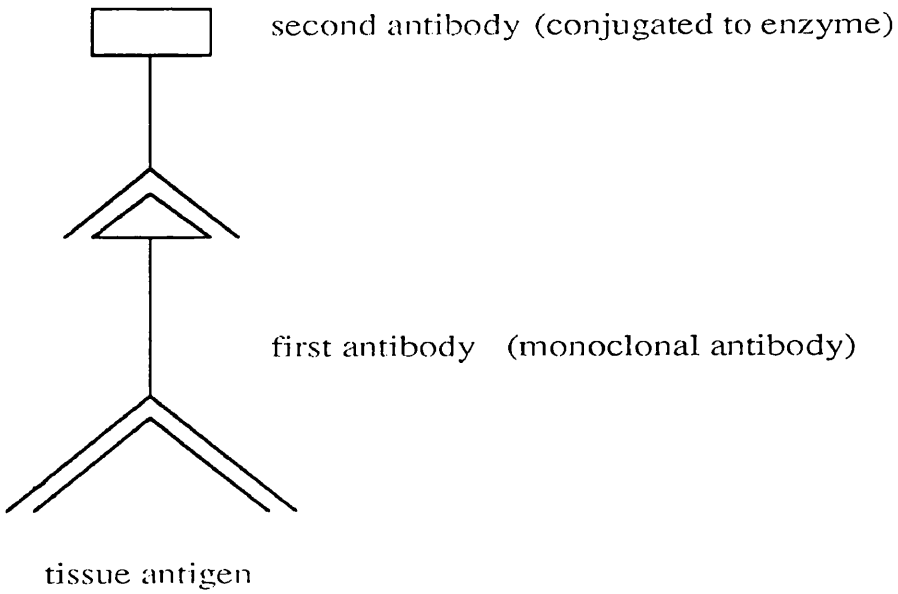


Figure 2 Diagramatic representation of an indirect immunocytochemical reaction

which one of the reactants is labelled with an enzyme and another is attached to a solid phase, commonly a 96 well plastic plate. The enzymes used should have the following characteristics: 1) the enzyme activity should be simple to quantitate, e.g. spectrophotometrically; 2) the turnover number should be high and the product of the enzymatic reaction should have a high-molar extinction coefficient, facilitating detection of small amounts of enzyme; 3) the enzyme should be stable and 4) the enzyme should be readily available in a purified state (Engvall & Carlsson, 1976). The enzymes used is typically a peroxidase (van Weemen & Schuurs,1971) or alkaline phosphatase (Engvall and Perlmann,1971) which react with a corresponding chromogenic substance to produce a colour which is then measured spectrophotometrically. The results are expressed numerically. The upper limit of the result is determined by a positive control (i.e. an antibody reacting positively with a target antigen), and the lower limit or negative reading is determined by a no first or an irrelevant antibody control. The presence of endogenous enzyme in the antigen being tested is detected by the addition of substract only to the plate to prevent false positive result from being recorded. The reactivity between an antibody and an antigen is judged against the upper and lower limits set by the controls. ELISA is more convenient than radioimmunoassay for large-batch analysis, as the procedures for the handling of reagents are simpler, and unlike radioimmunoassay, stable reagents which can be stored for a long period are used. The disadvantages with ELISA are the batch-to-batch, plate-to-plate and even well-to-well variability of the microtitre plates routinely used, producing the so-called edge effect (Chessum & Denmark, 1978; Kricka et al., 1980) which may give a false positive result.

iii Radioimmunoassay (RIA)

Radioimmunoassay was first developed for the measurement of protein and polypeptide hormones in the late 1950's and the 1960's (Parker, 1976), but has since been applied in a much wider context. The antigen-antibody reactions form the basis of the assay. Although originally radioiodinated antigens were used for such assay, many modifications have been made, and radiolabelled antibodies have also been used. In principle, it does not matter whether it is the antigen or the antibody that is labelled nor how the measurement is made as long as the reaction is specific, and there is sufficient difference between the free and bound reagents. In a solid phase radioimmunoassay, the antigens, for example crude membrane extract of colorectal cancer, are coated onto a polystyrene or polyvinylchloride (PVC) microtitre plate. The antibodies to be tested for reactivity with the antigens are then added. The reactive antibodies will be bound to the antigens on the plate. This reaction is detected by the addition of a radiolabelled second antibody which is raised against the test antibodies. The plate is washed to remove any unbound radioactivity. The bound radioactivity, indicating a specific reaction between an antibody and antigen, is then released from the plate by means of detergent and transfered to a scintillation tube to be counted in a gamma counter. The results are expressed numerically as count of radioactivity per minute (c.p.m.), and similar to ELISA, interpretation of results depends on the reading obtained from a positive and a negative control antibody. Radioimmunoassays are very sensitive and usually easy to perform. However, there are several disadvantages, for example, the use of unstable reagents, necessitating frequent preparation, the need for sophisticated equipment for measurement of the radioactivity, and the risk of handling radioisotopes and their disposal.

In the choice of an assay for detecting the reaction between an antibody

and an antigen, such as in the screening for reactive antibodies in the production of monoclonal antibodies, a sensitive and reproducible technique which is simple to perform is desired. It is necessary to test both the ELISA and RIA techniques for a suitable assay to be chosen. However, the advantages of ELISA have made it a more preferable assay to be used whenever possible.

iv Flow cytometry

In this technique the reaction between tumour cells and monoclonal antibodies is detected by means of a fluorescence activated cell sorter (Beverley, 1984). The cells to be tested are prepared as a single cell suspension and are then incubated first with the antibodies and then with an anti-mouse immunoglobulin conjugated to fluorescein. A positive reaction between the antibodies and the cells will therefore result in the coating of the cells with a layer of fluorescein. On passage through the cell sorter the fluorescein-coated cells are individually detected by being passed in a narrow fluid stream, past a source of light (usually a laser) and an appropriate detector. This signal generated by each cell is separately recorded and information on the intensity of fluorescence in many cells can be quickly accumulated.

The disadvantage of the fluorescence activated cell sorter is that it is a very expensive piece of equipment and is not generally available. However, it is a reliable and quantitive method of detecting the reaction between an antibody and a cell. Moreover, a positive reaction with live cells indicates that the reactive-antigen is present on the cell surface. Monoclonal antibodies reactive with cell membrane are necessary for the radioimmunolocalization of tumours. The flow cytometry technique can therefore be used to confirm the surface reactivity of a monoclonal antibody with tumour cells observed on immunocytochemical staining.

1.13 Radiolabelling of monoclonal antibodies

An increased understanding of radiochemistry, the availability of different radioisotopes and recent developments in the radiolabelling techniques for antibodies have all contributed to the advances in immunoscintigraphy of tumours using polyclonal and monoclonal antibodies.

i Radioiodination of antibodies

The first radioisotopes of iodine were available in the late 1940's. The first use of ^{131}Iodine labelled proteins of high specific activity was achieved by Berson et al. (1956) in an insulin assay. A large quantity of iodine was necessary for labelling. The use of ^{131}Iodine was made easier and safer by better and purer preparation of the radioisotope in the late 1950's and improvement in labelling technique (Hunter and Greenwood, 1962). Another isotope ^{125}Iodine which has a long half life of 60 days became available in 1963. Both ^{131}Iodine and ^{125}Iodine are now commonly used for the labelling of antibodies in clinical and laboratory studies.

Radioiodination of antibodies can be achieved by reacting the oxidised iodine in the form of hypoiodous acid with the tyrosine and histidine residues of the antibody molecule. Oxidation of the iodine can be rapidly achieved by using chloramine T (Greenwood et al., 1963). To prevent any reaction of the oxidised iodine with other co-existing proteins, sodium metabisulphite is added to reduce unreacted isotope back to iodide and to prevent the iodination of any carrier protein subsequently added. An alternative gentler method is the use of the solid phase 'iodogen' reagent (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril) which is pre-coated onto a vial and the iodination is completed by the addition of an aqueous mixture of radioiodine and antibody (Fraker and Speck, 1978). The stability and

poor solubility of this chloroglycoluril in water can account for the minimal damage to the proteins and even living cells in the iodination process and avoided the need for reduction at the end of the reaction. Another iodination method involves the use of a peroxidase and hydrogen peroxide oxidising agent. The oxidised iodide is attached to the active site of a peroxidase which then transfers it to the tyrosine on the antibody (Morrison et al., 1971). In the methods described above, various oxidising and reducing agents are added to the antibody iodine mixture. Although these agents are intended primarily for the iodine, they may damage the antibody in the labelling process. To prevent contact between the protein being iodinated and oxidising agents, reducing agents and potentially damaging contamination in the radioiodide solution, Bolton and Hunter (1973) descrided a method in which a ligand is first labelled. After purification it is then mixed with the target molecule to form amide bonds with the free amino groups.

ii Bifunctional chelating agents for radiolabelling

A more recent development in antibody labelling has been the use of bifunctional chelating agents (Sundberg et al., 1974). Such agents can be activated chemically for binding to protein through an amide bond and at the same time also possess a binding site for metal. Sundberg et al. (1974) described a method using an analogue of ethylenediaminetetraacetic acid (EDTA) to make specific metal chelating sites on macromolecules. A more comonly used method is that described by Krejcarek and Tucker (1977) to which diethylenetriaminepentaacetic acid (DTPA) is attached to proteins covalently using a mixed anhydride reaction. The technique produces hexadentate chelating sites for metal ions on proteins. A variety of metals with useful physical properties can be specifically bound to the DTPA-conjugated macromolecules. This technique has been used to produce

antibody conjugates (Stern et al., 1982; Fairweather et al., 1983). Other approaches have also been used (Pritchard et al., 1976; Yeh et al., 1979; Hnatowich et al., 1982). Hnatowich et al. (1983) have used cyclic-DTPA conjugates of monoclonal anti-CEA antibodies for xenograft localisation studies, without detecting any damaging effect on the immunoreactivity of the antibody on cell binding. Stern et al. (1982) have demonstrated the *in vivo* stability of similar preparation. For clinical application, the radioactive metals available are ^{67}Ga, ^{111}In and ^{99m}Tc. These isotopes are suitable for gamma imaging as they are readily available, have appropriate half lives and relatively low v-ray energy. The radiation doses they emit are not too high for diagnostic imaging. ^{111}In with a half life of 2.8 days has been used for immunoscintigraphy.

In clinical immunoscintigraphic studies indium-labelled antibodies detected more tumours than corresponding ^{131}I labelled antibodies, for example, in the detection of CEA producing tumours (Fairweather et al., 1983). A similar finding confirming the superiority of indium labelled antibody was reported by Rainsbury et al. (1983) using ^{111}In, ^{123}I and ^{131}I labelled monoclonal antibody M8 in the imaging of breast cancer. However, an important and significant disadvantage of indium-labelled antibodies is a high uptake of radioactivity by the liver, to such an extent as to prevent detection of hepatic metastases. The accumulation of radioactivity in the liver may be due to the release of indium-111 by the DTPA linker (Goldenberg, 1988). Although attempts have been made to address this problem, no effective solution has so far been found. New chelates are being developed to overcome this difficulty (Halpern & Dillman, 1987).

CHAPTER 2

AIMS OF THESIS AND PLAN OF INVESTIGATION

2.1 INTRODUCTION

Despite a reduction in operative mortality and morbidity through the availability of better antibiotics and methods of bowel preparation, the 5 year survival rate of colorectal cancer following surgery has remained between 20-50% in the past three decades. An important reason for this lack of progress is the extent of tumour dissemination at the time of diagnosis so that eradication of the disease is still not attainable. Various radical surgical techniques have been developed over the years but have not not been proved to increase survival. Recently, there has been a renewed interest in the the no-touch technique in the prevention of tumour dissemination but this is not widely practised and fails to show benefit in survival. Resection of the mesorectum may prevent recurrent rectal carcinoma and increase survival but more supportive evidence is required. Patients with hepatic metastases generally have a poor prognosis but there is evidence to suggest that surgery can increase the survival in some of these patients. Unfortunately, only a small number of patients are suitable for hepatic resection of colorectal cancer metastases, as only those with less than 4 tumour deposits and who at the same time do not have extra-hepatic disease are likely to do well. It is clear that following surgery on the primary cancer systemic therapy is needed to treat occult dissemination. Various adjuvant therapies have been developed, but the results of these treatments have been disappointing in terms of survival, although they may delay the onset of recurrent disease. To date there is still no effective chemotherapeutic agent against colorectal cancer.

Surgery is more likely to be curative in patients with Dukes' A tumour and emphasis is placed on early diagnosis of colorectal cancer. Screening for colorectal cancer with faecal occult blood testing may improve early diagnosis of

colorectal cancer, but such tests need better sensitivity and specificity for wider acceptance, without underestimating the problems of compliance and adequate resources and facilities to ensure the success of a screening programme.

It can be seen that an improvement in the prognosis of colorectal cancer may be achieved in three areas, namely that of early diagnosis, more accurate and early detection of metastatic tumour and more effective treatment for disseminated disease.

Monoclonal antibodies may have the potential to solve some of the problems outlined above, as they may be used as a means of detecting colorectal cancer following labelling with radioisotopes and for therapy of disseminated tumour when suitably armed with cytotoxic agents. These possibilities have initiated the present investigation into monoclonal antibodies. Although several monoclonal antibodies have been used in the radiolocalisation of colorectal cancer, antibodies with better specificity are still required, and more antibodies are needed to overcome the phenomenon of tumour heterogeneity and the human anti-mouse antibody (HAMA) response (1.10)

2.2 AIMS OF THESIS

A fundamental requirement in the application of monoclonal antibodies to colorectal cancer is the demonstration of their *in vivo* localisation to the tumours. The study performed here is therefore planned with this ultimate objective. However, an initial step is to search for colorectal cancer-reactive monoclonal antibodies, for which two approaches have been used. The first was the use of a crude membrane extract of colorectal cancer from several patients to produce new

monoclonal antibodies and the second was to screen monoclonals raised to other tumours for their cross-reactivity with colorectal cancer. Potentially suitable monoclonals were tested for *in vivo* localisation in a xenograft model before a clinical localisation study in patients with colorectal cancer was carried out.

2.2.1 Production of new monoclonal antibodies to colorectal cancer

To date monoclonal antibodies raised to colorectal cancer have cross-reactivity with other normal tissues and are not entirely tumour-specific. Although as already discussed, tumour-specific monoclonal antibodies to colorectal cancer is unlikely to be obtained, there remains a possibility that antibodies with a better reactivity to this tumour may be produced. The immunogens used for the production of colorectal cancer monoclonal antibodies have included cell lines (Herlyn et al., 1979; Koprowski et al., 1979) or tumour extracts (Finan et al., 1982). A cell membrane extract obtained from a pool of different patients with colorectal cancer has not previously been used before for this purpose.

The likely location of colorectal cancer-associated antigens is in the cell membrane. Such antigens may be expressed in varying quantities in different tumours as illustrated by tumour heterogeneity. A membrane extract from a larger number of different patients is potentially more likely to contain tumour-associated antigens than a smaller number as has been adopted by others (Finan et al.,1982; Thompson et al., 1983; Brown et al., 1983; Makin, 1986). A crude membrane extract of colorectal cancer resected from 13 different patients was therefore used as an immunogen in an attempt to produce more tumour-specific monoclonals. It was hoped that this preparation would provide a good representation of tumour

antigens present on the cell membrane and obviate the problem of inadvertantly raising antibodies to rare histocompatibility/blood group antigens inherent in the use of a single patient's tumour (1.7.iii).

An immunoglobulin non-secreting mouse myeloma cell line NSO (Clark et al., 1981) was used as the fusion partner to produce the monoclonal antibodies, thus avoiding the contamination with irrelevant immunoglobulins secreted by the myeloma. During the production of the monoclonals, an enzyme-linked immunosorbent assay (ELISA) using the immunogen as the target antigen was used as the initial screening method. ELISA was chosen as the screening assay after a comparison between ELISA and a radioimmunoassay showed that the two techniques gave equal sensitivity. Positive antibodies were then screened on frozen sections of colorectal cancers by means of immunocytochemistry. As the main objective of monoclonal antibody production was to select antibodies for immunoscintigraphy, characterisation of these antibodies was performed mainly with immunocytochemistry to define their reaction with colorectal cancer and other normal organs after their immunoglobulin classes had been determined. The reactivity with the surface of live colorectal cells was also tested with some monoclonals using flow cytometry.

2.2.2 Selection of monoclonal antibodies cross-reactive with colorectal cancer

In the late 1970's and 1980's, there was an expectation that monoclonal antibody technique might produce tumour-specific antibodies. This hope was fuelled by reports of several antibodies which appeared to fit this category. An example is the monoclonal antibody Ca1 which was raised against wheatgerm agglutinin-binding glycoproteins extracted from a human laryngeal carcinoma cell

line (Ashall et al., 1982; McGee et al., 1982). It was described as cancer specific because it reacted with the majority of cancers on immunocytochemical staining but apparently not with benign tumours or the majority of other normal tissues, (except the fallopian tube and transitional epithelium of bladder). However, more thorough immunocytochemical studies have shown that the monoclonal antibody Ca1 does in fact react with a number of normal tissues (Woodhouse et al., 1985), and is not different in this respect to a number of other monoclonals raised against tumour cells, and even normal tissue products such as milk fat globule membrane.

It is likely that cells from different tissues or tumours share a large number of surface antigens; tumour cells may show great heterogeneity and an ability to modulate their surface antigens (Fink & Clarke, 1984). The only truly tumour specific monoclonal antibodies are those produced to some lymphomas which have unique immunoglobulins on their cell surfaces acting as specific markers (Wiseman & Rao, 1986). However, the lack of tumour specific antigens in most tumours should not prevent the application of monoclonals for immunoscintigraphy or therapy, provided that their affinity for tumours is higher then normal tissues. The nature of the antigens recognised by many tumour-reactive monoclonal antibodies is still unknown.

In the characterisation of this type of monoclonals, it is commonly found that monoclonal antibodies to different human tumours may cross-react with corresponding normal tissues (Beverley, 1982). Antibodies produced against one cancer will also react with other tumours of another histological type although usually of similar cell origin i.e. carcinomas as distinct from sarcomas, although there are even exceptions to this general correlation. In the case of colorectal cancer, cross-reactivity is illustrated by the antibody B72.3 which was originally raised against a breast cancer. It has, in fact, a greater reactivity with colorectal

than breast cancer (Colcher et al., 1981). Another monoclonal antibody 791T/36 which was raised against an osteogenic sarcoma cell line, cross-reacts with colorectal cancer (Armitage et al., 1985). Both of these antibodies have been used for the immunoscintigraphy of colorectal cancer.

Therefore the properties of cross-reactivity of monoclonal antibodies may allow the recognition of more antibodies which are reactive to colorectal cancer. A panel of 25 monoclonal antibodies raised against breast, bladder and other cancers was screened for reactivity to colorectal cancer by immunocytochemistry. The indirect alkaline phosphatase and the indirect immunoperoxidase techniques were used and compared for this purpose as endogenous alkaline phosphatase in the colon may influence the staining reaction. Monoclonal antibodies showing the best reactivity with colorectal cancer were further tested for their reactivity to metastases in the liver and lymph nodes as well as normal colon. Antibodies with a greater affinity for colorectal cancer than normal colon were then prepared for immunoscintigraphy of colorectal cancer.

2.2.3 Localisation of radiolabelled antibodies to xenograft model

Immunocytochemistry is not the sole criterion in the selection of a monoclonal antibody for imaging. There are other important factors to consider. One is the immunoglobulin class as the bigger IgM molecule is less suitable than the smaller IgG antibody in crossing tissue compartments to reach the tumour (1.11). Another factor is the immunoreactivity of the antibody following radiolabelling which may damage the antibody. Successful application of these antibodies for immunoscintigraphy depends on their retention of immunoreactivity following radiolabelling. The radiolabelled antibodies must also be stable *in vitro* and *in vivo*. Instability of radionuclide bound to antibodies will result in

interference of scanning due to a high background from the free radioisotope in the circulation, unless it is cleared rapidly from the circulation. Various radiolabelling methods involving different isotopes have been developed to overcome these problems of which radioiodination has probably been the most widely used, and is certainly the earliest. As each monoclonal antibody is unique in its chemical reactions, generalisation cannot be made about immunoreactivity following radiolabelling by a particular iosotope or method. Radiolabelling of the antibodies was therefore performed with iodine-125 using both the chloramine T and iodogen methods, to determine which was a better technique, and also with indium-111.

The immunoreactivity of a selected antibody was tested with a cell binding assay (Rainsbury, 1984) and compared with the binding obtained when excess unlabelled antibodies was added. A radiolabelled monoclonal antibody non-reactive with the target cell line used for the binding assay was used to provide a control cell binding result.

The *in vivo* reactivity of the radiolabelled antibodies was tested by their ability to localise to tumours in xenograft models. Xenografts are human tumours transplanted to immune deficient animals, usually a mouse or rat. They are a convenient way of providing a human tumour in an *in vivo* setting for assessing the localising ability of radiolabelled antibodies. Immunocytochemistry was used to select an appropriate xenograft reactive with these antibodies. The radiolabelled antibody uptake in the tumour was measured by means of the tumour to blood ratios taken at various time points in several groups of animals. The uptake of radiolabelled antibodies by other organs of the animals was also measured. A radiolabelled antibody which was non-reactive with the xenograft was used in a control study.

2.2.4 Immunoscintigraphy of colorectal cancer in patients

The cell binding assay and the xenograft localisation studies have been employed to evaluate the immunoreactivity of the selected radiolabelled antibodies, before proceeding with a clinical immunoscintigraphy study.

Patients with primary colorectal cancers and a few with recurrent tumours were invited to take part in the investigation. The radiolabelled antibodies were administered by intravenous injections following a skin test in which unlabelled antibody was injected subcutaneously to exclude a hypersensitive reaction. At the beginning of the study a gamma camera scan was taken daily to determine the optimal scanning time. Later, scans were taken at the optimal time. The localisation of the antibodies were compared with other clinical parameters. Further confirmation of localisation was obtained by measuring the radiolabel uptake by the tumours and other normal tissues (pericolic fat, normal colon, muscle, subcutaneous fat and skin) as compared to blood. The activities of the radiolabelled antibodies in the circulation and in the urine were measured in a few patients to determine if clearance of the antibodies correlate with the optimal imaging time.

CHAPTER 3

PRODUCTION OF NEW MONOCLONAL ANTIBODIES TO COLORECTAL CANCER

3.1 INTRODUCTION

In the search for new colorectal cancer reactive monoclonal antibodies, two strategies were adopted in this thesis. The first, described in this chapter, was the production of new antibodies to this carcinoma using a crude membrane extract (CME) prepared from a pool of several colorectal cancers. This was an effort to generate colonic cancer-associated specificities not available in existing antibodies and, hopefully, better than those to be identified by the other approach involving the screening by immunocytochemistry for cross-reacting antibodies and described in the next chapter.

To date, the immunogens used for the production of monoclonal antibodies to colorectal cancer have included carcinoembryonic antigen (CEA) (e.g. Accolla et al., 1980; Hedin et al., 1982), cell lines (Herlyn et al., 1979; Koprowski et al., 1979; Voak et al., 1980; Arklie and Bodmer, 1981; Makin, 1986) or cells from freshly resected carcinomas (Koprowski & Steplewski, 1981), whole tumour cells (Thompson et al., 1983), and membrane preparations (Finan et al., 1982; Brown et al., 1983). The details of these monoclonal antibodies to colorectal cancer are shown in Table 1 (Chapter 1).

None of these monoclonal antibodies are colorectal cancer specific. Most show cross-reaction with normal tissues. The reactivity of some of these antibodies to certain normal tissues has not been reported. Not all of these monoclonal antibodies are suitable for immunoscintigraphy of colorectal cancer as the transport of a protein from the circulation into the extracellular fluid is inversely proportional to the molecular weight of the protein (Larson and Carrasquillo, 1987). Thus antibodies of the immunoglobulin class IgM, for example, the monoclonal antibody C14/1/46/10 (Brown et al., 1983) is usually too large for

efficient penetration into the tumour, because of their pentameric structures. The smaller IgG class antibodies are more suitable. Some antibodies react with cytoplasmic rather than surface membrane antigens (e.g. monoclonal antibody CAM5.2, Makin, 1986), and such intra-cellular antigens will not be able to react with the antibodies *in vivo*, other monoclonals cross-react with normal tissues or blood cells e.g. monoclonal antibodies 1/3.12, 1/1.1, 2/9.12 and 2/13.4 (Voak et al., 1980).

Some of the monoclonal antibodies recognising colorectal cancer antigens have, nevertheless, been used for immunoscintigraphy including 17-1A, 19-9 (Chatal et al., 1985) and YPC2/12.1 (Smedley et al., 1983). The monoclonal antibody 17-1A detected 54% of colorectal carcinoma (Mach et al., 1983) and monoclonal 19-9 detected 66% of the cancer (Chatal et al., 1984), while the monoclonal antibody YPC2/12.1 localised to 81% of colorectal cancer (Smedley et al., 1983). Other monoclonal antibodies used for immunoscintigraphy of colorectal cancer have been raised to carcinoembryonic antigen (CEA), such as Mab 23 (Mach et al., 1985) which detected 60% of colorectal cancer, and the monoclonal antibody C46 which localised to 83% of the primary and secondary tumours (Armitage et al., 1986).

Two monoclonal antibodies cross-reacting with colorectal cancer have been used with successful imaging of this carcinoma. The monoclonal antibody 791T/36 raised against an osteogenic sarcoma cell line detected 54% of primary colorectal cancer and 85% of metastatic carcinomas. The monoclonal antibody B72.3 raised against a breast carcinoma (Colcher et al., 1987) localised to 75% of colorectal cancer. The tumour detection rate of these different antibodies is in the region of 50 to 80%, irrespective broadly of the antigen with which they react or the tumour against which they were raised.

There is, therefore, still a need for more antibodies with greater tumour reactivity. Furthermore, the phenomenon of tumour heterogeneity (1.10) results in only a proportion of cells in a tumour reacting strongly with an antibody. Thus the generation of monoclonal antibodies to distinct epitopes of colorectal cancer antigens would provide a better choice of potential 'cocktail partners' for the improved imaging or targeting of this carcinoma. The production of new antibodies may also help overcome the human anti-mouse antibody (HAMA) response (1.10) which follows a repeated administration of a monoclonal. There is evidence to show that at least part of the HAMA response is directed against the idiotypic region (binding site) of the immunoglobulin (Chatenoud et al., 1989), reducing the interaction of an antibody with a target antigen. A panel of different monoclonal reagents would enable alternative antibodies to be administrated when a HAMA response has developed.

A CME of colorectal cancers resected from different patients was used as an immunogen. A pool of tumour extract such as this has not been used before in the production of monoclonal antibodies to colorectal cancer. It was hoped that this preparation would provide a good representation of tumour antigens present on the cell membrane and obviate the problem of inadvertantly raising antibodies to rare histocompatibility/blood group antigens inherent in the use of a single patient's tumour.

The fusion method used in this study was a modification by Edwards (1981) in which agar was used in the initial plating of cells following fusions of spleen cells from mice immunised with the crude membrane extract from colorectal cancers. This method facilitates the aggregation of cells into clones, enabling the picking of clones earlier, as opposed to the plating in liquid tissue culture medium in which mixing of clones can occur.

A radioimmunoassay and an ELISA were compared before either was chosen for screening the fusion products with CME as the target antigen. As a secondary screen the immunoperoxidase method was employed to check all CME-positive antibodies for their reaction with frozen sections of colorectal cancer. Frozen sections were used as cellular antigens might be damaged by tissue fixation and paraffin embedding, thus producing a false negative result.

As the purpose of the production of monoclonal antibodies to colorectal cancer was for tumour localisation, the characterisation of these antibodies concentrated on their reaction with different colorectal cancers, normal colon and other normal tissues. Frozen sections were used for this purpose, but the reactivity of the antibodies with formal saline-fixed and paraffin-embedded tissues was also tested, as the use of paraffin-sections would simplify immunocytochemical studies. As the antibodies used for immunoscintigraphy should react with the surface membrane of the colorectal cancer, the reaction of one of the antibodies with the cell surface of live colorectal cancer cell lines was also tested by means of fluorescence activated cell analysis to determine if membrane reactivity observed in immunocytochemical staining correlated with the finding in the live cells.

3.2 MATERIALS AND METHODS

3.2.1 Sources of tissue samples

Fresh tissues were required to prepare the CME and frozen sections for the screening and characterisation of monoclonal antibodies (3.1). Frozen sections were used to check the reactivity of CME-positive antibodies with colorectal cancer in the initial screening. For determining the tissue distribution of the monoclonal antibodies produced, frozen sections of colon, lung, liver, spleen, kidney, oesophagus, stomach, breast, adrenal, thyroid, ovary, testes, epididymis and skin were used.The reactivity of monoclonal antibodies with paraffin-embedded tissue sections were also studied.

For frozen sections, fresh tissues were collected from the operating theatre of University College Hospital London. They were taken immediately to the Histopathology Department for examination by a pathologist who then removed appropriate samples from the specimens to be snap-frozen in liquid nitrogen and stored in liquid nitrogen until use. In the preparation of CME, colorectal cancer specimens were obtained from 13 patients.

For paraffin-embedded tissue sections, different colorectal cancers, colonic polyps and normal colon were obtained from the Histopathology Department of University College Hospital London. These specimens were used for the characterization of the antibodies after the reactivity of the antibodies with paraffin-embedded tissue sections had been tested.

3.2.2 Antibody screening assays

In the selection of a screening assay to test for the production of reactive-antibody by the hybridomas, a radioimmunoassay was compared with

ELISA. Two colorectal cancer cell lines, Colo 205 and SW 480 (obtained from the American Tissue Culture Collection, Rockville, MD.), grown in 96 well plates and fixed with glutaraldehyde were used. The monoclonal antibodies used were M8 (Foster et al., 1982 a,b), 77-1, 8-30-3, 48-1 and 3-48-2 (Summerhayes et al., 1985) in the form of tissue culture supernatants. The culture supernatant from the myeloma cell line NSO was used as a negative control. Further control was provided by the addition of phosphate buffered saline / 0.5% w/v bovine serum albumin (PBS/BSA) (Appendix 2) to the wells. The reaction of these antibodies with CME-coated plates was also tested by means of ELISA.

i Preparation of cell line plates

The cell lines Colo 205 and SW 480 were grown to confluence in 96 well plates with DMEM and 10% FCS, buffered with 5% CO_2. The tissue culture medium was removed and the cells were fixed in 0.25% v/v glutaraldehyde for five minutes. They were washed three times with PBS and once with PBS/BSA. The plates were preserved in 200 µl of 0.2% w/v sodium azide in PBS/BSA and kept in a moist box at 4°C until use.

ii Preparation of crude membrane extract (CME) plates

CME (3.2.3.i) was coated onto 96 well microtitre plates (Falcon) using a carbonate-bicarbonate buffer (Appendix 2). To determine the optimal CME concentration for the ELISA, CME was diluted with the coating buffer at doubling dilutions up to 1: 1280. The diluted CME (200 µl) was added in triplicates to the wells and incubated overnight to ensure a permanent coating of CME to the wells. Reactive antibodies (obtained from sera of immunised mice) were used to test the different concentrations of the CME preparations and the optimal concentration

was selected for subsequent ELISA. The CME coated plates were covered with cling film and stored in a moist box at 4°C until use.

iii Enzyme-linked immunosorbent assay (ELISA)

The PBS/BSA was removed from the wells and in the case of CME plates they were washed with 200 µl PBS/BSA for a further hour to remove any residual coating buffer. Antibody supernatant (50 µl) was added to the wells in triplicate and incubated at room temperature for 1 hour. After washing three times with PBS/BSA and three times with PBS, 50 µl of rabbit anti-mouse Fab'2 conjugated to urease (Sera-Lab) diluted 1:100 in PBS/BSA was added to each well. Following one hour incubation at room temperature, the plates were washed three times with PBS/BSA, three times with PBS, and twelve times with distilled water. A volume of 50 µl of substrate solution (urea in bromocresol blue, Sera-Lab) was added to each well, and the plate was incubated at 37°C for 30 minutes. The optical density was read at 570 nm using a Dynatech Microplate reader MR600. Further readings were taken at 30 minute intervals to select an optimal reading which should give the highest difference between the positive readings and the negative controls.

iv Radioimmunoassay

The wells were emptied of the PBS/BSA, and 50 µl of the first antibodies were added to incubate for one hour. The wells were washed three times with PBS/BSA. The second antibody, a rabbit anti-mouse immunoglobulin (Sigma) labelled with iodine-125 was added (50 µl) and left for 1 hour. The second antibody was then removed and the wells were washed four times with PBS/BSA. A detergent consisting of 1% w/v sodium dodecyl sulphate (SDS)

(Sigma) and 0.1 M sodium hydroxide (NaOH) was then added. After five minutes the contents from the wells were transfered to scintillation vials for counting using a Packard Multi-Prias autogamma counter. Positive results were determined by comparing the readings with negative and no first antibody controls.

3.2.3 Production of monoclonal antibodies

The method used for the production of monoclonal antibodies was essentially similar to that used by Kohler and Milstein (1975). To facilitate clone selection following fusion, the modification adopted by Edwards et al. (1980) was used. In this method the fused cells are grown in semi-solid agar medium so that hybrids will grow as separate clones at the outset, but can be screened in bulk, following which individual clones from positive wells are rescreened after separately removing them from the agar and culturing them in liquid medium. Mouse thymocytes were used as 'feeders'. A schematic representation of the production of monoclonal antibodies is shown in Figure 3.

i Preparation of immunogen

CME was prepared from 13 different colorectal tumours essentially as described by Standring and Williams (1978), except that no detergent was used. A total of 10.2 gm of pooled tumour was diced dry on a petri dish on ice, and washed twice in cold PBS (pH 7.5). The mixture was filtered and rinsed several times with cold PBS, following which five volumes of cold Tris buffered saline (TBS) pH7.4 (Appendix 2) containing 1mM phenylmethylsulphonyl fluoride (PMSF) was added to the tumour pieces.

The tissue was homogenised on ice with a Potter-Elrhevm homogeniser, until almost all the tumour pieces were disaggregated. The mixture was then spun

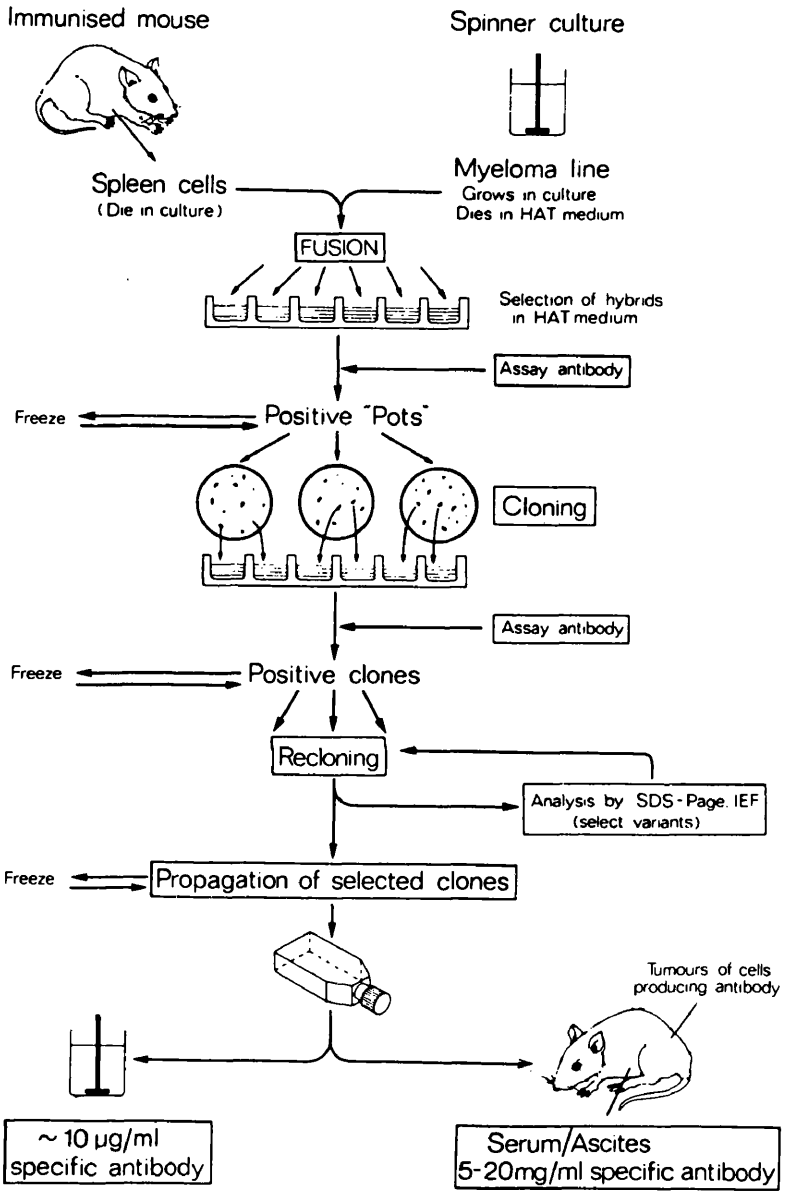


Figure 3 Schematic representation of the production of monoclonal antibodies. (From Milstein and Lennox, 1980)

at 3000 x g for 15 minutes to remove nuclei. The supernatant from the first centrifugation was spun further at 35,000 x g for 30 minutes at 4°C in a Sorvall RC-5 Superspeed refrigerated centrifuge to pellet mitochondria. The second supernatant was then spun at 100,000 x g for 60 mins at 4°C to pellet small membrane fragments. The resultant pellet, which constituted the CME, was resuspended in 5 ml of 10mM Tris buffer (Appendix 2), divided into 200 µl aliquots and stored at -20°C. The total protein content of the CME was determined by the method of Lowry et al. (1951) using dilutions of BSA at 100 µg/ml to 600 µg/ml as the standard.

An aliquot of CME (200 µl) of CME was transferred to a conical flask and frozen in ethanol / ice. The water in the CME suspension was removed by vaccum evaporation. The dry CME was weighed and re-suspended in 1 ml of 1% deoxycholate solution. Three dilutions of CME were used for protein estimation (100%, 50% and 25%). Reagents A and B (Appendix 2) were mixed at a ratio 50:1, and 1ml was added to each sample for 10 minutes at room temperature. A volume of 100 µl of Folin and Ciocalteu's phenol reagent (BDH) was added to each sample, and after 30 minutes the intensity of colour change of the CME and the BSA was read by absorbtion at 640 nm. A graph of the optical density values for the known BSA standards was used to determine the protein content of the CME.

ii Immunisation of mice

One 200 µl aliquot of CME prepared above was used to immunise four mice. The CME was diluted to 800 µl with PBS and female ex-breeder Balb/c mice (Olac) in groups of four were each given 0.2 ml of diluted CME intraperitoneally (ip) at weekly intervals for four weeks. Mice were tested for serum antibody

response using tail-bleed samples, by means of ELISA using CME coated plates. Reactive mice were given a booster injection ip. 3 days prior to sacrifice. To maintain antibody secretion in mice not yet used for fusion, immunisation was continued at weekly intervals.

iii Fusion method

a Preparation of spleen cells

A mouse showing significant anti-CME activity in the blood was sacrificed by cervical dislocation and the spleen was removed aseptically and placed in 3 ml of air-buffered Leibowitz L15 medium (Gibco-Biocult). The spleen cells were disaggregated by shredding the spleen through a sterile 20 gauge stainless steel grid, and resuspended in L15 medium. A sample was haemolysed in ammonium chloride solution (Appendix 2) and viable cells counted by trypan blue exclusion. A total of 10^8 cells were taken, resuspended in 5 ml of fresh L15 medium and maintained at 37°C to await fusion.

b Preparation of thymocytes

A thymus was removed aseptically from a 4 week old Balb/c mouse and the thymocytes were released from the thymic capsule by shredding it through a stainless steel grid. They were resuspended in L15 medium and counted, and 10^8 thymocytes were resuspended in 20 ml L15 hypoxanthine and thymidine (HT) medium (Appendix 2) containing 20% foetal calf serum (FCS).

c Culture of NSO myeloma cells

Cells of the non-secretor mouse myeloma line NSO obtained from Dr. C. Milstein, (Clark et. al., 1981) were grown as a spinner culture in Dulbecco's

modified Eagles medium (DMEM), 10% FCS and antibiotics (Appendix 2), with an atmosphere of 5% carbon dioxide in air at 37°C. Three days before fusion the concentration of the cells was adjusted to 10^5 cells/ml, and maintained at this density by adding more medium daily to ensure that the cells were in the exponential growth phase when harvested for culture. The cells were stained with trypan blue (0.1% w/v) to determine cell viability and10^7 cells were harvested; they were not used for fusion unless a viability of greater than 95% was obtained.

d Fusion protocol

The suspensions of NSO myeloma cells (10^7) and spleen cells (10^8) were mixed and pelleted by centrifugation at 300 x g for 5 minutes. The medium was decanted and the residual liquid was aspirated carefully with a Pasteur pipette. The pellet was loosened by tapping the tube, and transferred to a 37°C waterbath in a laminar flow hood. A volume of 0.8 ml of a solution of sterile polyethylene glycol 1500 (PEG) (Sigma) in L15 (50% v/v) (Appendix 2) was gradually mixed with the myeloma and spleen cells over 1 minute with stirring, using a glass 1ml pipette. The mixture was stirred for a further 1 minute and then diluted with a total of 10 ml of serum-free L15 medium over 5 minutes. The first 2 ml were added with stirring over 2 minutes, the next 2 ml over 1 minute, and the remaining 6 ml at a rate of 3 ml/minute. The cell suspension was then pelleted and resuspended in the 20 ml of L15 medium containing 10^8 thymocytes, HT and 20% FCS, prepared as described above.

e Plating of fusion products

Plastic 24-well tissue culture plates (Falcon) were coated with 100 µl/ well of 0.2% w/v agar in sterile distilled water and allowed to set overnight. A

soft agar solution in DMEM was prepared (Appendix 2) and kept at 52°C. To 9 ml of the agar in DMEM, 16.6 ml of L15 HT medium at was added to give a final solution of 0.75% (w/v) agar in culture medium at approximately 41°C. Eighteen ml of the cell suspension of fusion products was mixed with 9 ml of the 0.75%(w/v) agar to give a final agar concentration of 0.25% (w/v). This suspension was plated out at 0.5 ml per well into two 24 well plates pre-coated with agar. The agar was allowed to gel in a laminar flow hood at room temperature, following which 1.5 ml of 5% carbon dioxide buffered HAT (hypoxanthine, aminopterin and thymidine) DMEM medium (Appendix 2) was added above the agar in each well to give a final aminopterin concentration of 0.25 μg/ml. The plates were incubated at 37°C with 5% carbon dioxide in air and 100% humidity. Cultures were left undisturbed for about 2 weeks, when macroscopic clones of hybrids began to appear in the wells. The remaining 2 ml of fusion products were seeded into four control wells in medium without agar to check for cell viability. These wells were also fed with DMEM containing HAT.

iv Identification and selection of reactive clones

About 0.5 ml of supernatant was aspirated from each well containing macroscopic clones of hybrids. The presence of reactive antibodies was detected by means of ELISA using CME coated plates and also plates containing colorectal cell line Colo 205. Individual clones from ELISA-positive wells were picked with Pasteur pipettes and transferred to separate wells of 24 well plates containing 2 ml of DMEM HAT liquid medium and thymocytes at concentration of 10^6/ml. After about 7 days when the wells contained approximately 10^6 cells, the supernatants were again tested for the production of reactive antibodies using ELISA with the CME coated plates so that the individual antibody secreting clones could be

identified. The positive clones were then tested for colorectal cancer reactivity by means of immunocytochemistry.

a Immunocytochemistry

The indirect immunoperoxidase method was used for screening ELISA-positive antibodies. Frozen sections were cut at 8 μ and fixed in acetone for 15 to 20 minutes and washed in TBS. The sections were then incubated with 100 μl of monoclonal antibody as undiluted tissue culture supernatant for 1 hour in a moist chamber at room temperature. The sections were washed with TBS to remove unbound antibodies and 100 μl of the second antibody, a rabbit anti-mouse immunoglobulin conjugated to peroxidase (Dako) (1:100 dilution in TBS) was added and incubated for 30 minutes. The sections were washed with TBS and the reaction product was developed using 1:2000 dilution of 3'3' diaminobenzidine tetrahydrochloride (Sigma) in TBS for 10 minutes, following which the sections were washed in TBS and counterstained with Mayer's haemalum, differentiated in saturated lithium carbonate, and rinsed again in water. The slides were dehydrated in alcohol, cleared in xylene and mounted in D.P.X (Raymond Lamb). A positive reaction was indicated by a brown colour.

For indirect immunoperoxidase staining of paraffin sections used in the characterization of the antibodies, the same procedure was used, except that 5 μ paraffin embedded sections were dewaxed in xylene and rehydrated through a graded alcohol series. Endogenous peroxidase activity was inhibited with 0.5% (v/v) hydrogen peroxide in methanol for 10 minutes, followed by washing with distilled water. After washing with TBS (pH 7.6), the first antibody was added and the procedure completed as above.

A positive and a negative control was employed for all the

immunocytochemical reactions. The positive control was used to prove that the reagents used in a particular staining study were performing normally. It consisted of a colorectal cancer tissue section known to be reactive with a monoclonal antibody, and a positive staining reaction was always expected from the positive control section, unless there was a deterioration in the immunocytochemical reagents. The negative control consisted of a section to which only the buffer solution had been added to prove that a positive result was not due to a non-specific reaction between the second antibody and the tissue section. The interpretation of results was made with the assistance of a histopathologist.

The staining reaction of the monoclonal antibodies with normal tissues and carcinomas showed a heterogenous pattern, so that the staining might be positive on only a few up to a large proportion of cells in a section. The staining intensity of the antibodies with the sections was also variable ranging from a weak to a strong reaction. Any attempt at quantitive grading of the staining results is difficult as no standardised system has been developed. In this study, the staining reaction was classified as positive, weak and negative, based primary on overall intensity of reaction.

Positive hybrids from this screen were grown up for recloning and freezing.

b Cloning and freezing of hybrids

Positive hybrids were recloned by serial dilution in 96-well plates at a concentration of 5 cells/well in 200 µl of liquid DMEM-HAT medium with thymocytes (10^6/ml). Wells were examined microscopically and only those containing single clones were retested for the production of reactive antibodies using CME-ELISA. Positive clones were transferred to individual wells of a

24-well plate, grown on for a further 7 days, again tested for reactive antibodies using ELISA, and recloned a second time by serial dilution. At this stage the reactive clones were frozen in L15 medium plus 10% FCS and 10% (v/v) DMSO (Sigma) at 4 x 10^6 cells/ ml and stored in liquid nitrogen.

3.2.4 Characterization of monoclonal antibodies

i Determination of immunoglobulin class

The class and subclass of the monoclonal antibodies selected from the fusions were determined by double diffusion in agar (1% w/v) (Ouchterlony 1962), using a rosette template. Supernatants from monoclonal cultures in which cells had grown to saturation density were concentrated using a Minicon A25 filter. A volume of 10 µl of concentrated supernatant was added to the central well of the rosettes. Undiluted class and subclass-specific antisera (10 µl) to mouse immunoglobulin (Serotec) were added to the surrounding wells, and incubated for 48 hours at room temperature in a moist chamber. The gels were removed from the plates, washed overnight in pH7.5 PBS, dried onto gel-bond film (Miles Lab.), and stained for 30 minutes with 0.2% (w/v) Coomassie blue in 40% methanol /10% glacial acetic acid/50% distilled water. They were then destained in 90% methanol / 2% acetic acid / 8% distilled water. The reaction between the monoclonal antibody and the specific anti-immunoglobulin antiserum formed a precipitating band between the two wells.

ii Immunocytochemistry of tissue distribution

Reactive antibodies were further tested with frozen sections of normal human tissues of colon, lung, liver, spleen, kidney, oesophagus, stomach, breast,

adrenal, thyroid, ovary, testes and epididymis. The antibodies were also tested for their reactivity with a panel of formal-saline fixed and paraffin-embedded normal colon (7), colorectal cancer (16), and polyps (15).

iii Flow cytometry

To evaluate if the observed immunocytochemical staining reaction between antibodies and colorectal sections was a geninue membrane reactivity, flow cytometry was used to test the monoclonal antibody with four live colorectal cancer cells (LoVo, Colo 205, Colo 320, SW 480 - obtained from the American Tissue Culture Collection, Rockville, MD.), a breast cancer cell MCF7 (Soule et al., 1973), a fibroblast line WI 38 (from M O'Hare, Institute of Cancer Research, London) and blood from group A and O subjects. One ml of cell-free hybridoma supernatant was added to samples of 2.5 x 10^6 freshly trypsinised or freshly prepared blood cells and incubated at 4°C for 40 minutes with constant gentle rocking. The cells were washed three times with cold L15 containing 10% FCS and goat anti-mouse Ig conjugated to fluorescein (GAM/Ig/FITC) (Nordic) was then added to each sample (0.5 ml of 1: 20 dilution) and incubated for 40 minutes at 4°C. The first antibody was omitted from control samples for each cell line. After staining with the fluorescein conjugate, the cells were washed three times in L15 and finally resuspended in 0.5ml of L15 and kept on ice until analysed by flow cytometry using an Ortho 50 H cytometer.

3.3 RESULTS

3.3.1 CME protein content

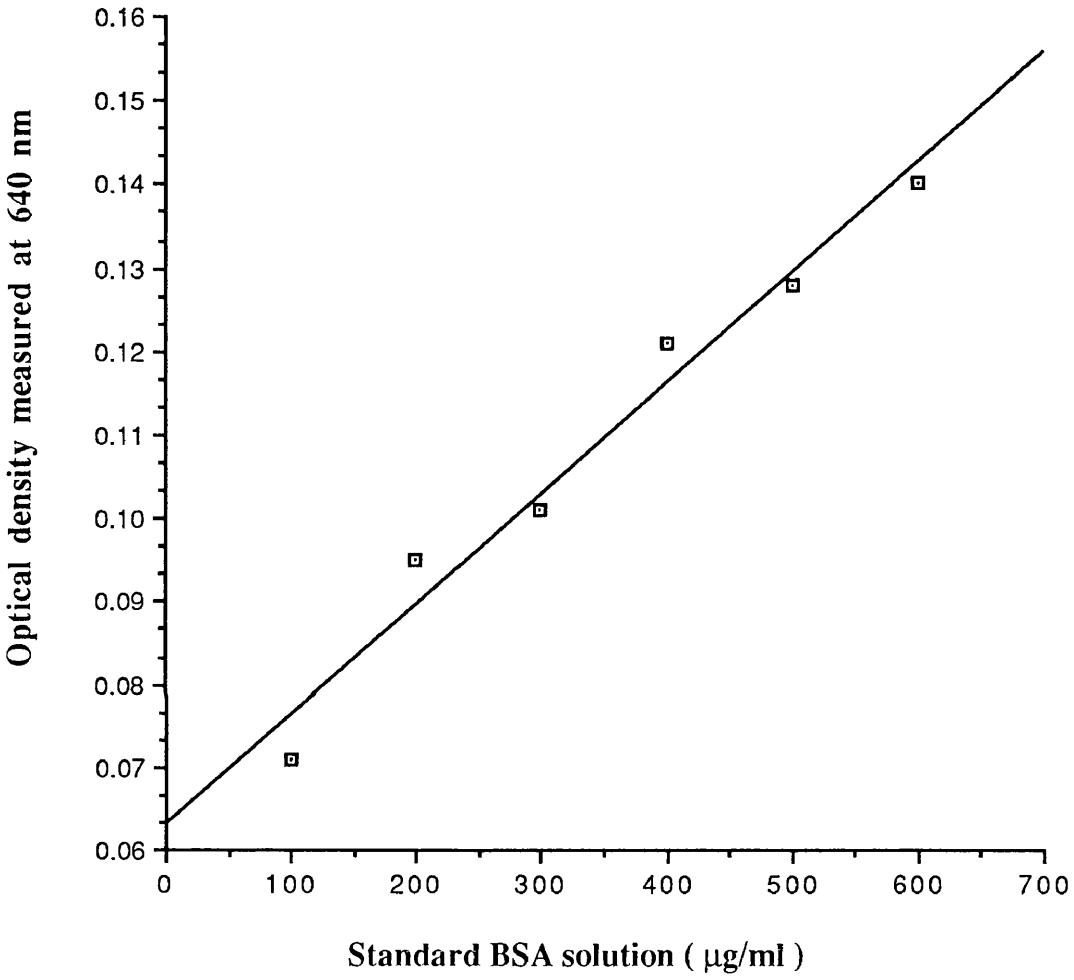
The optical density (measured at 640 nm) of the CME at concentrations of 100%, 50% and 25% were 0.145, 0.116 and 0.056 respectively. This corresponded to a protein content of 700 μg per mg (or an aliquot of 200 μl of CME) as estimated from the standard curve (Figure 4) generated by the bovine serum albumin. As 200 μl of CME were used to immunise four mice, the dose of CME injected per mouse was 175 μg.

3.3.2 Antibody screening assays

A positive reaction between Colo 205 and the monoclonal antibodies 48-1 and 3-48-2 was detected by both radioimmunoassay and ELISA and these antibodies were therefore subsequently used as controls for the cell line screening assay. None of the antibodies reacted with the SW480 cell line in either assay. As the sensitivity of these two assays was similar, the ELISA was selected for subsequent antibody screening to avoid the radiation hazards of radioimmunoassay.

When the CME-coated plates were tested with the antibodies by means of ELISA, 48-1 gave a positive result and this antibody was used as a positive control for subsequent CME-coated ELISA.

Figure 4 Standard curve for CME protein estimation



3.3.3 Fusions and cloning

An antibody response to the CME immunogen was not obtained from the immunised mice before 4 weeks. This immune response was confirmed on ELISA and immunoperoxidase staining of colorectal cancer sections using serum from the mouse before the spleen was removed for fusion.

A total of 10 successful fusions was performed (Table 3). The initial screening with ELISA on the crude membrane extract (CME) showed that 191 wells (40%) were positive out of 480 wells plated in the twenty 24-well plates. The first few fusions were also screened in ELISA using the Colo 205 cell line. This did not, however, identify more reactive antibodies than were detected by the CME plates. The CME-ELISA assay was, therefore, used to screen all later fusions.

Eight hundred and ninety three clones were picked from the positive wells, each of which contained several clones. Further ELISA screening of these 893 clones when they became established showed that 245 (27%) were positive. Supernatants from these 245 ELISA positive clones were tested on frozen sections of two individual cancers. Only 67 clones (27%) produced antibodies also reactive with tissues on the frozen sections. This represented 7.5% of the original 893 clones. However, only 22 out of these 67 hybridomas secreted antibodies which did not also react with tissue stroma. In summary, from a total of 893 clones generated from the 10 fusions, 22 hybridoma clones (2.5% of the total) produced antibodies which reacted predominantly with colorectal tissues.

Seventeen of the 22 initial reactive hybridomas were lost on cloning, due to contamination, loss of antibody secretion, or a failure to re-establish hybridoma cultures from thawed cells. Five hybridomas were successfully cloned twice to ensure monoclonality and stability for antibody secretion. The antibodies

were designated UC-ICR abbreviating for University College and the Institute of Cancer Research. The first number signified the original well from which the hybridoma was picked and the next number indicated the clone from within that well. Three of these cell lines were from fusion number eight, they were designated UC-ICR 12.1, 20.3 and 40.1 and represented clones picked from separate wells and thus were independently generated hybrids. Two hybridoma cell lines were obtained from fusion number 10 designated UC-ICR 41.2 and 41.6; these were clones picked from the same well.

Following cloning to the monoclonal stage, the reaction of these 5 antibodies with frozen sections of colorectal cancer was checked to confirm that the hybridomas still secreted the antibodies. A positive staining was obtained from all 5 monoclonals.

3.3.4 Characterisation of monoclonal antibodies

i Ig class and subclass determination

The Ig class and subclass of the five colorectal cancer-reactive monoclonals was determined by Ouchterlony (1962) double agar diffusion. All antibodies were of the IgG subclasses except UC-ICR 20.3 which was an IgM. The results are shown in Table 4.

ii Reaction with colorectal cancer

These five antibodies all reacted with formal-saline fixed paraffin-embedded sections, although UC-ICR 40.1 had a better reactivity with frozen sections. The reaction of these antibodies with a larger number of colorectal

cancer sections, polyps and normal colon was determined using fixed material and the results are summarized in Table 5.

A panel of 16 colorectal cancers with varying degrees of differentiation was used. UC-ICR 12.1 was positive with 9 of these tumours (56%). The staining reaction was strong in all these sections, and staining occurred mainly on the luminal cell surface. With the exception of one section which showed staining of 20% of the cells, all other sections showed positive staining with 50 to 100% of the cells. Three sections showed staining with polymorphs and blood vessels in areas of high background staining activity, but on the same section in another areas there was no reaction between 12.1 and these structures.

UC-ICR 20.3 reacted with 12 of the sections (75%). The staining was luminal in pattern. The antibody reacted with only a few cells in three tumours, but with between 50% to 80% of the cells in the other 9 tumours tested. Exclusion of the former would give the positive staining frequency of 56%. Although the initial staining with frozen sections showed that there was no reaction with the blood vessels, UC-ICR 20.3 was found to react with blood vessels and sometimes with red blood cells in 6 of the paraffin sections.

UC-ICR 40.1 was positive in 3 out of 9 tumours (33%). This antibody reacted with approximately 20% of the cells in all the tumours tested. The staining reaction occurred mainly in the cytoplasm. However, the staining reaction of 40.1 with frozen sections of colorectal cancer was much stronger than the paraffin-embedded tissues.

UC-ICR 41.2 reacted positively with 15 cancers (94%). The staining reaction was on the tumour cell surface and intense (Figures 5). There was staining of the cytoplasm in normal colonic cells in areas corresponding to the mucin vacuoles. The antibody reacted with between 20% and 90% of the tumour

cells. There was no reaction with the connective tissues or blood cells.

UC-ICR 41.6 also reacted with 15 (94%) of the sections. The pattern of reactivity with the tumours (Figure 6) was similar to that of 41.2. These two antibodies had the best reactivity with colorectal cancer.

iii Reaction with colonic polyps

The reaction of these antibodies was tested with 15 polyps which consisted of tubular and tubulo-villous adenomas (Table 5). UC-ICR 12.1 reacted with 13 polyps (87%). The staining was mainly on the cell surface and involving the apical sections of the tumour rather than the lower part of the crypts where in general the staining was negative or minimal and weak. Staining of some polymorphs was noted in one section. There was no reaction with other cells of the connective tissue.

UC-ICR 20.3 reacted with 4 (14%) of the polyps and only with a few percents of the cells in these sections. There was staining with the blood vessels and red blood cells in all the sections.

UC-ICR 40.1 was not tested for reactivity with polyps as it only reacted with 20% of the paraffin-embedded colorectal cancer sections.

UC-ICR 41.2 reacted with 13 (87%) of the polyps. However, in most of the sections the antibody reacted with only a few percent of the cells. In only one section was staining observed in over 90% of the cells (Figure 7). The pattern of staining was on the luminal surface as well as in the cytoplasm. There was little staining of the cells in the crypts and the reaction occurred mainly with cells in the upper parts of the polyps. The reaction with UC-ICR 41.6 was similar to that of 41.2, reacting with 14 (93%) of the polyps. There was no reaction with the connective tissue or blood cells.

iv Reaction with normal colon

A total of 7 sections from different patients was used (Table 5). The sections were obtained from the 'normal' colon of resected tumours or from diverticular disease specimens. UC-ICR 12.1 reacted weakly with three sections and strongly with one i.e. with 57% of the normal colon samples. UC-ICR 20.3 reacted weakly with one and strongly with another normal colon (29%). UC-ICR 41.2 reacted with 6 sections (86%) and UC-ICR 41.6 with similar number. The reaction between the antibodies and the normal colon occurred on the more superficial part of the mucosa. The reaction between UC-ICR 41.2 and normal colon is shown in Figure 8. UC-ICR 40.1 reacted with all seven sections (100%), although the staining reaction was weak in three sections. In general the staining pattern of these antibodies with the normal colon was similar. The staining was on the cell surface and cytoplasm. It was heterogenous and occurred mainly on the apical sections of the colonic mucosa. The basal cells were rarely stained and little reaction was observed in the bottom of the crypts.

v Normal tissue distribution

There was no reaction between the parenchyma cells of the liver or bile ducts and UC-ICR 41.2 (Figure 9), 41.6 and 20.3. UC-ICR 40.1 reacted with bile canaliculi while UC-ICR 12.1 reacted with the liver parenchyma. There was no reaction between any of these antibodies and the spleen. The staining reaction between UC-ICR 41.2 and the spleen is shown in Figure 10, the dark staining areas showed endogenous peroxidase in white blood cells. There was no reaction between UC-ICR 41.2 (Figure 11) and 41.6 and the tubules or glomeruli of the kidney. The other three reacted with distal tubules of the kidney but not the glomeruli, and 12.1 and 20.3 only reacted with a few cells of the tubules. The

monoclonal 40.1 reacted with the alveoli of the lung, while UC-ICR 12.1, 20.3, 41.2 and 41.6 did not. None of these antibodies reacted with the thyroid or the adrenal glands. All antibodies stained the luminal content of a large breast duct but not many other small ducts in one frozen section; the stained duct was at the edge of the section, indicating that the reaction might be due to edge effect in which a non-specific staining reaction may occur on structure near the edge of a section.

In the gastrointestinal tract all the antibodies except UC-ICR 12.1 reacted with the mucosal cells of the stomach. UC-ICR 20.3 stained the membrane of the cells in the base of the crypts (Figure 12) ; in contrast 41.2 (Figure 13) and 41.6 stained the cells in the more superficial parts of the mucosa with no staining of basal cells; 40.1 stained the cytoplasm of all the epithelial cells of the gastric mucosa. The monoclonal UC-ICR 40.1 did not react with the oesophagus, but all the other antibodies reacted with the more superficial but not the most basal layers of the squamous epithelium of the oesophagus. The reaction between the oesophagus and UC-ICR 41.2 is shown in Figure 14. All antibodies except 20.3 reacted with the surface of epithelial cells of the gall bladder but 40.1 showed cytoplasmic staining. The reaction between UC-ICR 41.2 and the gall bladder is shown in Figure 15. Two of the antibodies (UC-ICR 12.1 and 40.1) reacted with the tubules of the epididymis. There was no reaction between any of the antibodies and the testes or the ovary. There was no reaction between the skin with any of the antibodies. Figure 16 showed the lack of reaction between UC-ICR 41.2 and skin.

vi Flow cytometer analysis

To correlate the observed membrane reactivity of these monoclonal antibodies on immunocytochemistry, a flow cytometer analysis was performed on one of the antibodies (UC-ICR 20.3). This antibody was selected because at the

initial screening when a smaller number of colorectal cancers were used it gave the best reactivity with colorectal cancer and no reaction was observed with blood cells, although later it was found to react with blood cells in some tissue sections.

Flow cytometry showed that it reacted with three out of four colorectal cancer cell lines tested (LoVo, Colo 205 and SW 480 but not SW320). It also reacted with a breast cancer cell line MCF 7, confirming that membrane reactivity observed on immunocytochemistry reflected true cell surface reaction. However, it did not react with human blood cells (groups A and 0) or with a fibroblast cell strain WI 38. Figure 32a shows the reaction between UC-ICR 20.3 with MCF7 cells and Figure 32b shows the reaction between Colo 205 but not with Colo 320.

Table 3 Summary of fusion results

Fusion No	Wells +ve on ELISA	Clones picked from wells	Clones +ve on ELISA	Clones+ve on staining
1	14/48	212	12/212 (5.7%)	0/12 (0%)
2	15/48	61	3/61 (4.9%)	0/3 (0%)
3	28/48	127	52/127 (40.9%)	8/52 (15.4%)
4	29/48	113	36/113 (31.9%)	1/36 (2.8%)
5	6/48	6	2/6 (33.3%)	0/2 (0%)
6	31/48	102	47/102 (46.1%)	2/47 (4.3%)
7	15/48	56	23/56 (41.1%)	17/47 (36.2%)
8	12/48	55	16/55 (29.1%)	12/55 (21.8%)
9	13/48	52	16/52 (30.8%)	12/16 (75%)
10	28/48	109	38/109 (34.9%)	14/38 (36.8%)
Total	191/480	893	245/893(27%)	67/245 (27%)

Table 4 **Characterisation of colon-reactive monoclonal antibodies**

Monoclonals	Fusion number	Well number	Ig class
UC-ICR 12.1	8	12	IgG1
UC-ICR 20.3	8	20	IgM
UC-ICR 40.1	8	40	IgG3
UC-ICR 41.2	10	41	IgG2b
UC-ICR 41.6	10	41	IgG2b

Table 5 Reactions of monoclonals with colorectal cancer, polyps and normal colon

Monoclonals	CRC (16)	Polyps (15)	Colon (7)
UC-ICR 12.1	9 (56%)	13 (87%)	4 (57%)
UC-ICR 20.3	12 (75%)	4 (27%)	2 (29%)
UC-ICR 40.1	33%	-	7 (100%)
UC-ICR 41.2	15 (94%)	13 (87%)	6 (86%)
UC-ICR 41.6	15 (94%)	14 (93%)	6 (86%)

Table 6 Tissue distribution of monoclonal antibodies

Tissue	Antibody designation				
	12.1	20.3	40.1	41.2	41.6
Lung					
(alveoli)	-	-	+	-	-
Liver (cells)	+	-	-	-	-
(b. canaliculi)	-	-	+	-	-
spleen	-	-	-	-	-
oesophagus	+	+	-	+	+
stomach	-	+	+	+	+
gall bladder	+	-	+	+	+
kidney					
(glomerulus)	-	-	-	-	-
(tubules)	+	+	+	-	-
adrenal	-	-	-	-	-
thyroid	-	-	-	-	-
breast	+	+	+	+	+
ovary	-	-	-	-	-
testes	-	-	-	-	-
epididymis	+	-	+	-	-
skin	-	-	-	-	-

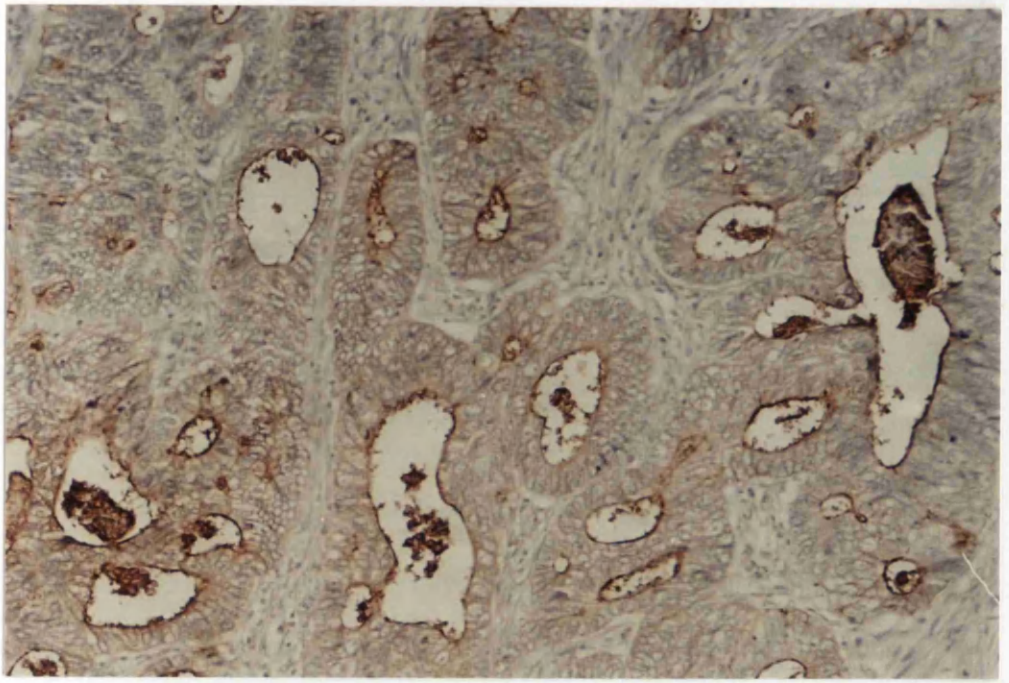


Figure 5 Immunoperoxidase staining of colorectal cancer with UC-ICR 41.2 showing the reaction on the luminal surface of the tumour x 150

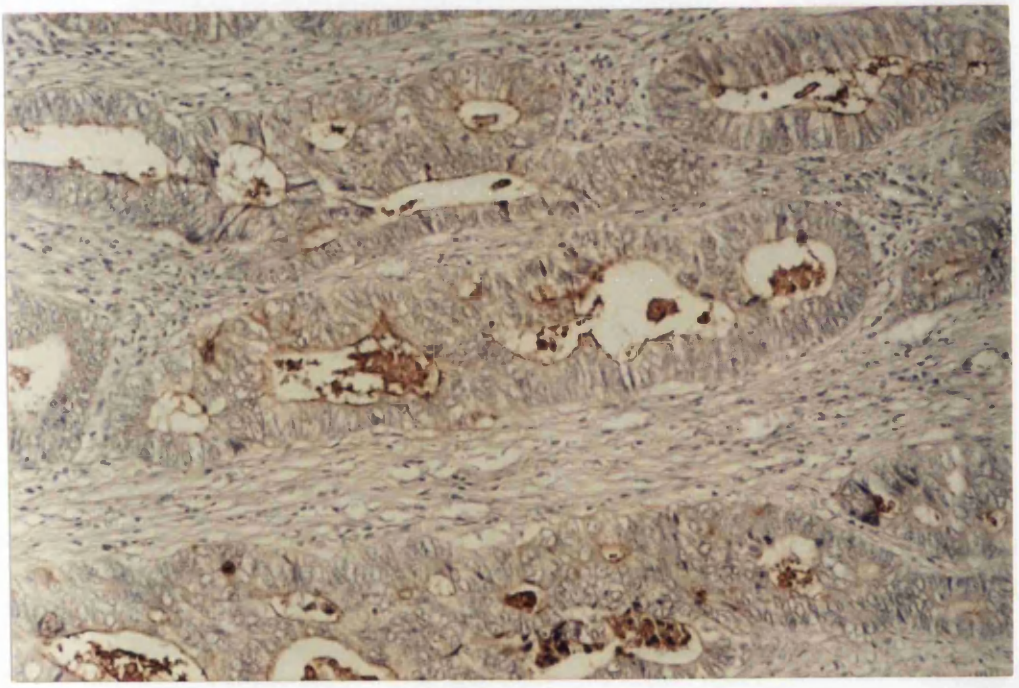


Figure 6 Immunoperoxidase staining of colorectal cancer with UC-ICR 41.6 showing the reaction on the luminal surface of the tumour x 150

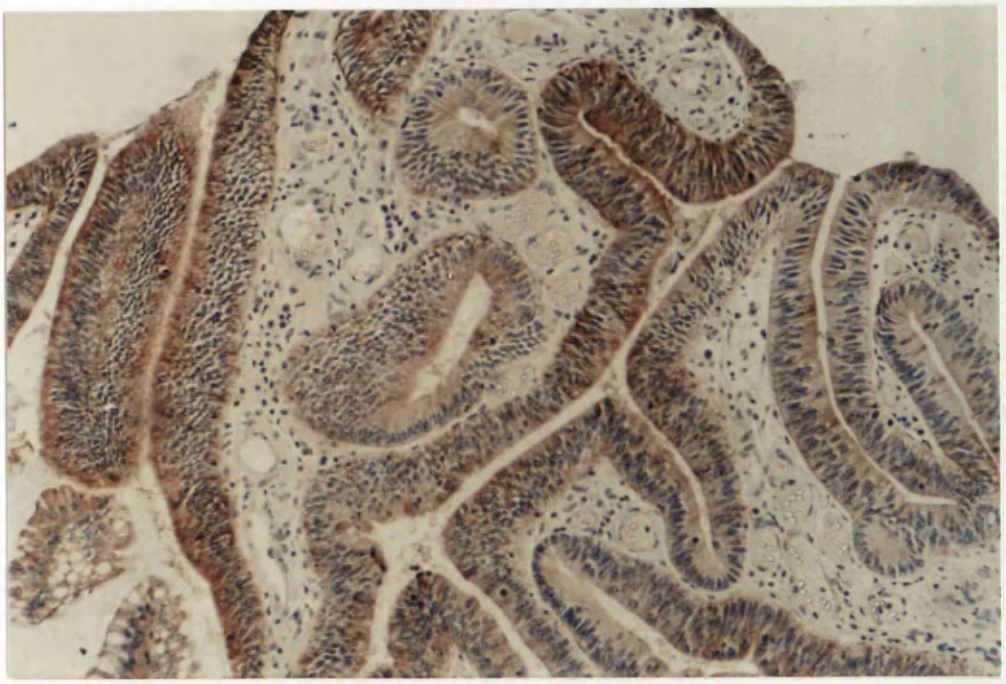


Figure 7 Immunoperoxidase staining of colonic polyp with UC-ICR 41.2 showing surface as well as cytoplasmic reaction x 150

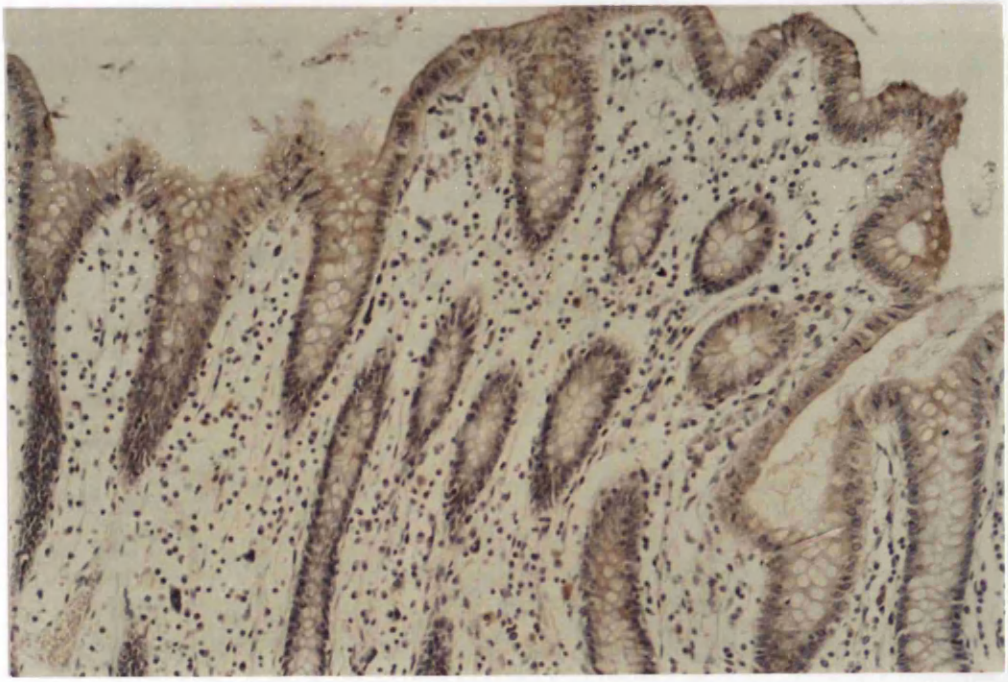


Figure 8 Immunoperoxidase staining of normal colon with UC-ICR 41.2 showing reaction with cells on the more superficial part of the mucosa x 150

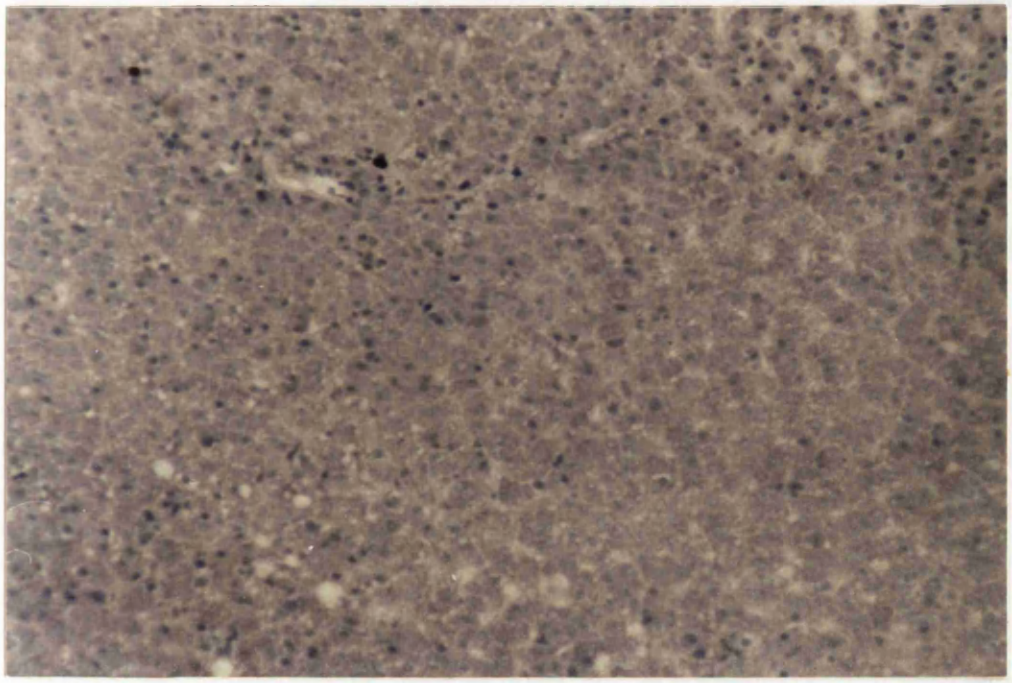


Figure 9 Immunoperoxidase staining to show that UC-ICR 41.2 does not react with either the cells of the liver parenchyma or bile ducts x 150

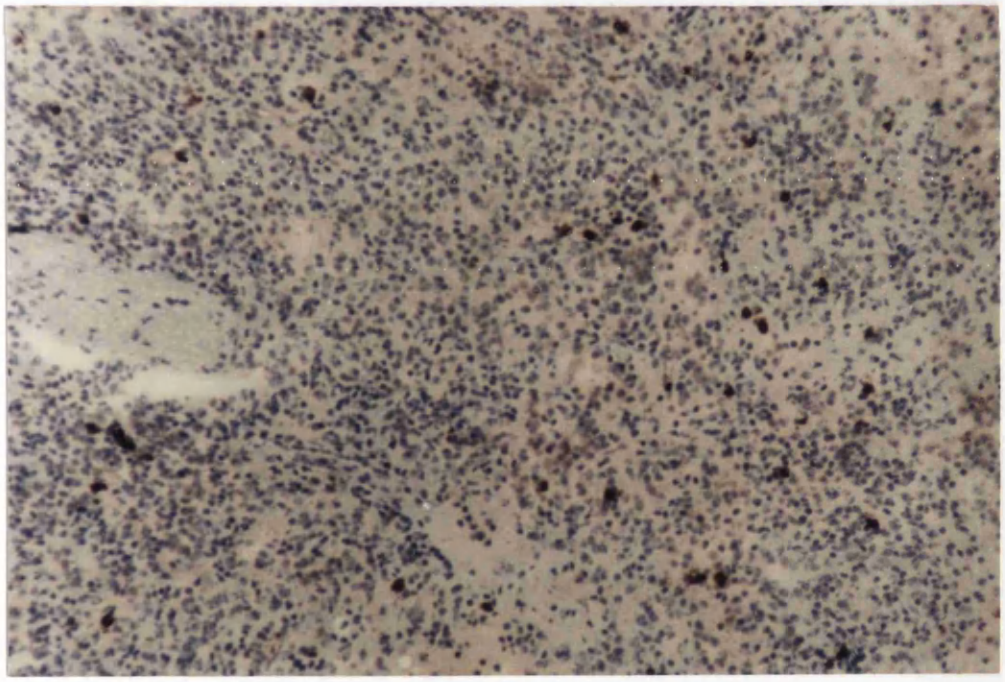


Figure 10 Immunoperoxidase staining to show that UC-ICR 41.2 does not react with spleen cells. Note endogenous peroxidase staining in white blood cells x 150

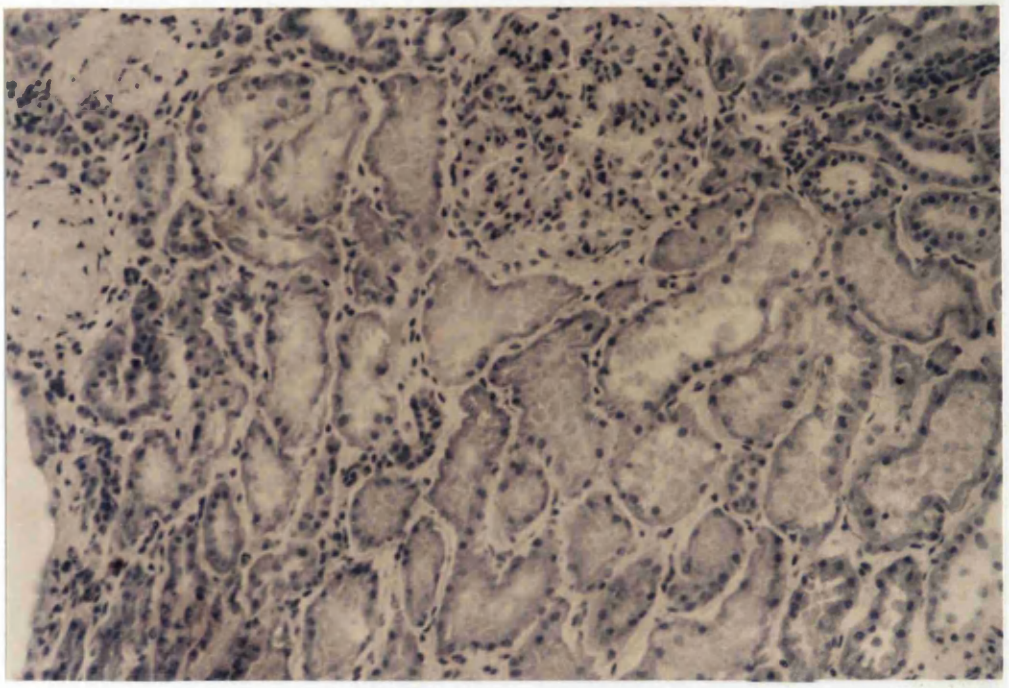


Figure 11 Immunoperoxidase staining to show that UC-ICR 41.2 does not react with either the tubules or the glomerulus of the kidney x 150

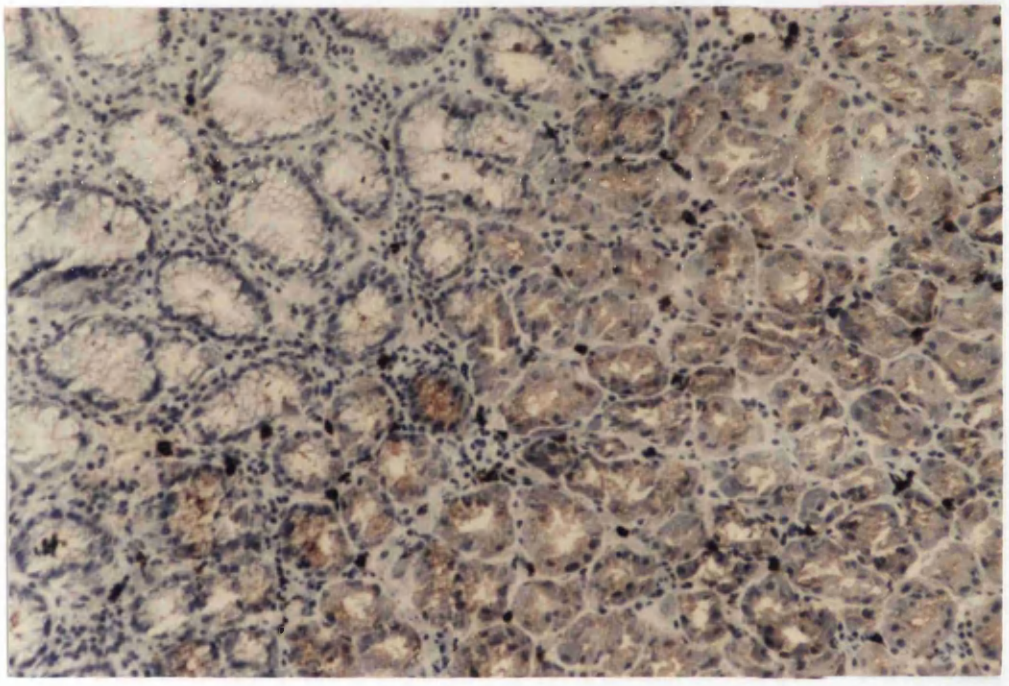


Figure 12 Immunoperoxidase staining of stomach with UC-ICR 20.3 showing reaction with cells on the basal part of the mucosa x 150

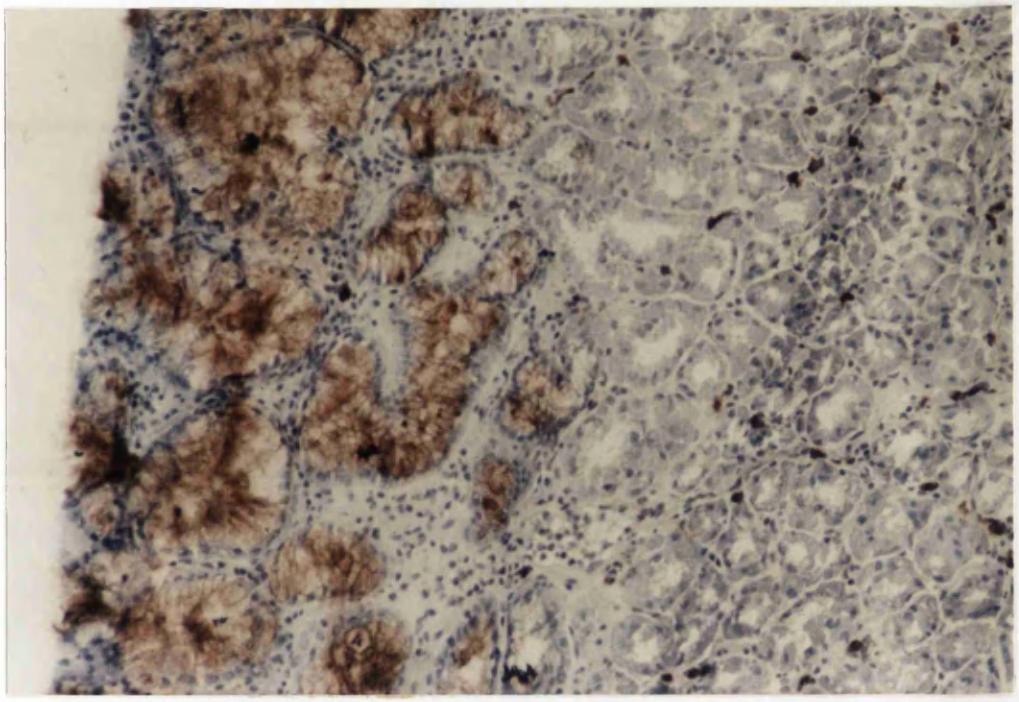


Figure 13 Immunoperoxidase staining of stomach with UC-ICR 41.2 showing reaction with cells on the upper part of the mucosa x 150

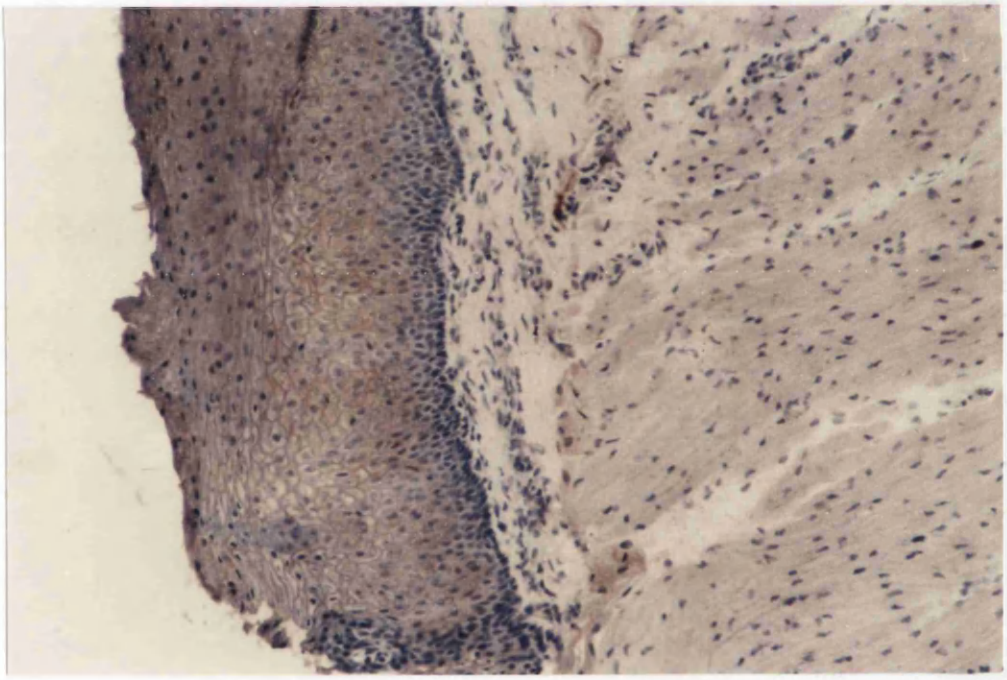


Figure 14 Immunoperoxidase staining of oesophagus with UC-ICR 41.2 showing reaction with cells in the more superficial but not the most basal layer of the mucosa x 150

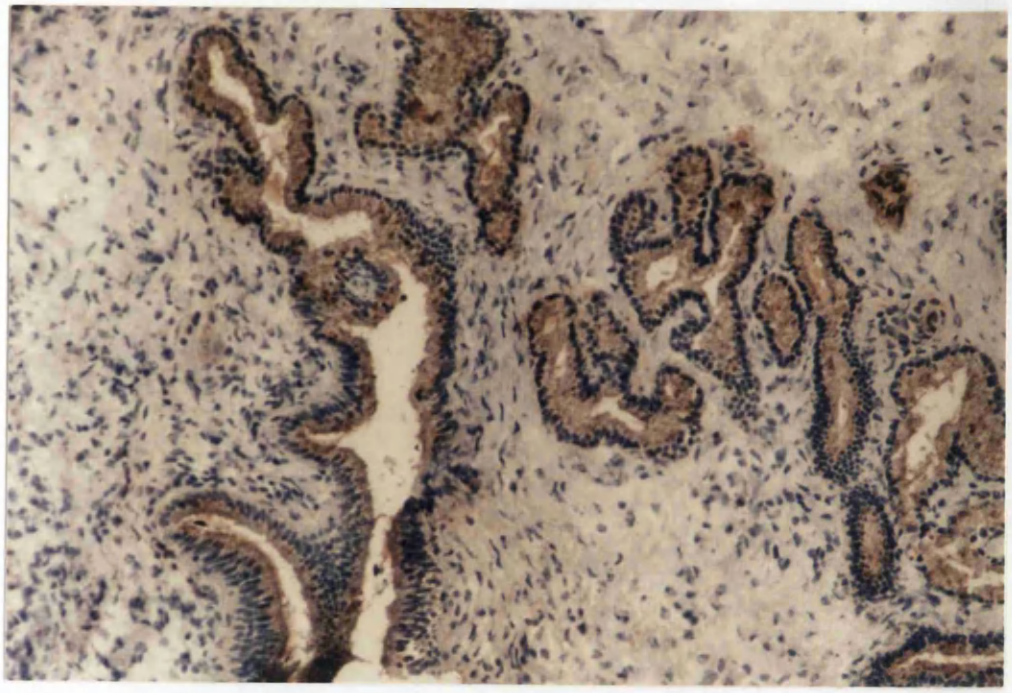


Figure 15 Immunoperoxidase staining of gall bladder mucosa with UC-ICR 41.2 showing reaction on the apical part of the cells x 150

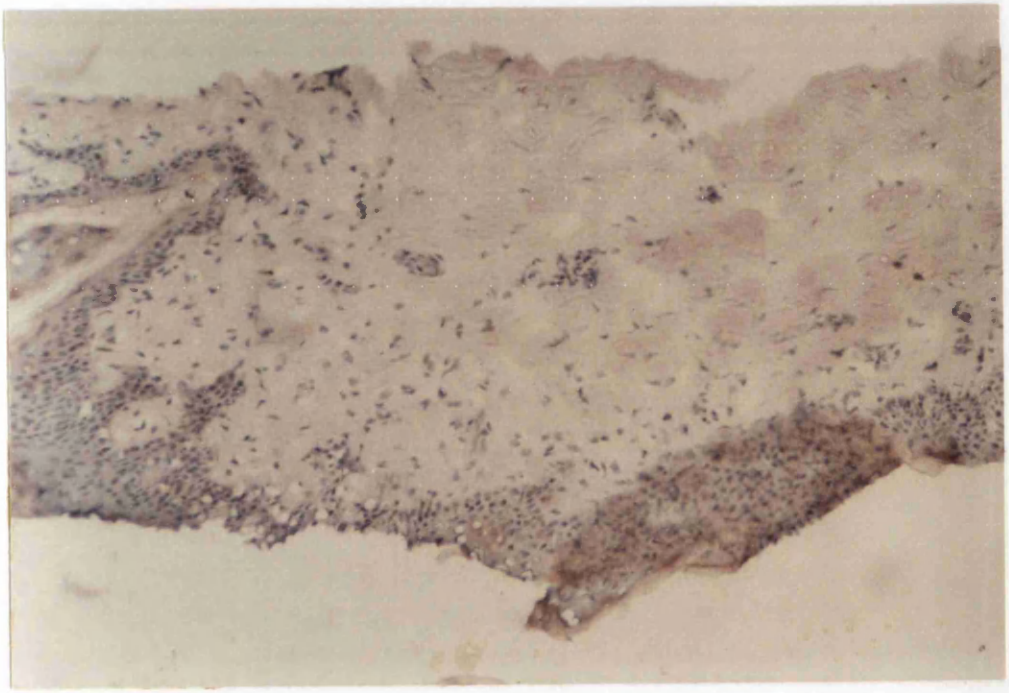


Figure 16 Immunoperoxidase staining of skin with UC-ICR 41.2 showing no reaction with the cells x 150

3.4 DISCUSSION

A crude membrane extract of colorectal cancer from several patients was used as an immunogen in this study because it was hoped that there would be a greater chance of obtaining tumour-associated antigens from this preparation. This type of immunogen has not been used before for raising monoclonal antibodies against colorectal tumour. It yielded a total of 5 reactive antibodies which survived cloning and two of these (UC-ICR 41.2 and 41.6) seemed potentially suitable for imaging purposes. The screening strategy was aimed at identifying membrane reactive antibodies with minimal delay. Thus, the crude membrane extract was used as the target antigen for the initial ELISA screening. In the first four fusions, screening was also performed with Colo 205 cells to determine if this would identify colorectal cancer reactive antibodies missed by the CME assay. However, no additional reactive antibodies were selected by the cell line assay. Immunocytochemistry was used as the next screen to examine the reaction of the antibodies with frozen sections of colorectal cancer, while the hybridomas were still maintained in culture. ELISA positive but colorectal cancer frozen section negative hybridomas were discarded. This avoided the culture and freezing of unwanted cells.

The ELISA was chosen as the routine primary screen because it was simple to perform and gave an equal sensitivity as radioimmunoassay, and avoided the hazards of radiation. This initial screening identified antibodies produced in response to the immunising antigens, and yielded a total of 245 clones. The interpretation of ELISA results was guided by the readings obtained with the diluted sera from the immunised mice. The same concentrations of these sera stained colorectal cancer sections with the immunoperoxidase technique. However,

there was no correlation with the intensity of the ELISA readings and the eventual staining reaction on tissue sections, or with colorectal cancer in particular. This was probably because the immunogen contained antigens other than those on the colorectal cancer cell surface, or in which the conformation and antibody accessibility and reactivity had been altered by extraction.

A secondary screening was necessary to detect those antibodies having tumour-membrane reactivity appropriate to imaging i.e. external membrane components. Immunocytochemistry was chosen for this secondary screen to eliminate antibodies reacting with ubiquitous membrane components, covert antigens, or simply "sticky" antibodies, which might have reacted principally with the BSA on the coated plates rather than the CME.

Frozen sections were used to avoid the potential damage to the tumour antigens by a tissue fixation and paraffin embedding technique, which might hinder the antigens recognition by the antibodies. Only two colorectal cancers were used for preparing frozen sections for this screen because of the quantity of material required. There was therefore a possibility that some antibodies, which could have reacted with other colorectal tumours, might have been inadvertently rejected. Nevertheless 67 clones were identified that secreted antibodies which reacted with some component of the frozen sections. However, of these only 22 were found to react with the colorectal cancer and not the stroma, and 5 of these were cloned successfully to the monoclonal stage.

The characterisation of these antibodies was performed mainly with immunocytochemistry as the objective was to identify suitable antibodies for immunoscintigraphy. Paraffin-embedded sections were used in some of these studies as the antigens recognised by the antibodies survived formalin fixation, with the exception of UC-ICR 40.1 which appeared to have a better reaction with

frozen sections. A better assessment of the reaction of 40.1 with colorectal cancer can only be obtained with another study using frozen sections. The results showed that as expected there was no absolute selectivity for cancer, as all these antibodies also reacted with either polyps or normal colon. Of the five monoclonal antibodies produced, UC-ICR 41.2 and 41.6 were probably the most suitable for immunoscintigraphy. It is most likely that these two antibodies were in fact the same as they were picked from the same well originally following fusion. They had very similar pattern of reactivity, and both belonged to the IgG2b immunoglobulin class. They reacted with the cell surface of (16/17) 94% of the colorectal cancers and a similar percentage of polyps. However, the reaction with polyps was often weaker and with a much smaller percentage, usually less than five percents, of the cells in each individual tumour. Reactivity with polyps should not be considered a disadvantage, as polyps are regarded as precursors of colorectal carcinoma, in the adenoma-carcinoma sequence (Morson & Day, 1981), and their earlier detection may be of value as their elimination reduces the incidence of colorectal cancer (Gilbertsen & Nelms, 1978). The reactivity of UC-ICR 41.2 and 41.6 to cancer compared favourably with other monoclonal antibodies raised to colorectal carcinoma. For example, the monoclonal antibody 19-9 reacted with 59% of colorectal cancer (Atkinson et al., 1982), the monoclonal antibody 250-30.6 reacted with 79% (Thompson et al., 1983) and MoAb F4/2E10 reacted with 92%. A monoclonal antibody B72.3 which was raised to breast cancer metastases reacted with 82% of colorectal cancer (Stramignoni et al., 1983). The monoclonal antibody C14/1/46/10 (Brown et al., 1983) reacts with 96% of colorectal cancer and 100% of colorectal adenomas, but it belongs to the IgM class and is therefore not suitable for immunoscintigraphy. The staining of the membrane by UC-ICR 41.2 and 41.6 indicated that the antigens on the tumour

recognised by the antibodies would be accessible to the immunoglobulins in the *in vivo* situation.

UC-ICR 41.2 and 41.6 reacted with up to 86% of normal colon samples stained. However, the number used (seven) was small, and a study with a larger panel of normal colons might be more appropriate to determine the frequency of reactivity between the colon and these antibodies. The staining reaction with colorectal cancers was on the cell membrane, as distinct from normal colon in which there was also cytoplasmic staining. The staining reaction in the cytoplasm probably represented a secretory product of the cells and was most likely to be mucins as many anti-epithelial monoclonal antibodies have recently been shown to react with the same group of molecules (Burchell et al., 1987). The antigen recognised by these antibodies was probably a glycoprotein, as many of the monoclonal antibodies raised to different carcinomas or human milk fat globule membranes have been shown to recognise epitopes present on large molecular weight glycoproteins (Burchell et al., 1987; Edwards, 1985). The reaction of these antibodies with normal colon would not necessarily preclude them from imaging colorectal cancer as an anti-CEA monoclonal antibody C46 detected about 90% of colorectal cancer when the radiolabelled antibody was administered intravenously (Armitage et al., 1986). This antibody reacts with over 90% of normal colon sections (Davidson et al., 1989). Other anti-colorectal cancer monoclonal antibodies are also known to react with normal colon, for example, the monoclonal antibody 19-9 reacts with 60% of normal colon (Arends et al., 1983a), the monoclonal 250-36.6 with 100% (Thompson et al., 1983). Reaction with normal colon has also been observed with the monoclonal antibody CCOL1 (Kaszubowski et al., 1984), the antibodies CAM5.2 and CAM17.1 (Makin, 1986) and MoAb LOVO F4/2E10 (Drewinko et al., 1986), although weakly.

Immunocytochemistry showed that there was no reaction between UC-ICR 41.2 and 41.6 and the connective tissue. This is an essential requirement for imaging. The lack of reaction with lung tissue, the liver, the kidney and spleen (Table 4) was also an advantage. The reaction with the epithelial cells of the oesophagus and stomach would not prevent their application for immunoscintigraphy as a number of antibodies used for this purpose have similar reactivity, for example, M8 (Foster et al., 1982a; Rainsbury, 1984). Cross-reactivity to normal tissues was also observed with other monoclonal antibodies raised to colorectal cancer. For example, the monoclonal antibody 19-1 reacts with normal pancreas, stomach mucosa, salivary gland, gall bladder and endocervical mucosa (Arends et al., 1983a), the antibody 250-30.6 reacts with secretory epithelium of the gastrointestinal tract, respiratory system and the urinary system, as well as the liver. The monoclonal antibody CCOL1 reacts with the bronchial epithelium, the oesophageal mucosa, and the mucosa of the stomach and pancreas (Kaszubowski et al., 1984). The monoclonal antibody MoAb LoVo F4/2E10 reacts with sections of ulcerative colitis and Crohn's disease, but there is no mention of reaction with normal liver, kidney or lung (Drewinko et al., 1986). The monoclonal antibody CAM5.2 reacts with all epithelial cells, whether normal or malignant, as it recognises cytokeratin proteins (Makin, 1986).

The monoclonal antibody UC-ICR 12.1 reacted with 59% of the colorectal cancers, 87% of the polyps and 67% of the normal colon sections. Its reaction with normal colon was weak. However, in consideration for clinical application, a significant concern is the observed reaction of 12.1 with the polymorphs in some histological sections. Two anti-CEA monoclonal antibodies 065 and 326 failed to localise to colorectal cancer in patients due to their cross-reaction with granulocytes and red blood cells (Dillman et al., 1984). This

reaction of 12.1 with polymorphs suggests that the antigen determinant recognised may be an epitope of CEA included in the Class I category by Reynoso et al. (1985).

The monoclonal antibody UC-ICR 20.3 showed a good reactivity with staining of 88% of the colorectal cancers. It reacted with red blood cells and blood vessels in some sections but the reaction with red blood cells was not confirmed on flow cytometer when blood groups A and O were used. There was also no reaction between UC-ICR 20.3 and the fibroblast cell line WI 38 on flow cytometry. Although it is possible that 20.3 reacted with blood group antigen as reported by Voak et al. (1980) when membrane was used as immunogen, the reaction with blood cells may also be due to a non-specific 'stickiness' of IgM class immunoglobulin in an immunocytochemical reaction. The observed membrane reaction between UC-ICR 20.3 and colorectal cancer sections on immunocytochemistry was confirmed by flow cytometry results when live colorectal cell lines were used. Although the UC-ICR 41.2 and 41.6 were not tested for their reaction with live cells by flow cytometry, it can be expected that like UC-ICR 20.3 they too would react with live cells, as they showed membrane staining on immunocytochemistry.

The immunoglobulin subclasses of these 5 monoclonal antibodies were of interest as only one was of the IgM subclass. For immunoscintigraphy with antibody, an IgM would be at a disadvantage as it would not be able to penetrate tissue compartments as readily as the smaller antibodies of other subclasses (Schlom, 1986). The mice used for these fusions (fusion 8 and 10) had been immunised for between 2-3 months with a weekly injection of crude membrane extract. An extended immunisation schedule may encourage the emergence of immunoglobulins of the IgG subclasses, as following antigenic stimulation, B

cells mature into antibody-secreting cells which usually secrete IgM. Days or weeks after the initial response, there is a shift towards secretion of other classes, particularly IgG (Goding, 1983). The use of adjuvant in immunisation may also influence the class of antibody produced. Handman and Remington (1980) show that mice which are chronically infected with Toxoplasma gondii make substantial quantities of IgG2 and IgG3 antibodies but virtually no IgG1. Hybridomas generated from these spleen cells show similar antibody class distribution. Johnson et al. (1981) immunised mice with the same parasite in Freund's adjuvant and obtained a substantial IgG1 response and predominantly IgG1 hybridomas. Freund adjuvant was not used in the immunisation here as the CME was sufficiently immunogenic. There is some correlation between the quantity of immunogen injected and the affinity of monoclonal antibodies produced (Foster, 1982). Although as little as 20 - 30 μg of an immunogen may be sufficient to produce an antibody response, 175 μg of CME were used for immunisation in this study to ensure an adequate immune response.

In the production of monoclonal antibodies for clinical application or in cases when antibodies with the IgG subclasses is desired, an augmented immunisation programme should be considered. However, in a situation where an equivalent IgG monoclonal antibody to one belonging to an IgM subclass is required, the antibody-secreting hybrid can be converted to an IgG secreting one by a process of repeated cloning, to identify rare IgG secreting mutants which are then propagated. This process, also known as isotype switching has been performed successfully by Bosslet et al. (1986) and Spira et al. (1987), and is important in enabling the clinical application of an IgM monoclonal antibody which would otherwise be inappropriate. In the present study, however, the properties of the IgM antibody (20.3) were not of sufficient interest to warrant this

labour-intensive step.

In summary, the antibodies produced in this study indicated that the crude membrane extract of colorectal cancer did not contain tumour associated antigens significantly different to other immunogens used previously. Two monoclonal antibodies, UC-ICR 41.2 and 41.6, produced by this immunogen may, however, have the potential for clinical application by virtue of their lack of reactivity with connective tissue and vital organs such as liver, lung and kidney combined with their reactivity with the great majority of colorectal cancers tested.

CHAPTER 4

SELECTION OF MONOCLONAL ANTIBODIES REACTIVE WITH COLORECTAL CANCER

4.1 INTRODUCTION

The phenomenon of the cross-reactivity with unrelated tumours by monoclonal antibodies originally raised against other tumours (2.2.2) prompted a search for monoclonal antibodies reactive with colorectal cancer from a range of antibodies produced with non-colonic immunogens. Some of these antibodies have not been fully characterised as yet, but all were raised against human cells, tumour membrane, or secreted products of epithelial cells (Table 7). For immunoscintigraphy, antibodies of the IgG classes may be more suitable than IgM (Schlom, 1986). The antibodies tested included different immunoglobulin classes (Table 7). However, as described previously, it is possible to convert an IgM antibody with a strong affinity with colorectal cancer to the more suitable IgG classes by the isotype switch technique (Bosslet et al., 1986; Spira et al., 1987) if required.

Anti-human milk fat globule membrane antibodies

A number of monoclonal antibodies have been raised against human milk fat globule membrane (HMFGM) (Foster et al., 1982 a,b). HMFGM was used as an immunogen to raise monoclonal antibodies reactive with the membranes of breast epithelial cells because membranes are sequestered from the cytoplasmic component and luminal plasma membrane of epithelial cells during the process of milk synthesis and secretion. A total of 19 monoclonal antibodies that reacted with the luminal epithelial cells of the breast were produced using this immunogen. Four monoclonal antibodies (M3, M8, M18 and M24) were identified which were directed against four different determinants (McIlhinney et al., 1985). A normal tissue screen, however, showed that these antibodies were not breast specific but

exhibited a complex and heterogenous distribution of staining throughout other normal epithelial tissues (Foster et al., 1982 a). One of these monoclonal antibodies (M8) binds to a component of human milk fat globule membrane called epithelial membrane antigen (EMA), also known as polymorphic epithelial mucin (PEM) previously identified with the use of polyclonal antisera (Ormerod et al., 1983; McIlhinney et al., 1985). These four antibodies reacted with the surface epithelium of the colon. However, their reaction to colorectal cancer is unknown.

Anti-bladder cancer antibodies

Another series of monoclonals were raised to human bladder carcinoma (Summerhayes et al., 1985). A crude membrane extract prepared from two metastatic tumours was used as the immunogen and three antibody groups were identified from the fusion. Group I (4-72-2) was urothelial specific and stained the basal and intermediate cells in normal urothelium; group II (3-48-2, 48-1, 3-50-3) reacted with intermediate and superficial cells; group III (8-30-3, 77-1, 2-94-2, 3-71-1, 94-3) reacted only with antigens on the luminal membrane of superficial cells (Summerhayes et al., 1985). The group III antibodies showed staining characteristics similar to conventional anti-epithelial membrane antigen (EMA) and monoclonal antibodies 77-1 and 8-30-3 competed with anti-EMA antisera in a competitive well binding assay. Western blot analysis showed that group I antibody did not react with any membrane component, group II antibodies recognised a glycolipid antigen with a molecular weight of 300,000 and group III with that having a molecular weight of 250,000 belonging to the neutral and ganglioside fraction. Other unpublished antibodies were also obtained from the same fusion (2-5-3, 93M2, 3-11-3 and 1-III-89). None of these anti-bladder antibodies have been previously characterised with respect to their reactivity to

colorectal cancer.

Other monoclonal antibodies

Other antibodies screened included those raised to membrane preparations of breast cancer 20.3, 22.2, 29, 36, 39-8-3 (Skilton, personal communication), and to subfractions of HMFGM which include H10A (Earl, 1987), and TW19 (Gore, 1985). Monoclonal antibody AS1748 was raised to colorectal cancer (McKenzie, personal communication).

4.2 SCREENING OF ANTIBODIES FOR USE WITH COLORECTAL CANCER

Several methods are available for identifying colorectal cancer-reactive monoclonals, for example, radioimmunoassays, enzyme linked immunosorbent assay (ELISA) or immunocytochemistry (1.11). The choice of screening method is dictated by the intended utility of the antibodies. For immunoscintigraphy of colorectal cancer, while entirely colorectal cancer specific antibodies are unlikely to be obtained, those with a greater affinity for the tumour than other tissues are essential. A screening technique that provides information about the relative reactivity of a monoclonal antibody to colorectal carcinoma and other tissues is therefore required. Immunocytochemistry fulfils this requirement as the reaction of a monoclonal antibody to these tissue components can be visualised on histological sections. Techniques such as radioimmunoassay (1.12) or ELISA which use tumour cells or their extracts as target antigens would not be as informative as they do not directly reveal the antibody reaction with other tissues.

The reaction of the antibodies listed in Table 7 with colorectal cancer was therefore tested by immunocytochemistry. In the selection of an appropriate staining procedure, the indirect alkaline phosphatase technique was first compared with the indirect immunoperoxidase method to determine whether the endogenous alkaline phosphatase in colonic cells is a source of staining error. As an initial screen sections from three different formalin-fixed, paraffin-embedded colorectal cancers were used. Antibodies with cell surface reaction with any of these tumours were further evaluated by a second detailed screen in which their reaction with a larger number of colorectal cancers and metastases in the liver and lymph nodes, with polyps as well as normal colon were examined.

Antibodies with the best selectivity to colorectal cancer were considered for further examination. Paraffin embedding may damage cellular antigens, and none of these 25 antibodies have been tested with frozen sections. In order to ensure that the selected antibodies did not react with other cells or connective tissues, their reactivity with frozen sections of some organs were examined. Normal lung, kidney, liver and spleen, and bone marrow were used for this purpose.

Table 7 Monoclonal antibodies screened for colorectal cancer reactivity

	Antibody	Immunogen	Class	Reference
1	M3	HMFG	IgM	Foster et al. (1982 a,b)
2	M8	HMFG	IgG1	Foster et al. (1982 a,b)
3	M18	HMFG	IgM	Foster et al. (1982 a,b)
4	M24	HMFG	IgM	Foster et al. (1982 a,b)
5	48-1	bladder cancer	IgG1	Summerhayes et al. (1985)
6	3-48-2	bladder cancer	IgG1	Summerhayes et al. (1985)
7	77-1	bladder cancer	IgG2a	Summerhayes et al. (1985)
8	8-30-3	bladder cancer	IgG2b	Summerhayes et al. (1985)
9	2-94-2	bladder cancer	IgG3	Summerhayes et al. (1985)
10	3-71-1	bladder cancer	IgG3	Summerhayes et al. (1985)
11	94-3	bladder cancer	IgG3	Summerhayes et al. (1985)
12	3-50-3	bladder cancer	IgG1	Summerhayes et al. (1985)
13	4-72-2	bladder cancer	IgG1	Summerhayes et al. (1985)
14	2-5-3	bladder cancer	NK	Summerhayes et al. (1985)
15	93M2	bladder cancer	NK	Summerhayes (unpublished)
16	3-11-3	bladder cancer	NK	Summerhayes (unpublished)
17	1-III-89	bladder cancer	NK	Summerhayes (unpublished)
18	22.2	breast cancer	IgM	Skilton (unpublished)
19	39-8-3	breast cancer	IgM	Skilton (unpublished)
20	20.3	breast cancer	NK	Skilton (unpublished)
21	36	breast cancer	NK	Skilton (unpublished)
22	29	breast cancer	NK	Skilton (unpublished)
23	H10A	HMFG fractions	IgG1	Earl (1988)
24	TW19	HMFG fractions	IgM	Gore (1985)
25	AS1748	colorectal cancer	NK	McKenzie (unpublished)

NK = not known

4.3 MATERIALS AND METHODS

4.3.1 Sources of tissues

Routine formal-saline fixed and paraffin-embedded histological sections of colorectal cancer, polyps, liver and lymph node metastases as well as normal colon were obtained from the Pathology Department, University College Hospital London. Specimens of primary carcinoma and lymph node metastases were obtained from five patients. A comparison of the reactivity between the primary and metastatic tumours with the antibodies was therefore possible. Fresh operative specimens of colorectal cancer, and normal lung, liver, kidney and spleen were collected from the operating theatre of University College Hospital London. They were examined by a pathologist who then provided samples to be snap-frozen in liquid nitrogen and stored for frozen section screening. Normal bone marrow specimens were provided by Dr C Coombes of the Ludwig Institute for Cancer Research (London Branch) and they were obtained from normal donors giving marrow for transplantation.

4.3.2 Sources of monoclonal antibodies

A total of 25 monoclonal antibodies were screened for reactivity to colorectal cancer. Details of these antibodies are described in 4.1.

The four monoclonal antibodies raised against HMFGM (Foster et al., 1982 a,b) were provided by the Ludwig Institute for Cancer Research (London Branch) as were the seven monoclonal antibodies 22.2, 20.3, 39.8.3, 36, and 29 (R. Skilton, personal communication), TW19 (Gore, 1985) and H10A (Earl, 1987). The 13 monoclonal antibodies raised against bladder cancer (Summerhayes et al., 1985) were provided by the Institute of Cancer Research London.

The monoclonal antibody AS1748 was a gift from Dr ICF Mckenzie of the University of Melbourne.

All antibodies were used in the form of tissue culture supernatants with the exception of AS1748, which was obtained as purified ascites using the Protein A method described in 5.2.1.

4.3.3 Screening procedure

All antibodies were applied to paraffin sections of colorectal cancer from three different patients in the initial screen. Antibodies which showed surface membrane reaction with any of these sections were selected for further evaluation. The second screen involved paraffin sections from 17 colorectal carcinomas, 12 adenomatous polyps, 10 'normal' colons taken 10 to 15 cms from the tumour or the site of diverticular disease, 10 lymph node secondaries and 6 liver metastases. Antibodies selected as a result of these screenings were then examined for reactivity with frozen sections of normal lung, liver, kidney and spleen.

4.3.4 Immunocytochemistry

Two different indirect enzyme immunocytochemical techniques were used, the alkaline phosphatase (Foster et al., 1982a) and the immunoperoxidase method described in 3.2.3.iv.a.

i Indirect alkaline phosphatase method

Five microns paraffin embedded sections were dewaxed in xylene, and rehydrated through a graded alcohol series. The endogenous alkaline phosphatase in the tissue was blocked by 15% v/v glacial acetic acid in distilled water for 15 minutes and washed in PBS. The sections were then incubated with 100 µl of

monoclonal antibody as undiluted tissue culture supernatants for 1 hour in a moist chamber at room temperature. After washing with PBS and distilled water, 100 µl of second antibody, a rabbit anti-mouse immunoglobulin conjugated to alkaline phosphatase (Type VII, Sigma) at a concentration of 1 : 20, was added to the sections and incubated for another hour, after which the sections were washed with PBS and distilled water. One ml of Fast Red substrate (Appendix 2) was added to the sections and incubated for 10 minutes at room temperature. The slides were finally washed in tap water, counterstained with Mayer's haemalum (BDH) and mounted in Hydromount (Raymond Lamb).

ii Bone marrow staining

Antibodies selected for potential immunoscintigraphy of colorectal cancer were tested for their reaction with bone marrow cells. Two ml of bone marrow aspirate were obtained through a Salah needle (size 16G). The red blood cells were removed by density separation in Ficoll and sodium metrizoate at a density of 1.077 ('Lymphoprep'; Nyegaard). The sample was spun at 400g for 20 minutes, and the cells were retained at the interface. They were washed, pelleted and then smeared onto 15-20 slides and fixed in absolute alcohol. The slides were stained with the alkaline phosphatase technique.

The interpretation of the immunocytochemical results was similar to that of 3.2.3.iv.a, and the results were classified as positive, weak and negative. However, the staining of a few cancer cells on a section by an antibody or a weak reaction with the antibody were regarded as negative, as the purpose of this screening was to select monoclonal antibodies with a strong affinity to colorectal cancer.

4.4 RESULTS

4.4.1 Comparison of immunocytochemical techniques

The comparison between the two indirect enzyme immunocytochemical techniques in the initial screening of colorectal cancer-reactive antibodies showed that the staining pattern and the sensitivity of both methods were similar. A red colour (Figure 17) was obtained with the alkaline phosphatase technique, while a brown colour resulted from the immunoperoxidase method (Figure 18). However, there was a possibility that the endogenous alkaline phosphatase present in the colon might give a false positive result with the alkaline phosphatase technique, despite the use of blocking reagents (see 4.3.4.i). The immunoperoxidase method was therefore used for subsequent staining. The colour reaction of this procedure, unlike that of the alkaline phosphatase technique, is not water soluble and can be mouted with D.P.X. which provides a better protection of the staining reactions.

4.4.2 Initial screening with colorectal cancer sections

i Monoclonal antibodies to HMFGM

Of the 4 antibodies (M3, M8, M18, M24) screened from this fusion, only M8 showed luminal membrane staining in the majority of the cells in the cancer sections. Three other monoclonals M3, M18 and M24 showed surface and cytoplasmic staining but with a far fewer number of colorectal cancer cells. Monoclonal antibody M8 (IgG1) was therefore selected from this series for further evaluation. It showed no reaction with connective tissue cells or blood cells.

ii Monoclonal antibodies to bladder cancer

Four monoclonals from this series reacted strongly with the cell surface membrane of most of the cells in the colorectal cancer and showed no reactivity with connective tissue or white and red blood cells. These were 48-1, 3-48-2, 77-1 and 8-30-3. Two antibodies, 3-71-1 and 3-11-3, stained a few cells only. Another three antibodies, 2-5-3, 3-50-3, and 93M-2 stained some of the cancer cells and also the connective tissues, while the remaining four monoclonals, I-III-89, 2-94-2, 4-72-2 and 9-4-3 did not react with the colorectal cancer. Monoclonal antibodies 48-1, 3-48-2, 77-1 and 8-30-3 were selected for further study. The immunoglobulin subclasses of these antibodies were IgG1, IgG1, IgG2a and IgG2b respectively. The remaining two antibodies, 22.2 and 39.8.3 did not react with colorectal cancer.

iii Other monoclonal antibodies

The anti-colorectal cancer monoclonal antibody AS1748 reacted with the cancer but also with the connective tissues. Two anti-breast monoclonals, TW19 and H10A, reacted with only a few colorectal cancer cells. Monoclonal 29 reacted with the cytoplasm of the colorectal cancer and the remaining three antibodies, 20.3, 36 and 39-8-3 did not show any reaction.

The initial screening of these 25 monoclonal antibodies therefore identified five antibodies with a strong reactivity to colorectal cancer cell membrane and with no cross reactivity with connective tissues or blood cells. The reaction of these antibodies to a larger number of different colorectal cancers, normal colon and the metastatic tumour to liver and lymph nodes is described below and listed in Table 8.

4.4.3 Secondary screening

i Colorectal cancer

A total of 17 different colorectal cancer sections were used to study the reaction of these five monoclonal antibodies to the tumour. The staining reaction of these antibodies with colorectal cancer was heterogenous, varying from a few to the majority of the tumour cells.

M8 reacted with 16 out of the 17 sections. Two of these sections showed staining of only a small percentage of the cells (less than 5%) and one section had a weak staining reaction. These sections were counted as negative, thus giving a positive result of 13 out of 17 (76%). The reaction between M8 and colorectal cancer is shown in Figure 19.

Monoclonal antibody 48-1 reacted with 16 / 17 sections (94%) while 3-48-2 reacted with all 17 sections (100%). Both these antibodies reacted with the majority of the cancer cells on the sections. Figures 20 and 21 show the reaction between colorectal cancer and 48-1 and 3-48-2 respectively.

Monoclonal antibody 77-1 reacted with all 17 sections, although one of the section was equivocal and another reacted with a few cells, and these were excluded, giving a positive score of 15 / 17 (88%). Monoclonal antibody 8-30-3 reacted with 16 sections with one section having a few cells stained only and it was discounted giving a similar result to 77-1 i.e. 15 out of 17 positive (88%). An example of the reaction between 8-30-3 and colorectal cancer is shown in Figure 22.

ii Polyps

Twelve adenomatous polyps were used for this study. The intensity of

the staining reaction and the number of cells in the polyps stained by the monoclonal antibodies M8, 77-1 and 8-30-3 were less than with the monoclonals 48-1 and 3-48-2.

Monoclonal antibody M8 reacted with a small percentage (< 5%) of cells in one and more strongly with another polyp i.e. two out of 12 polyps or 17%. Monoclonal antibody 77-1 reacted with a small number of cells in two polyps (17%). Figure 23 shows the minimal reaction occurring between 77-1 and a polyp. Monoclonal 8-30-3 reacted with 6 polyps (50%), two of which showed only weak reaction.

In constract, monoclonal antibodies 48-1 and 3-48-2 reacted with all the polyps (100%) and with most of the cells within an individual tumour.

iii Metastases

All five antibodies reacted with all six colorectal liver metastases (100%), again with a heterogenous staining pattern. None of the primary tumours from these metastases were available for comparison. Figures 24 and 25 show the reactions between colorectal liver metastases and M8 and 77-1 respectively. Of the 10 lymph node metastases M8 reacted with six (60%), and 77-1 and 8-30-3 with eight or 80% of the tumours. Figures 26 and 27 show the reactions between colorectal lymph node metastases and M8 and 77-1 respectively. Monoclonal antibody 48-1 reacted with six (60%) and 3-48-2 with seven (70%) of the tumours. The primary cancers from five of these metastases had been already examined. A comparison of staining results between the primary and secondary tumours was thus possible. The monoclonal antibodies M8 and 48-1 in one case reacted with a primary cancer but not the metastatic tumour. Similarly, the antibodies 77-1 and 3-48-2 reacted with another primary tumour but not with the

secondary deposit. The antibody 48-1 reacted in a similar fashion with another set of primary and secondary tumours. However, it stained the lymph node metastasis from one patient but reacted negatively to the primary cancer. These results indicated a difference in the antigenic expression between several primary cancers and their metastases.

iv Normal colon

With normal colon, monoclonal M8 reacted with 6 (60%) of the 10 different sections (an example is shown in Figure 28). Monoclonal 77-1 reacted with three (30%) and 8-30-3 with one (10%). Figure 29 shows the reaction between a normal colon and 77-1. The reaction of these antibodies with normal colon was often weak and occurred with a small percentage of cell usually in the crypt of the mucosa in each individual specimen. In contrast, 48-1 and 3.48.2 reacted with all 10 sections (100%) of the normal colon strongly and with nearly all the cells present on the different sections. Figure 30 shows the reaction between 48-1 and normal colon.

4.4.4 Frozen sections tissue distribution

The results of the staining of M8 and 77-1 with frozen sections of the liver, lung, kidney and spleen were the same as with paraffin sections. Thus, M8 reacted with the parenchyma and the bile duct while 77-1 only reacted with the bile duct. Both M8 and 77-1 reacted with alveoli of the lung and the distal tubules of the kidney. There was no reaction between the two antibodies and the spleen. There was no staining of the the bone marrow cells by either M8 or 77-1.

A summary of the tissue distribution of monoclonal antibodies M8, 77-1 and 8-30-3 is shown in Table 9.

Table 8 **Summary of the reactivity of the selected monoclonal antibodies with colorectal cancer, metastases, polyps and normal colon**

Tissue		Monoclonal antibody designation				
		M8	**48-1**	**3-48-2**	**77-1**	**8-30-3**
CRC	(17)	13 (76%)	16 (94%)	17 (100%)	15 (88%)	15 (88%)
Polyps	(12)	2 (17%)	12 (100%)	12 (100%)	2 (17%)	6 (50%)
N colon	(10)	6 (60%)	10 (100%)	10 (100%)	3 (30%)	1 (10%)
LN mets	(10)	6 (60%)	6 (60%)	7 (70%)	8 (80%)	8 (80%)
Liver mets	(6)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)

Table 9 Tissue distribution of monoclonal antibodies M8*, 77-1 and 8-30-3****

	M8	77-1	8-30-3
Gastrointestinal tract			
oesophageal epithelium	-	+	+
gastric epithelium	+	++	++
ileal epithelium	+	+/-	+/-
colonic epithelium	+	+	+
liver			
parenchyma	+	-	+
bile duct	+	+	+
gall bladder	NA	-	+
pancreas			
islet	-	-	-
duct	-	+	+
Spleen	-#	-	-
Respiratory system			
lung alveoli	+	+	+
Genitourinary system			
kidney		+	+
glomeruli	-		
proximal tubules	+/-		
distal tubules	+		
ureter	NA	+	+
bladder	NA	++	++
uterus	+	+	++
Fallopian tube	+	-	-
ovary	-	-	-
testis	+	-	-
prostate	-	+/-	+/-
Nervous & endocrine system			
brain -	-	-	
adrenal	-	-	-
thyroid	+	-	-

NA = not available ++ = strong positive + = positive +/- = weak
# = author's observation

References: *Foster et al., 1982a **Summerhayes et al., 1985

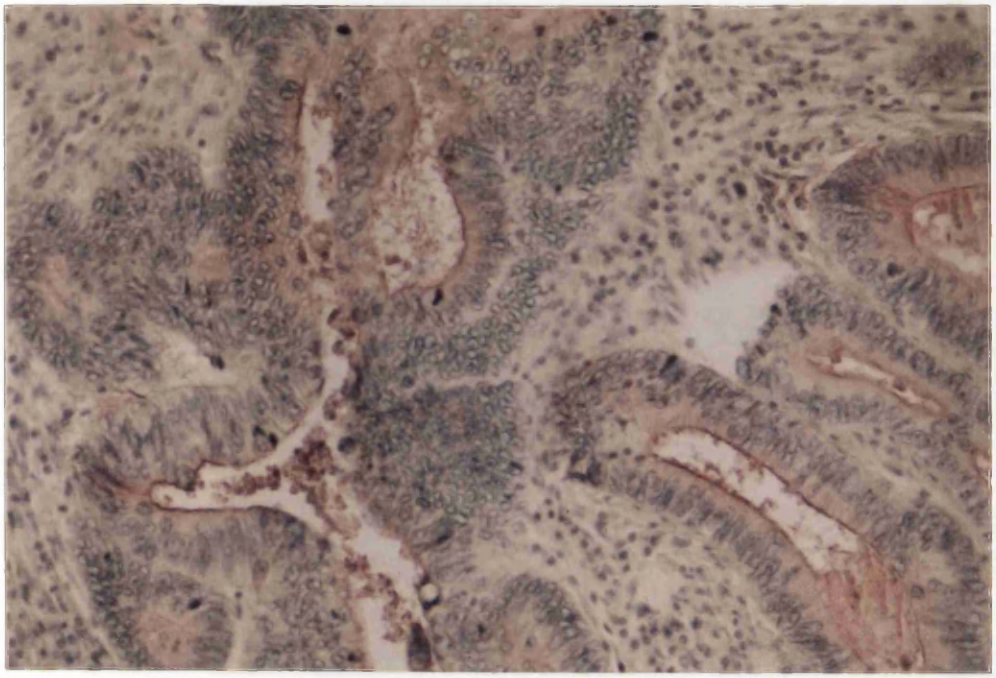


Figure 17 Alkaline phosphatase staining of colorectal cancer with 77-1 showing reaction on the luminal surface of the cells and a red colour x 150

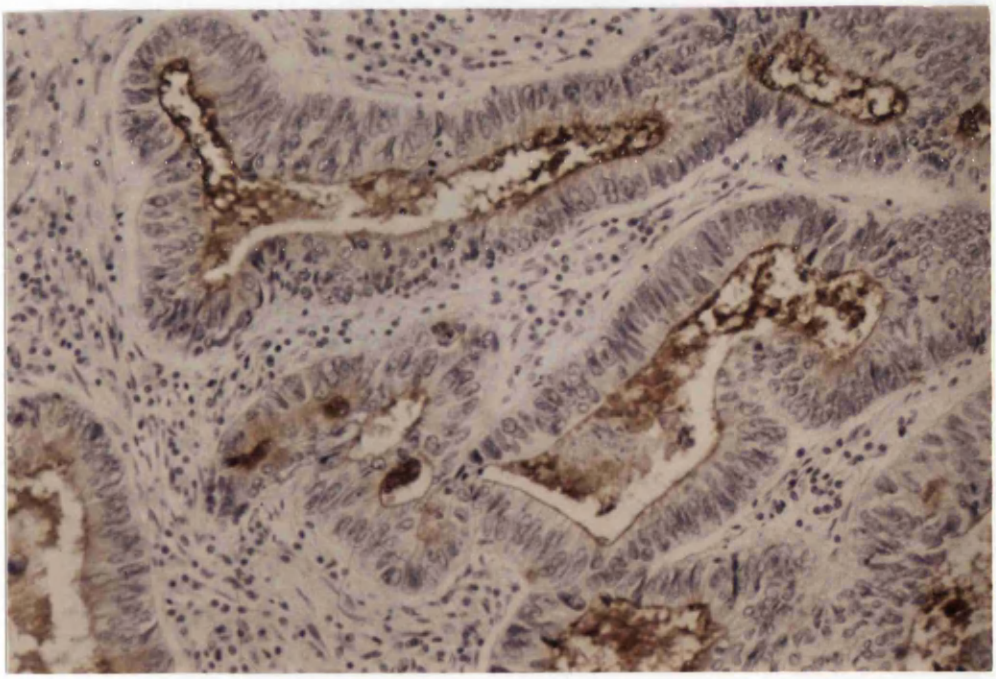


Figure 18 Immunoperoxidase staining of colorectal cancer with 77-1 showing luminal surface reaction with the cells and a brown colour x 150

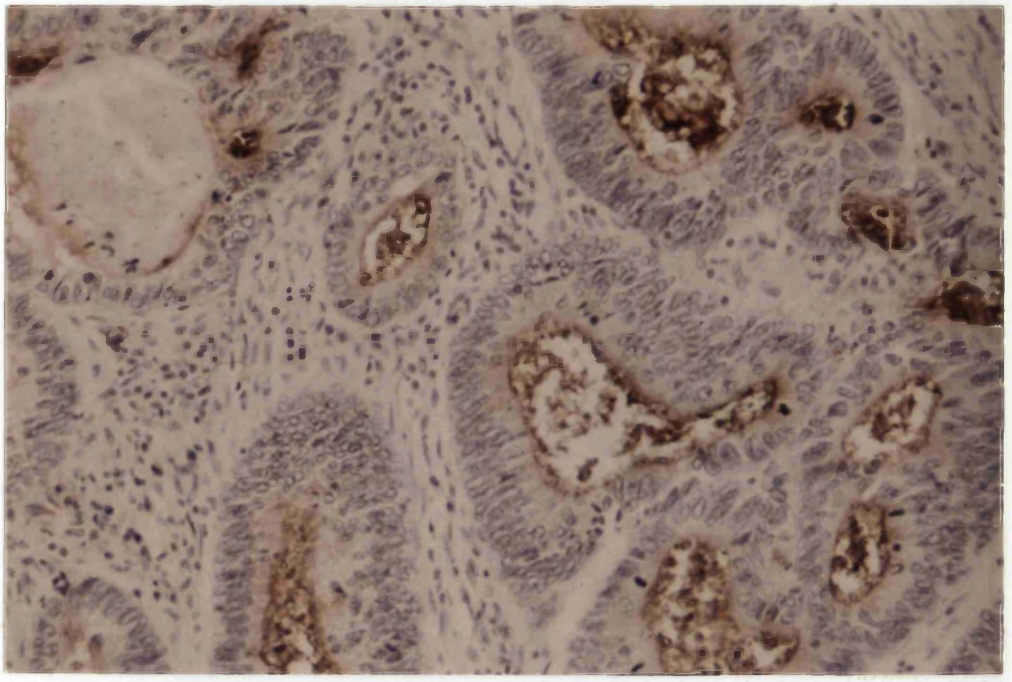


Figure 19 Immunoperoxidase staining of colorectal cancer with M8 showing reaction on the luminal surface of the cells x 150

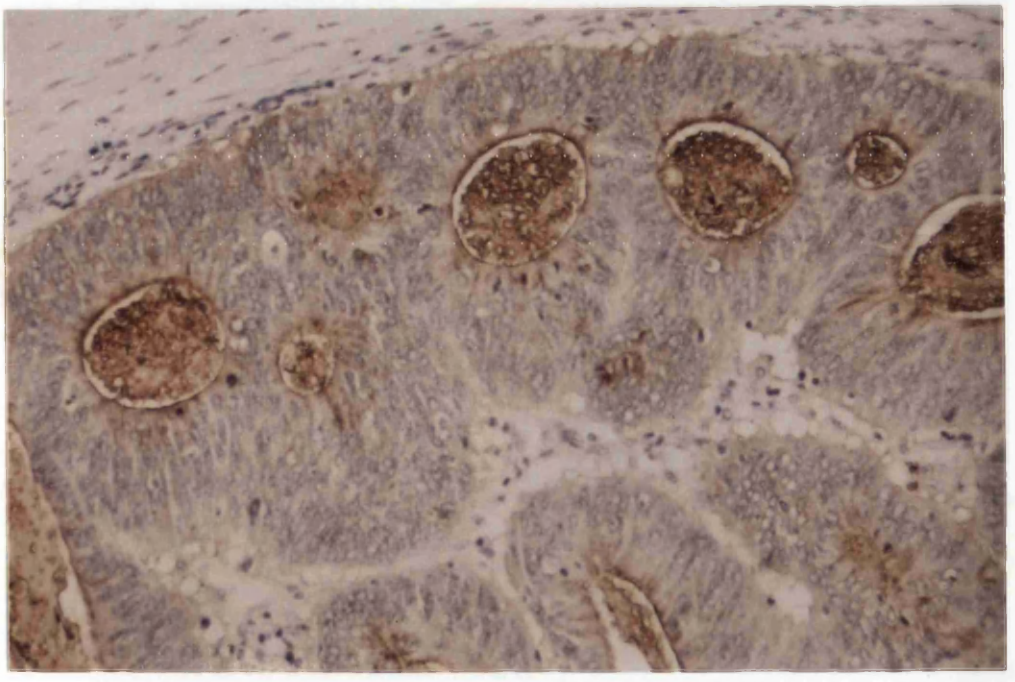


Figure 20 Immunoperoxidase staining of colorectal cancer with 48-1 showing reaction on the luminal surface of the cells x 150

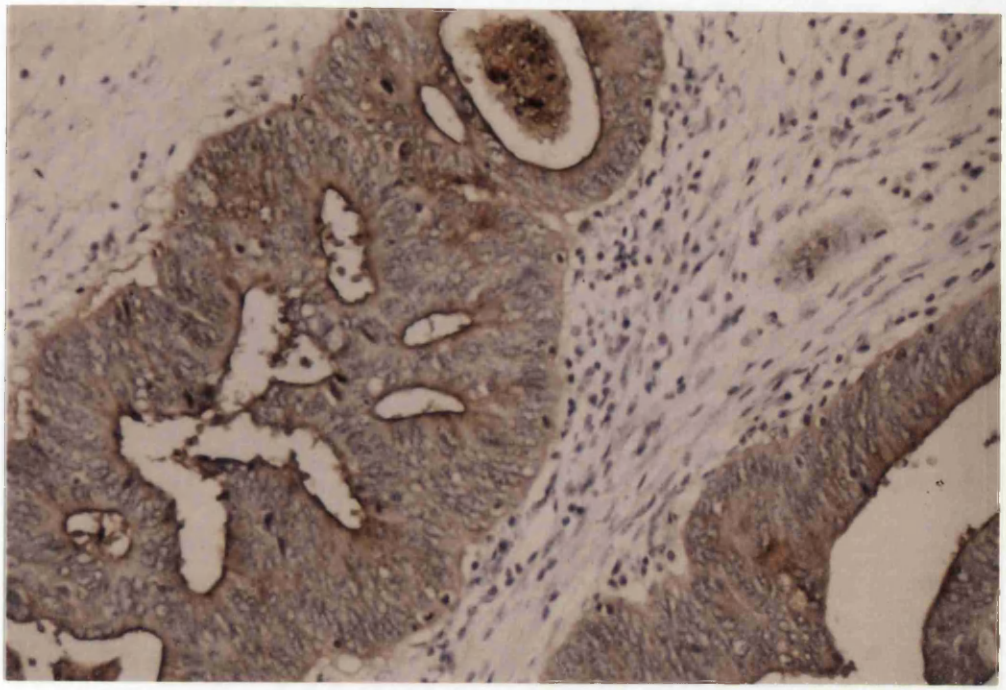


Figure 21 Immunoperoxidase staining of colorectal cancer with 3-48-2 showing reaction on the luminal surface of the cells x 150

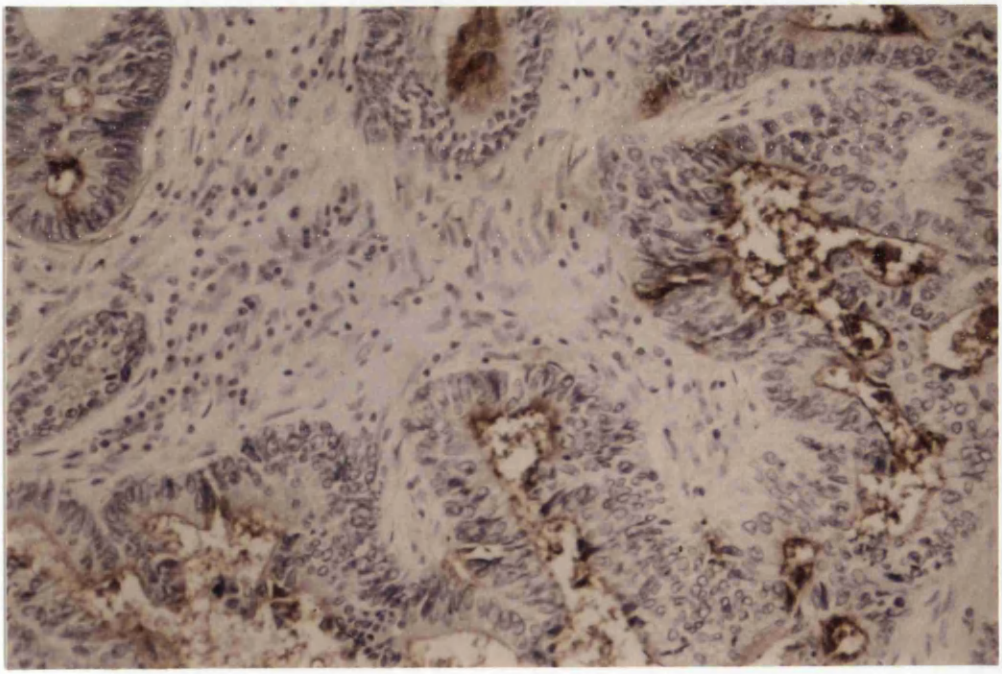


Figure 22 Immunoperoxidase staining of colorectal cancer with 8-30-3 showing reaction on the luminal surface of the cells x 150

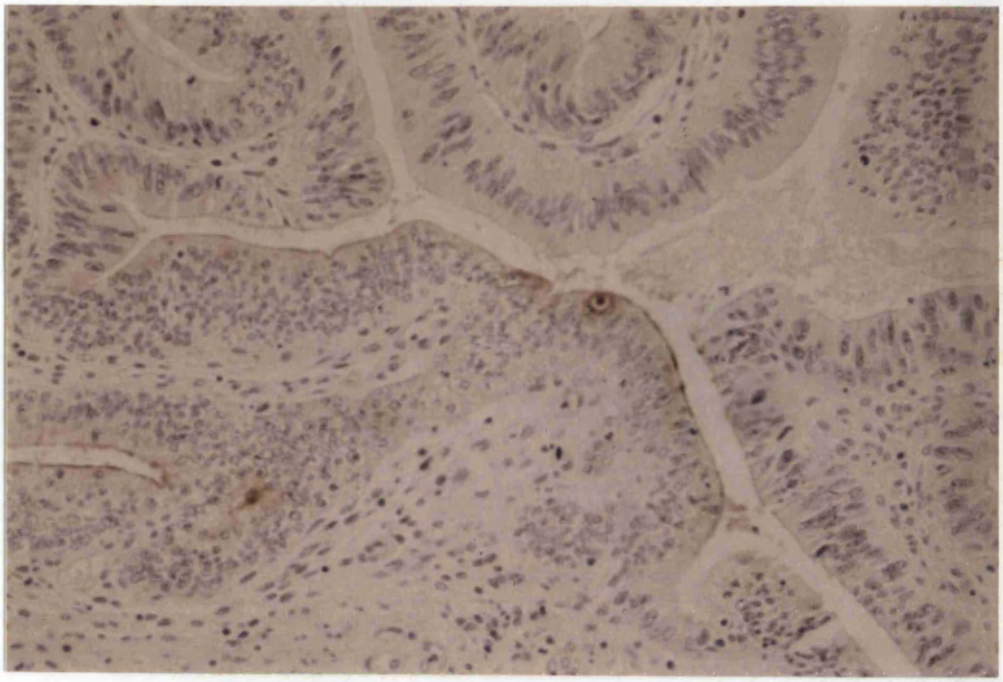


Figure 23 Immunoperoxidase staining of polyp with 77-1 showing minimal reactivity x 150

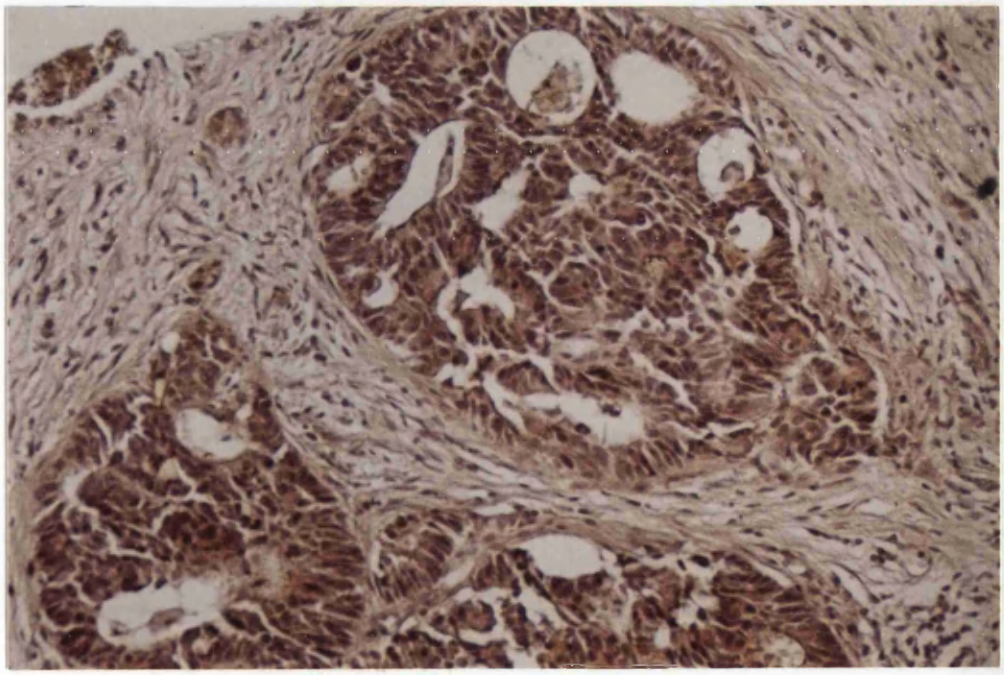


Figure 24 Immunoperoxidase staining of colorectal liver metastasis with M8 x 170

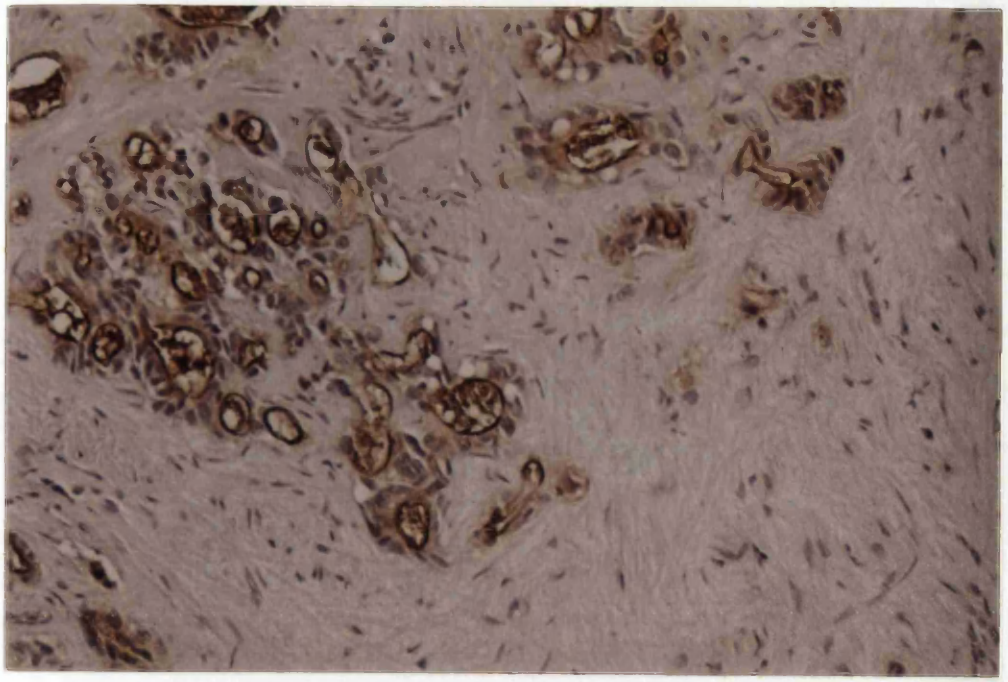


Figure 25 Immunoperoxidase staining of colorectal liver metastasis with 77-1 x 150

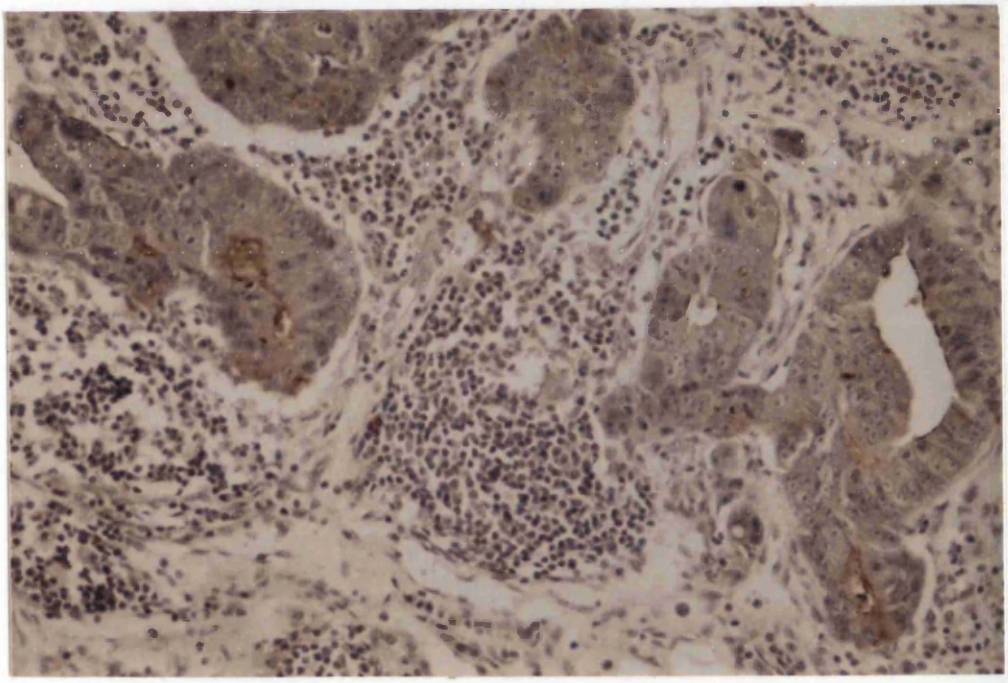


Figure 26 Immunoperoxidase staining of colorectal lymph node metastasis with M8 x 150

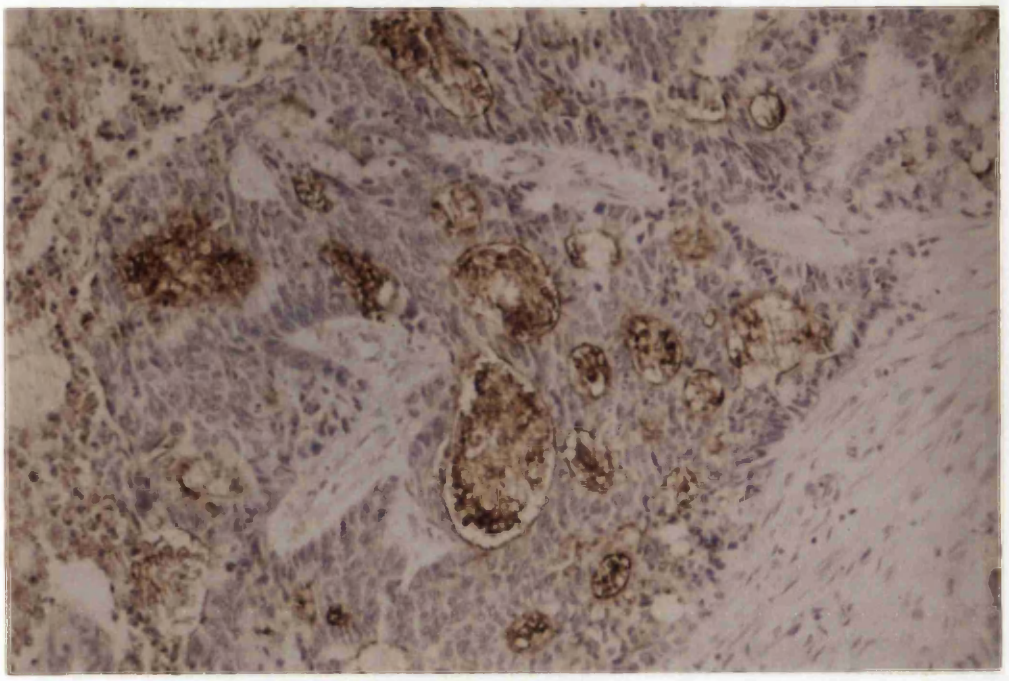


Figure 27 Immunoperoxidase staining of colorectal lymph node metastasis with 77-1 x 150

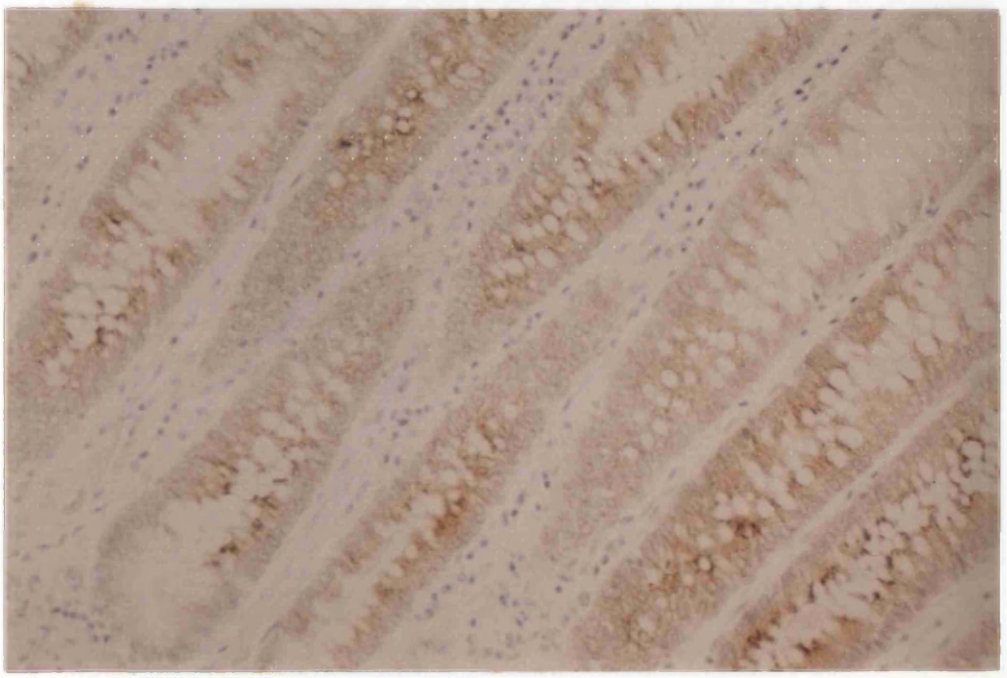


Figure 28 Immunoperoxidase staining of normal colon with M8 x 150

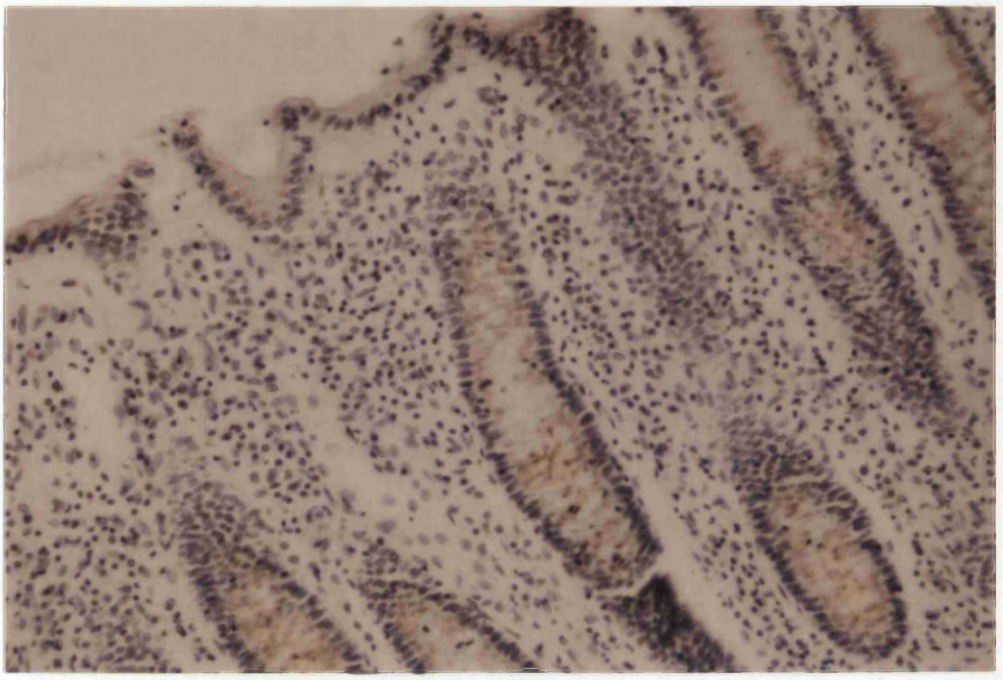


Figure 29 Immunoperoxidase staining of normal colon with 77-1 showing reaction with cells at the more basal part of the mucosa x 150

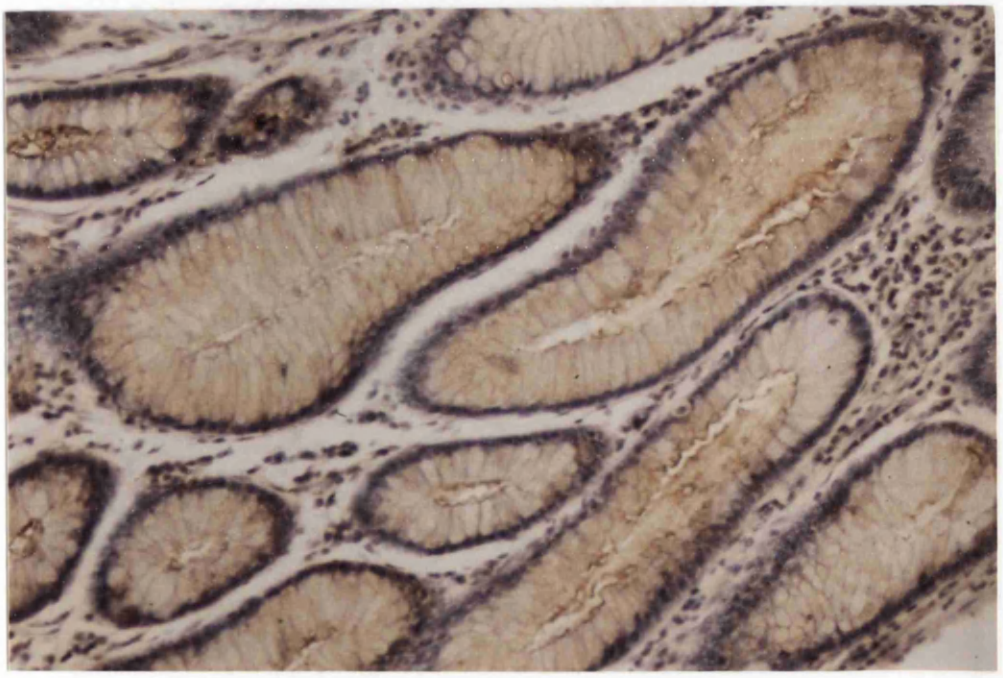


Figure 30 Immunoperoxidase staining of normal colon with 48-1 showing reaction with the cytoplasmic content x 170

4.5 DISCUSSION

The cross-reactivity of monoclonal antibodies described in this part of the study forms the basis of a plan to select antibodies suitable for the immunoscintigraphy of colorectal cancer by screening monoclonals raised to other tumours. In this study, 25 monoclonal antibodies raised against human milk globule membrane (HMFGM), breast cancer extracts, bladder cancer and one raised against colonic cancer were screened for reactivity to colorectal cancer. The screening was performed in two stages: an initial screening which was to identify colorectal cancer-reactive antibodies, and a secondary screening which was aimed at determining the relative reactivity of these antibodies with a larger number of colorectal carcinomas, metastatic carcinomas, polyps, and normal colon.

The results obtained in this study confirmed the phenomenon of the cross-reactivity of monoclonal antibodies, and showed that colorectal cancer-reactive antibodies could be obtained by screening existing antibodies.

The comparison between the alkaline phosphatase and immunoperxidase techniques had shown that the staining pattern and the sensitivity were the same with either method. Endogenous alkaline phosphatase in the colon did not appear to give rise to false positive results. However, a disadvantage of the alkaline phosphatase technique is that the staining reaction is water soluble and requires a water soluble medium for mounting. Consequently, the histological sections may not be as durable as those stained with the immunoperoxidase method, the colour product of which is not water soluble, and in which tissue sections are mounted in D.P.X. which affords a better preservation of the slides. A potential hazard of the immunoperoxidase method is the use of diaminobenzidine tetrahydrochloride which may be a carcinogen, and precautions have to be taken in its use and

disposal. A combination of these two techniques, however, may be used to demonstrate the reaction of two different antibodies on the same tissue section e.g. in the selection of antibody 'cocktail partners'. This double staining technique had been used successfully to show the reaction between monoclonal antibody 8-30-3 and 3-48-2 and a number of other antibodies on colorectal cancer by the investigator. The immunoperoxidase technique was performed first followed by the alkaline phosphatase method.

The initial screening showed that 15 monoclonal antibodies reacted with colorectal cancer: all four antibodies of the HMFGM series, seven from the bladder cancer series, and four from the remaining monoclonals. Only those antibodies which showed the best membrane reactivity with colorectal cancer and which at the same time did not react with connective tissues were considered for further evaluation. Antibodies showing cytoplasmic reactivity or the staining of a small number of cells in the colorectal cancers were also rejected. This selection criteria resulted in the identification of five monoclonal antibodies with strong colorectal cancer surface membrane reactivity, one of these antibody (M8) was raised against HMFGM while the other four (48-1, 3-48-2, 77-1, 8-30-3) were raised against bladder cancer.

In the secondary screening, the five monoclonal antibodies selected were tested against 17 different colorectal cancer sections, and their affinity to the cancer was confirmed. The percentages of different colorectal carcinomas reacting with these five antibodies (76-100%) compare favourably with other monoclonals currently used for the immunoscintigraphy of colorectal cancer. For example, B72.3 reacts with 82% of colorectal cancer (Stramignoni et al., 1983), an anti-CEA antibody C46 which reacts with over 90% % of the tumours (Davidson et al., 1899), and the monoclonal antibody 19-9 which reacted with 59% of the

colorectal cancers tested (Atkinson et al., 1982). The two new monoclonal antibodies (UC-ICR 41.2 and 41.6) produced by the author in this investigation (Chapter 3) showed better reactivity than the antibodies M8, 77-1 and 8-30-3, as they reacted with 94% of colorectal cancer.

All five antibodies reacted with the six liver metastases tested (100%), but with a smaller number of lymph node metastases, 60% for M8 and 48-1, 70% for 3-48-2 and 80% for 77-1 and 8-30-3. A comparison of the reactivity between the antibodies and the primary tumours and their lymph node metastases was possible in five patients. It was interesting to note that in several instances the primary tumours stained positively with the antibodies but the metastatic tumours were negative. In one patient, 48-1 reacted with the lymph node secondary but not the primary cancer. This difference in antigen expression between the primary and secondary cancer has been reported by others (Chatal et al., 1985), and is of relevance in the application of monoclonal antibodies in the radioimmunolocalisation or targeting of tumours.

The reaction of these antibodies to adenomatous polyps and normal colon separated them into two groups. M8, 77-1 and 8-30-3 reacted with 10-60% of normal colon and 17-50% of polyps, indicating their preferential reaction with colorectal cancer, while 48-1 and 3-48-2 did not show this selectivity and reacted with 100% of normal colon and polyps. The antibodies UC-ICR 41.2 and 41.6 reacted with 86% of normal colon sections. However, their reaction with the colon was not as strong as that with 48-1 and 3-48-2, which stained all cells in the colonic mucosa, but UC-ICR 41.2 and 41.6 stained mainly cells on the apical portion of the colonic crypt. The reaction of 48-1 and 3-48-2 with normal colon may not prevent their application for immunoscintigraphy, as an anti-CEA monoclonal antibodies C46 used for the imaging of colorectal cancer also reacts

with a large proportion of normal colon (Davidson et al., 1989).

The apparent preferential reactivity of the first group of monoclonal antibodies (M8, 77-1 and 8-30-3) to colorectal cancer, although reacting with a slightly smaller percentages of tumours compared to the second group (48-1 and 3-48-2), nevertheless made them more suitable for the immunoscintigraphy of this tumour. Therefore from 25 antibodies initially screened, three were finally selected for preparation for the radiolocalisation of colorectal cancer (M8, 77-1 and 8-30-3).

The method of tissue processing can affect the reactivity of antibodies with tissue sections as antigens may be altered or destroyed in the process. Frozen section staining was necessary to confirm that the pattern of reaction of these antibodies observed with the paraffin sections reflected their likely reactivity *in vivo*. The tissue distribution of these antibodies (M8, 77-1 and 8-30-3) was characteristic of epithelial membrane antigen (EMA), and they reacted with most epithelial cells. However, despite this fact, the monoclonal antibody M8 has been used for the radioimmunodetection of breast cancer secondaries with success (Rainsbury, 1984). There are therefore grounds for expecting that the antibody 77-1 might also be successful. The tissue distribution of the UC-ICR 41.2 and 41.6 was more selective than the anti-EMA antibodies, as they did not react with the lung, liver, kidney and the reproductive organs.

All antibodies reacted heterogeneously with the tumours, the staining reaction involved a few to most of the cells in the sections and the intensity of the staining also varied from strong, moderate to weak. Many attempts have been made to classify such staining reaction, for example, the use of different dilution of antibodies (Colcher et al., 1981), or a semi-quantitative (+++ > ++ > + > -) grading system to indicate the intensity of the staining reaction (Schlom et

al.,1984). Ellis et al. (1985) and Muir et al. (1987) use a scoring system to express the intensity of staining between the monoclonal antibody NCRC 11 and breast cancer sections. Staining of 1-25% of tumour cells on a section = + (grade 1); staining of 26-50% of tumour cells = ++ (grade 2); staining of 51-75 % of tumour cells = +++ (grade 3) and staining of 76-100% of tumour cells = ++++ (grade 4). Thus, staining intensity may be used by some to mean the intensity of colour, and by others to mean the number of cells stained. In general, the differential interpretation of immunocytochemical results is subjective and to some degree difficult to quantify.

In the assessment of staining reactions in this part of the study, a weak reaction or the staining of a few cells on a section were classified as negative. This led to lower positive staining scores for the antibodies and colorectal cancer sections, affecting mainly the monoclonal antibodies M8, 77-1 and 8-30-3. However, this criterion was chosen so as to select monoclonal antibodies with strong reactivity with colorectal cancer. It can be argued that such strict criterion may not be necessary, as specimens from tumours which scanned positively with radiolabelled antibodies may reveal a very low antigen concentrations when subsequently stained (Halpern & Dillman, 1987). Furthermore, the antigen concentration on a tumour can vary from site to site within the same patient, and from section to section in the same tumour (Halpern & Dillman, 1987).

However, in several studies there has been a good correlation between immunocytochemistry and imaging so that an antibody that reacts with a tumour on section can also be expected to scan the tumour on immunoscintigraphy (Larson and Corresquillo, 1987). It can therefore be expected that the radiolabelled M8, 77-1 and 8-30-3 have a good chance of localising to colorectal cancer in a clinical study.

The immunoglobulin subclasses of these three antibodies IgG1 (M8), IgG2a (77-1) and IgG2b (8-30-3) were also appropriate for immunoscintigraphy as being smaller molecules they can cross tissue compartments more easily than the larger IgM immunoglobulin. Monoclonal antibodies of the IgG2a subclass may have cytotoxic properties (Hellstrom et al., 1986), and monoclonal antibody 77-1 may have this potential.

CHAPTER 5

LOCALISATION OF RADIOLABELLED ANTIBODIES IN XENOGRAFT MODELS

5.1 INTRODUCTION

In the search for monoclonal antibodies for immunoscintigraphy of colorectal cancer two approaches were used. The first was the production of new monoclonal antibodies to colorectal cancer, using a crude membrane extract and five new monoclonals were characterised in detail. Two of these monoclonal antibodies UC-ICR 41.2 and 41.6 may have the potential for immunoscintigraphy. However, while they reacted with 94% of colorectal cancer, like the anti-CEA monoclonal antibody C46, which reacts with over 90% of normal colons (Davidson et al., 1989), UC-ICR 41.2 and 41.6 reacted with 83% of normal colons (Chapter 3). The reaction with normal colon, however, has not prevented C46 from localizing to colorectal cancer (Armitage et al., 1986a). The second approach was the screening of 25 monoclonal antibodies raised predominantly to breast secretary products and bladder cancer by means of immunocytochemistry (Chapter 4). This revealed three monoclonal antibodies M8, 77-1 and 8-30-3. Similarly, these antibodies are not tumour-specific, as they recognise epitopes of epithelial membrane antigen (EMA) (Ormerod et al., 1983; McIlhinney et al., 1985; Summerhayes et al., 1984) which are present in normal epithelial tissues. However, they showed selectivity for colorectal cancer when compared with normal colon and polyps and were therefore chosen for initial clinical study in preference to the new antibodies.

The monoclonal antibody M8 has been used for the immunoscintigraphy of breast cancer with success, and did not show any side-effects following intravenous administration to patients (Rainsbury, 1984). The tissue distribution of monoclonal antibody 77-1 is similar to that of M8 with the further advantage that it does not react with hepatocytes (Chapter 4) and it was therefore reasonable not to

expect significant adverse reactions following its administration to patients. The monoclonal antibody 77-1 has not been used before for the immunoscintigraphy of human cancer. Antibody 8-30-3, while fulfilling most of the criteria for selection for imaging did react with the hepatocytes and was therefore not chosen for initial clinical use. For these reasons it was felt that it was appropriate to investigate the antibodies M8 and 77-1 for the imaging of colorectal cancer.

For immunoscintigraphy, a radioisotope has to be attached to an antibody so that its localisation can be detected by an external gamma camera. The two most commonly used radioisotopes for antibody labelling are iodine-131 and indium-111. The relative merits of the two radionuclides have been discussed in 1.11. The immunoreactivity of an antibody, however, may be damaged by the radiolabelling process. Therefore in the preparation of an antibody for immunoscintigraphy, the *in vitro* immunoreactivity of both the iodinated and indium-111 labelled antibodies has to be assessed, as either procedure may have a damaging effect on antibody reactivity. In the radiolabelling with iodine-125 in this study, the chloramine T method was also compared with the iodogen method. The localisation of an immunoreactive radiolabelled antibody to the target tumour may be interfered with by the metabolic events in the circulation or in the tissues following its administration. Thus a xenograft model is commonly used to demonstrate the retention of its *in vivo* immunoreactivity by its localisation to the tumour. The immunoreactivity of the radiolabelled monoclonal antibody M8 had already been demonstrated (Rainsbury, 1984) but this has not been done previously for 77-1. It was therefore necessary to demonstrate that the radiolabelled 77-1 remained immunoreactive by a cell binding assay before its application to patients and to confirm that the radiolabelled antibody could localise to the target tumour as demonstrated in a xenograft model.

5.1.1 *In vitro* immunoreactivity of radiolabelled antibodies

The *in vitro* immunoreactivity of a radiolabelled antibody can be demonstrated by its binding to a target antigen such as a cell extract (Colcher et al., 1984), or a tumour marker such as CEA (Halpern et al., 1983) using a solid phase radioimmunoassay (Colcher et al., 1984), or preferably to live cells (Chatal et al., 1984; Rainsbury, 1985) using a cell binding assay (Chatal et al., 1984; Rainsbury, 1985). The radiolabelled M8 was tested on a breast cancer cell line MCF7 (Soule et al., 1973) by means of a cell binding assay (Rainsbury, 1985). As monoclonal antibody 77-1, like M8 reacts with epitopes of epithelial membrane antigen (EMA) (Ormerod et al., 1983; McIlhinney et al., 1985), it was anticipated that 77-1 would also react with MCF7. The live cell binding assay was chosen for testing the immunoreactivity of the radiolabelled 77-1, and flow cytometry was used to select an appropriate target cell from a panel of several cell lines, including MCF7.

5.1.2 *In vivo* immunoreactivity of radiolabelled antibodies

The *in vivo* immunoreactivity of the radiolabelled 77-1 was tested in xenograft localisation studies. Xenografts are human tumours transplanted to immune deficient animals, usually a mouse or rat. Toolan (1951) was the first to describe human tumours transplanted to artifically immune suppressed mice. Immunosuppression became better established with the technique of thymectomy and total body irradiation followed by bone marrow reconstitution (Castro, 1972; Cobb et al., 1973). Steel et al. (1978) used cytosine arabinoside (Ara-C) to protect the bone marrow instead of marrow reconstitution following irradiation.

Congenital athymic nude mice (Pantelouris, 1968) are an alternative to immune suppressed mice for transplantation establishment of human xenografts with the first successful transplantation of human tumour to nude mice reported by

Rygaard and Povlsen (1969), followed by many others (Giovanella et al., 1974; Sordat et al., 1974). Tumours established in this manner can, in some cases, be serially passaged to provide large quantities of material for study.

In essence, a xenograft is a convenient way of providing a human tumour in an *in vivo* setting for assessing the localising ability of radiolabelled antibodies. Antibodies raised to human tumours in mice are unlikely to cross react with the host mouse tissues. However, the behaviour of the antibodies when administered to human subjects cannot be confidently predicted, as the antibodies will cross react with normal human tissues. For localisation study, an essential criterion is a xenograft which expresses the target antigen that can be recognised by the antibody. Several xenografts were therefore tested for reactivity with 77-1 by means of immunocytochemistry before one was selected for the purpose of this investigation.

Several methods can used to demonstrate the localisation of a radiolabelled antibody to a xenograft. A higher level of radiolabelled antibody should be shown to be present in the tumour compared to normal tissue, and to be shown to be due to an immunological reaction and not to a non-specific deposition of immunoglobulin (Pimm & Baldwin, 1985). Pressman (1957) used two different isotopes of iodine to label the test antibody and the control antibody, and injected these radiolabelled reagents simultaneously into the animal. The uptake by the tumour of the specific antibody was compared to the non-specific immunoglobulin. Instead of using a control antibody, a control xenograft may be used. The uptake of antibody to the tumour may be measured as a ratio to that of the liver (Mach et al., 1974); to blood; as a percentage of the injected dose per gram of tissue (Colcher et al., 1984; Rainsbury, 1985); or as a localisation index which is a ratio of the tumour to blood ratio of the specific antibody compared to a

similar ratio obtained with a control antibody (Moshakis et al., 1981c). The tumour / tissues to blood ratios were used in this study.

The tumour to blood ratio was used as a measurement of antibody uptake by the xenograft in this study as it is commonly used to demonstrate the localisation of an antibody to a tumour. Apart from demonstrating the localisation of the radiolabelled M8 to a xenograft (Rainsbury, 1984), the tumour to blood ratio has also been used for the same purpose for other monoclonal antibodies, for example, the localisation of an anti-osteogenic sarcoma monoclonal antibody 791T/36 to an osteogenic sarcoma xenograft 791T (Pimm & Baldwin, 1985), the monoclonal antibody B72.3 to a colon carcinoma xenograft LS-174T (Colcher et al., 1984), and the monoclonal antibody fragment $F(ab')_2$-ICI2 to another colon cancer xenograft MAWI (Rogers et al., 1986).

5.2 MATERIALS AND METHODS

5.2.1 Production and purification of monoclonal antibodies

The antibodies were produced as ascites in Balb/c ex-breeder mice (Olac 1976 Ltd., Oxon., U.K.). The M8, 77-1 and 48-1 hybridoma cells were stored in liquid nitrogen until used. The cells were thawed and grown in 5% CO_2 buffered DMEM containing 10% FCS and antibiotics, with Balb/c mouse thymocytes as feeders (see 3.2.2.iii.b). Antibody secretion from the thawed hybridomas was confirmed by means of ELISA on the crude colorectal cancer membrane extract (see 3.2.2.iv.a). To facilitate the formation of ascites the mice were given 0.2ml of 'Pristane' (2,6,10,14-tetramethylpentadecane) intraperitoneally, four days before the intraperitoneal injection of 10^6 hybridoma cells. Ascites usually developed in 10 to 14 days. The mice were sacrificed by cervical dislocation, and 3 to 5 ml of ascitic fluid were collected from each animal. The fluid was centrifuged at 300 x g for 5 minutes to remove the cells. The protein fraction in the supernatant was precipitated with a dropwise addition of saturated ammonium sulphate solution to a give final concentration equivalent to 55% (v/v), followed by mixing for 1 hour at 4°C. The protein was then collected by centrifugation at 2000 x g for 5 minutes. The protein pellet was resuspended in 50 mM Hepes buffer (pH 8.1), and dialysed overnight at 4°C against 50mM Hepes to redesolve the proteins.

A 7 mm x 100 mm column of staphylococcal Protein-A, covalently linked to Sepharose CL-4B, (Protein-A Sepharose, Pharmacia Fine Chemicals, U.K.) was used to extract the antibody from the protein solution. The column was pre-washed with 50 mM Hepes buffer (pH 8.1). It was then loaded with 5ml of the protein solution at a rate of 0.2 ml per minute. The column was further washed with 50 mM Hepes buffer until the optical density of the eluate at 280nm (A_{280})

was less than 0.02, indicating that all protein had passed through it. The column was eluted with 0.1 M sodium citrate-citric acid buffers at pHs 5.5 and 4.5 to release the different Ig subclasses. M8 and 48-1 (IgG1) was eluted at pH 5.5 and 77-1 (IgG2a) at pH 4.5. The immunoglobulins were identified and collected by monitoring the optical density of the eluate from the column at a wavelength of 280 nm. On completion of this procedure, the column was washed with 200 mM glycine (pH 2.8) containing 0.01% (w/v) sodium azide. The antibodies were dialysed against Hepes buffer (pH 7.5) and stored at 4°C until use. The concentration of monoclonal antibody thus obtained was about 1-2 mg/ml.

5.2.2 Radiolabelling of monoclonal antibodies

i ^{111}Indium labelling

This procedure involves the conjugation of the antibody to the bifunctional chelating agent DTPA followed by the labelling of the conjugate with ^{111} indium.

A high concentration of antibody is necessary for conjugation. A volume of 10 to 15 ml of antibody solution from the Protein-A column was concentrated to 1 ml using a Minicon B125 concentrator (Amicon Corp., Danvers, MA 01923, U.S.A.), giving a final concentration of 20-25 mg/ml. The bicyclic anhydride of DTPA (Sigma) was dissolved in dimethylsulphoxide (DMSO) (Sigma) at 1 mg/ml. A molar ratio of 2-3:1 of DTPA to antibody was used for conjugation. The DTPA solution was added to the antibody and mixed by vortexing for 1 minute. The conjugate was then dialysed against 100 mM glycine (pH 3.5) to remove excess DTPA. A 1.6 cm x 50 cm Sephadex G-25 column was used to remove any remaining DTPA by elution with 100 mM glycine (pH 3.5).

Aliquots of 1 ml were collected and the antibody fractions identified by measurement of optical density at 280nm. The conjugated antibody was then filtered with a Millipore 0.22 um filter (Millipore S.A., Molsheim, France) and stored at 4°C.

An activity of 3-36 MBq of indium chloride, ($^{111}InCl_3$, INSIP, Amersham International, U.K.) was added to a volume equivalent to 100 μg of antibody conjugate in 100 mM glycine buffer (pH 3.5). Free indium was removed from the radiolabelled antibody by means of a 1.6 cm x 20 cm Sephadex G-25 column pre-washed with 2 ml of 1% bovine serum albumin (BSA). The eluting agent was 100 mM glycine buffer (pH 3.5) containing 1% (w/v) BSA. One ml fractions of eluate were collected and the labelled antibody fraction identified by counting 10 μl aliquots with a Packard Multi-Prias autogamma counter. The radiolabelled antibody forms the first peak of radioactivity to emerge from the column. The more efficient the labelling process the less free radioisotope there will be in the eluates which follow the radiolabelled antibody fractions. This can be seen in a graph which shows the activities of the 10 μl aliquots of the different chromatography fractions of indium-111 labelled 77-1 (Figure 32) which shows little radioactivity to follow the radiolabelled antibody fraction. However, in a less efficient labelling procedure another peak of radioactivity, indicating the presence of free radioisotope, will form after the radiolabelled antibody fraction (Figure 34).

ii) ^{125}Iodine labelling

The antibodies were labelled with iodine (^{125}I IMS 30, Amersham International, U.K.) using the chloramine-T method of Greenwood et al. (1963) and iodogen method. They were dialysed against 0.1M phosphate buffer (pH 7.5) before iodination.

a Chloramine T method

A dose of 0.5 mCi of ^{125}I was added to a volume of antibody equivalent to 25 µg, followed by 50 µl of 0.4M phosphate buffer (pH7.5), and the mixture was stirred. A volume equivalent to 3.8 µg from a stock solution of 1mg/ml in water of chloramine-T (Sigma) was added and the solution mixed for a further 1 minute. The reaction was terminated with 100 µg from a stock solution of 10 mg/ml in water of sodium metabisulphite (BDH) and free iodine was then removed by gel chromatography using Sephadex G-25 loaded into a 10 ml disposable column which was pre-washed with PBS/BSA 0.5% (w/v) before adding the labelled antibody which was eluted with PBS. One ml fractions were collected and the labelled antibody fraction was identified by counting 10 µl aliquots in a gamma counter. It usually emerged at fraction four or five.

b Iodogen method

Iodogen (Sigma) was dissolved in chloroform at concentration of 12.5 mg/50ml and 100 µl of the solution was added to 2 ml plastic vials. The chloroform was evaporated with nitrogen to form a coating of iodogen on the vials. To 25 µg of antibody in an iodogen-coated vial, 0.5 mCi of ^{125}I and 45 µl of 0.4M phosphate buffer (pH7.5) was added. After 15 minutes the labelling reaction was terminated by the addition of 100 µg of sodium metabisulphite (BDH) prepared freshly at 10 mg/ml of water for one minute. The labelled antibody was separated from the free iodine using a Sephadex G25 column as above.

Ten µl of the eluate were taken from each fraction collected from the Sephadex G25 column and the radioactivity counted.

5.2.3 Cell binding assay

An initial step in this procedure was the selection of a cell line reactive with 77-1 so that the immunoreactivity of the radiolabelled 77-1 could be tested. Three colorectal cancer cell lines Colo 205, SW 320 and SW 480, and the breast carcinoma cell line MCF7 were tested for reactivity with 77-1 by means of flow cytometry using the method described in 3.2.3.iii.

The selected cells were grown in monolayer tissue culture using 5% $C0_2$ buffered DMEM supplemented with 10% (v/v) FCS. Cells from stock culture were harvested using trypsin/versene and plated at approximately 10^4 cells/well into a 24 well polystyrene culture plate (Costar U.K.) in 2 ml medium and maintained in an incubator at 37°C in a humid atmosphere of 5% CO_2/air until the wells were 80% confluent.

The wells were washed once with fresh medium. Four different concentrations of radiolabelled antibodies were added to the wells in duplicate. A sample of radiolabel for each of the different concentrations added was kept as a standard for calculation of the cell binding activity of the radiolabelled antibodies. The plate was kept at 4°C for 1 hour on a rocking platform, following which the cells were washed three times with 1 ml of PBS. They were then lysed with 0.5 ml of 1M NaOH added to each well and left for five minutes. This procedure was repeated to ensure the complete removal of radioactivity from the cells. The lysates, which contained the bound radioactivity, were counted in a gamma-counter. The results were plotted as a graph and the linear part of the slope was used to estimate the binding activity of the radiolabelled antibodies with the cells (i.e. cell binding activity = radioactivity bound to the cells / radioactivity added to the cells x 100%). The counts bound in the presence of an excess of unlabelled antibody (in a competitive assay) were determined. Finally, the binding

results obtained with a control antibody were also estimated.

5.2.4 Xenograft localisation

i Preparation of xenograft

CBA/Ca Ola mice (Olac 1976 Ltd., Oxon., U.K.) were rendered immune-deprived by thymectomy and total body irradiation as described by Castro (1972), Cobb et al. (1973) and Steele et al., (1978). Ether-anaesthetised mice (at 6 to 8 weeks) were thymectomised using a modified Pasteur pipette. This was attached to a tap suction pump via a glass collecting jar and was inserted into the mediastinum, via a midline division of the sternum, to aspirate the two lobes of the thymus. The wound was closed with a Michel clip. The animals were given acid water (addition of HCl to pH 2.8) and Mouse Number 1 modified diet (Lillico and Son Ltd., Surrey, U.K.) ad libitum, and were grown to a weight of 20 gm which usually took a further 6 weeks. They were then injected intraperitoneally with 0.2 mg/g body weight of Cytosar (Upjohn) three days before being irradiated with 900 rads with a ^{60}Cobalt source. The localisation of the iodine-125 labelled 77-1 was performed using the xenografts in these thymectomised mice. However, an outbreak of mouse hepatitis prevented their use for the indium-111 labelled 77-1 study. Nude mice had to be used instead. They were the MF1-nu/Ola strain (Olac 1976 Ltd., Oxon., U.K.) and were used without treatment at 6 weeks of age.

Cell lines may not necessarily grow in immune suppressed mice. Two colorectal cancer xenografts, MAWI and Taf (from the Department of Oncology, Charing Cross Hospital), and two xenografts which react with M8 - a breast cancer xenograft HX99 and a renal carcinoma xenograft XK1 (from the Institute of Cancer Research) were tested for reactivity with 77-1. An appropriate xenograft

was selected on the basis of its reaction with 77-1 using the immunoperoxidase method described in 3.2.2.iv.d.

The animals were anaesthetised with ether and the selected tumour implanted subcutaneously into the both flanks, using freshly-removed tumours which had been cut into 3 mm^2 pieces, and the wounds were closed with Michel clips.

ii Radiolocalisation of xenograft

The animals were selected for experiments when the xenografts were about 3-7mm in diameter (50-200mg). Radiolabelled antibodies (10-20 μCi, 2-5 μg) were injected intraperitoneally into groups of animals, each consisting of 4 mice. The groups were sacrificed at 24 and 48 hours for the indium-111 labelled 77-1 and also up to 72 hours for the iodine-125 labelled antibody. A radiolabelled control antibody was administered to comparable groups of animals. The animals were anaesthetised with ether and blood samples were collected by cardiac puncture. They were sacrified by exsanguination. The tumours together with specimens of skin, muscle, lung, heart, small intestine, colon, bone, brain, kidney, liver, spleen, pancreas and fat were removed from the animals, weighed, and counted in a Packard Multi-Prias autogamma counter.

5.3 RESULTS

5.3.1 Production and radiolabelling of monoclonal antibody

The concentration of monoclonal antibody extracted from the Protein-A column was between 0.5 to 1 mg/ml and the antibody remained immunoreactive after storage at 4°C for 6 months. A typical spectrometry tracing showing the elution of 77-1 at pH 4.5 from the Protein-A column is shown in Figure 31. The antibody-DTPA conjugation process started with 20 mg of monoclonal antibody in 1 ml and gave a yield of 5 mg of DTPA-conjugate or 20-30% of the initial amount of antibody.

The monoclonal antibody 48-1 was selected as a control antibody in the *in vitro* and *in vivo* tesing of the radiolabelled 77-1 because it did not react with MCF7 on flow cytometry or the xenograft XK1 on immunocytochemistry (5.3.2 and 5.3.3).

The labelling efficiency of indium-111 was high as most of the activity was taken up by the antibody fraction. Figure 32 shows that there is little radioactivity in the fractions which follow the radiolabelled 77-1 (fraction five), indicating that the majority of the indium has bound to 77-1-DTPA conjugate.

The comparison between the iodogen and chloramine T methods showed that the iodogen technique was the more efficient iodination procedure as most of the radioactivity was taken by the antibody (compare Figure 33 with Figure 34). There is little free iodine to follow the I-125 77-1 fraction (fraction 5, Figure 33) after labelling with the iodogen technique, but a larger amount of free iodine can be seen to emerge after the radiolabelled antibody fractions (fractions 5 and 6, Figure 34) in the chloramine T procedure.

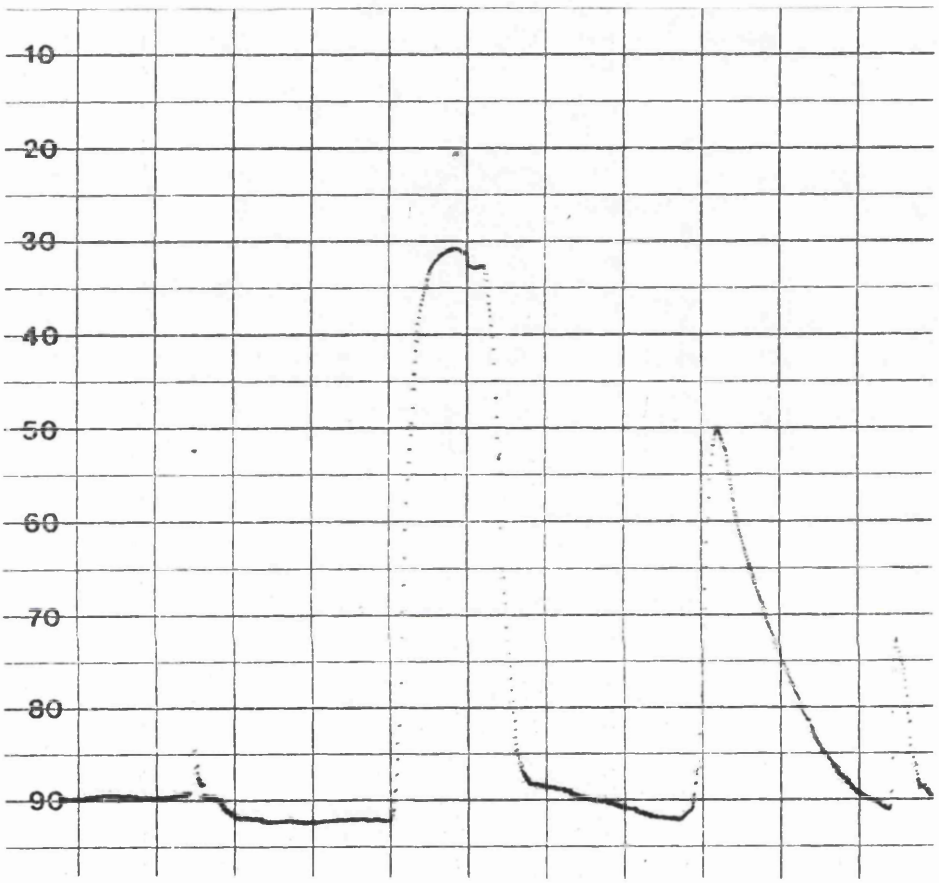


Figure 31 Protein A sepharose elution of 77-1 at pH 4.5. The first peak represents the protein solution loaded into the column. The second peak is the 77-1 fraction eluted at pH 4.5 and the third peak is eluted at pH 2.8 and contains other immunoglobulins

Figure 32 G-25 chromatography profile of 77-1 after labelling with indium-111 demonstrating an efficient labelling as most radioactivity is present in the antibody fraction in number 5

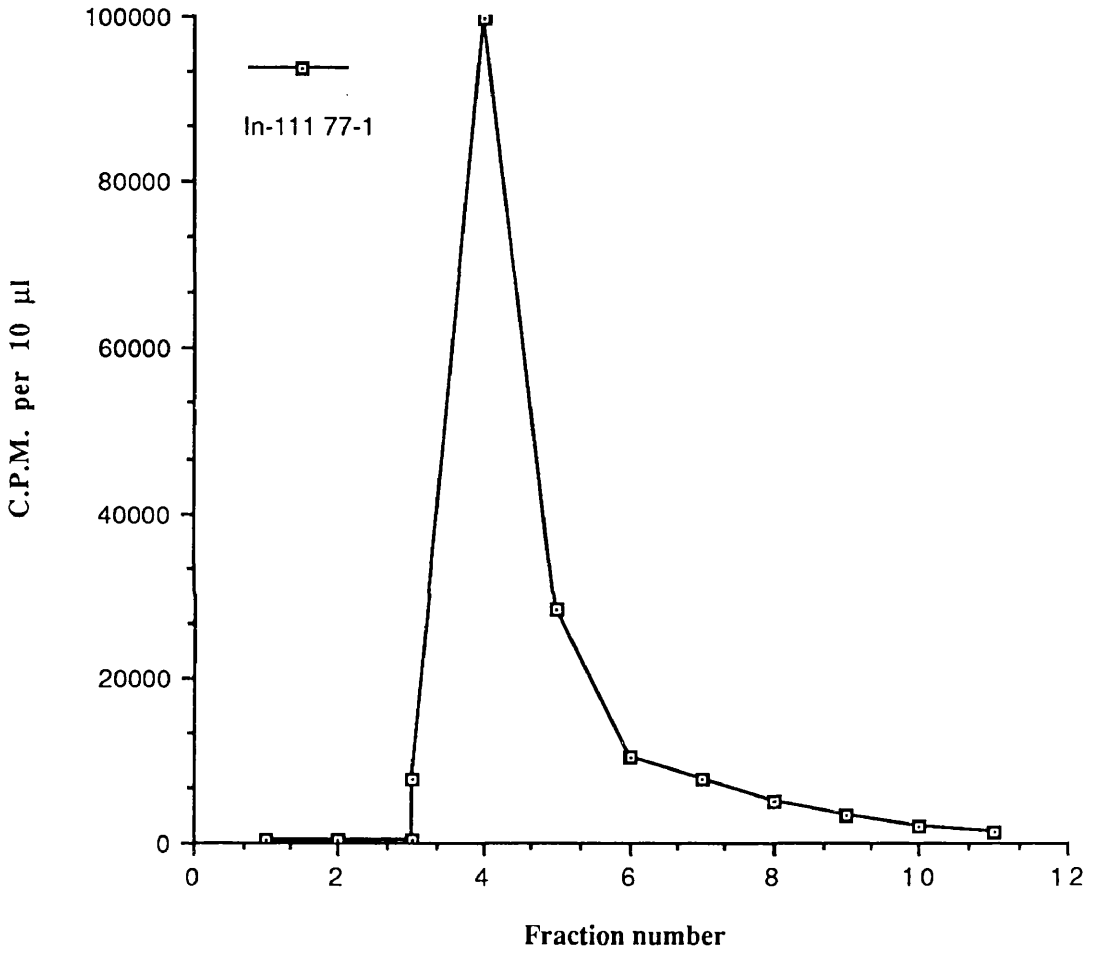


Figure 33 G-25 chromatography profile of 77-1 after labelling with iodine-125 with the iodogen method showing an efficient labelling as most of the radioactivity is taken up by the antibody fraction in number 5

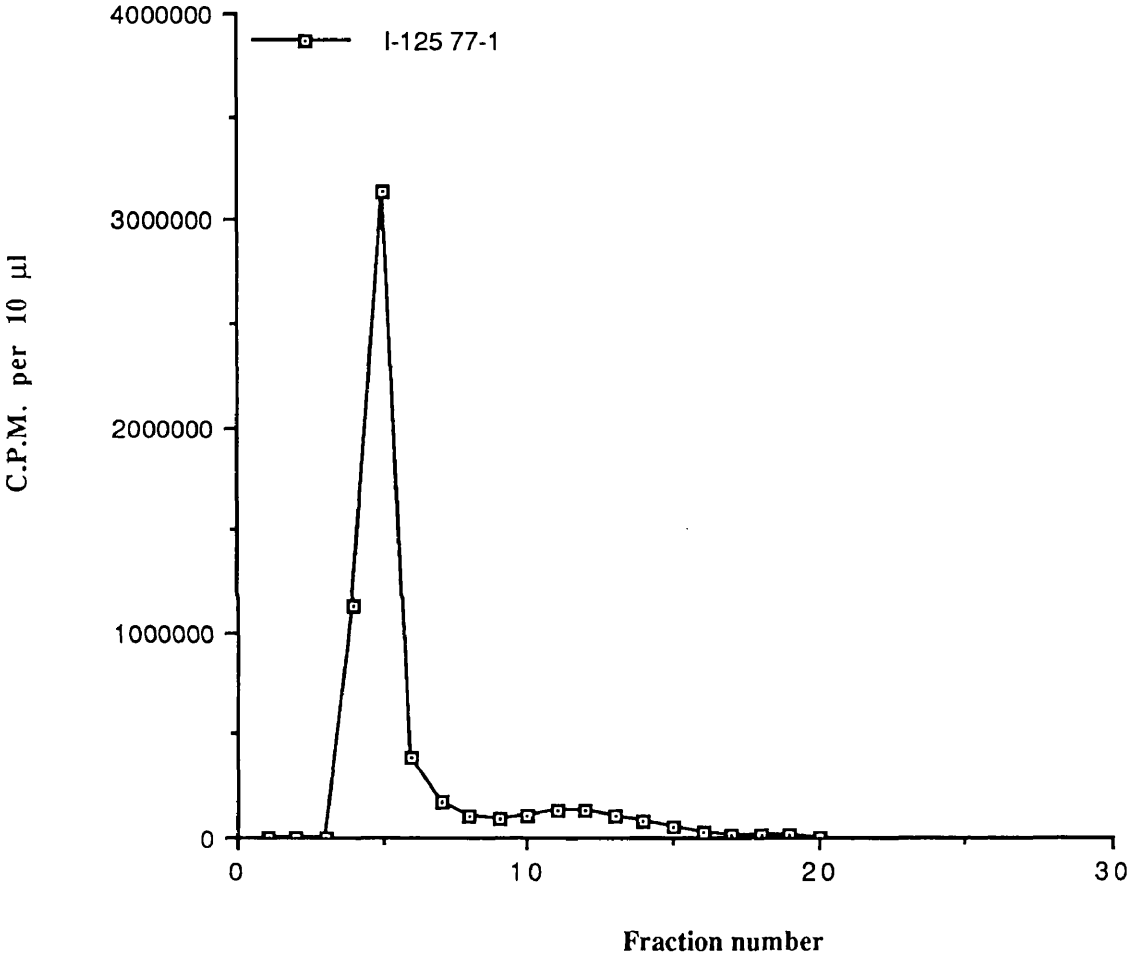
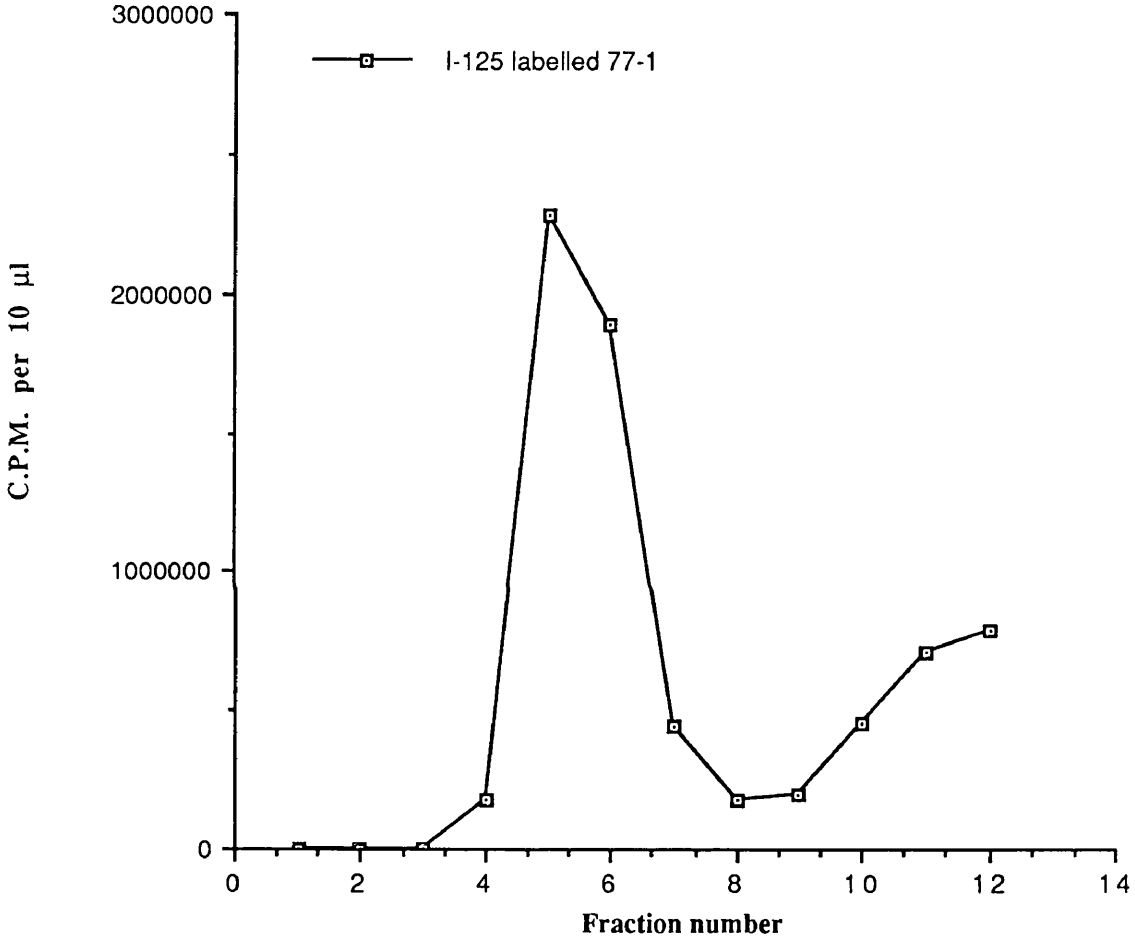


Figure 34 G-25 chromatography profile of 77-1 after labelling with iodine-125 with the chloramine T method. Fraction number 5 represents the antibody fraction and the radioactivity from fraction number 9 onwards is due to free iodine-125 not used in the radiolabelling process, indicating a less efficient radiolabelling than the iodogen method



5.3.2 Cell binding assay

Of the four cell lines tested for reactivity with 77-1 by means of flow cytometry only MCF7 gave a positive reaction. Figure 35 shows the binding between 77-1 and MCF7 cells as illustrated by the fluorescence activity in the horizontal axis. No activity was detected in the control when 77-1 was not added (no first antibody). The monoclonal antibody UC-ICR 20.3 acts as a positive control as it also reacts with MCF7 cells (3.3.4.vi). MCF7 was therefore selected for testing the immunoreactivity of the radiolabelled 77-1.

When the iodine-125 labelled 77-1 was incubated with monolayers of MCF7, a binding of up to 18% was obtained (Figure 36). The same cell binding results were obtained with either the chloramine T or the iodogen method, although the iodogen method was more efficient (5.3.1). The iodogen method was therefore used for the 125-I labelling of 77-1 for localisation studies. The cell binding results obtained with the indium-111 labelled 77-1 were generally lower than the iodinated 77-1 averaging about 8-12%. When a 30 fold excess of unlabelled 77-1 was added, the cell binding was depressed by 10 fold to less than 1% (Figure 37). The control radiolabelled antibody (48-1) also gave a binding of less than 1%. Thus labelling with iodine-125 and indium-111 routinely yielded the antibody 77-1 which showed specific binding to a reactive cell-line.

5.3.3 Xenograft localisation

Immunoperoxidase staining showed that there was no reaction between 77-1 and the two colorectal cancer xenografts MAWI and Taf. However, 77-1 reacted with the xenografts HX99 and XK1. Figure 38 shows the membrane reaction between 77-1 and XK1. The XK1 xenograft was selected for the *in vivo* immunoreactivity study of the radiolabelled 77-1.

Figure 35 Flow cytometry showing the binding between 77-1 and MCF7 cells as illustrated by the fluorescence activity in the horizontal axis. UC-ICR 20.3 acts as a positive control. There was no fluorescence activity when no first antibody was added

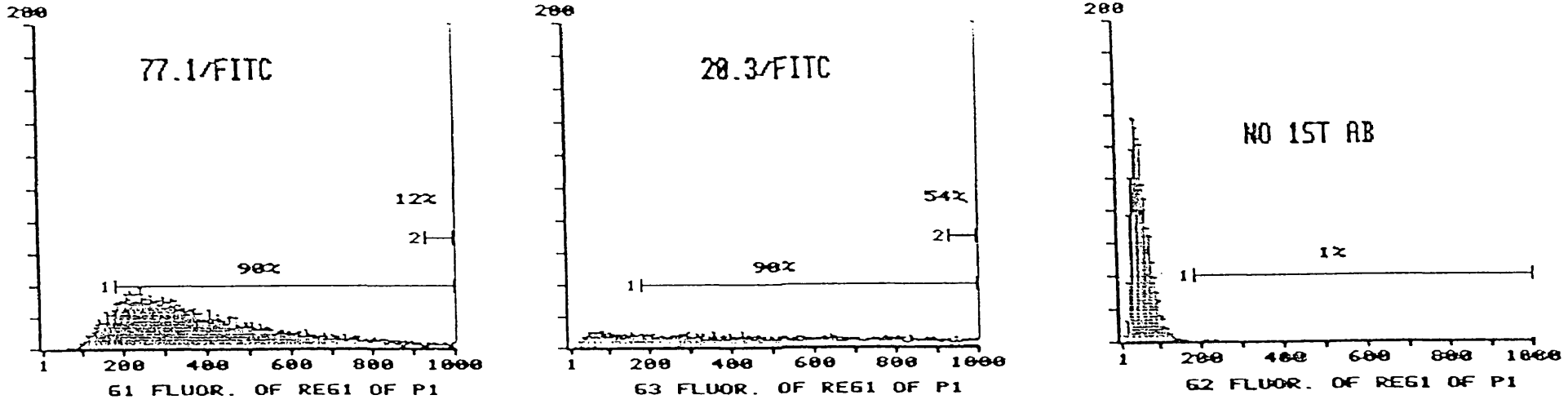


Figure 36 The binding of I-125 labelled 77-1 with MCF7 cells

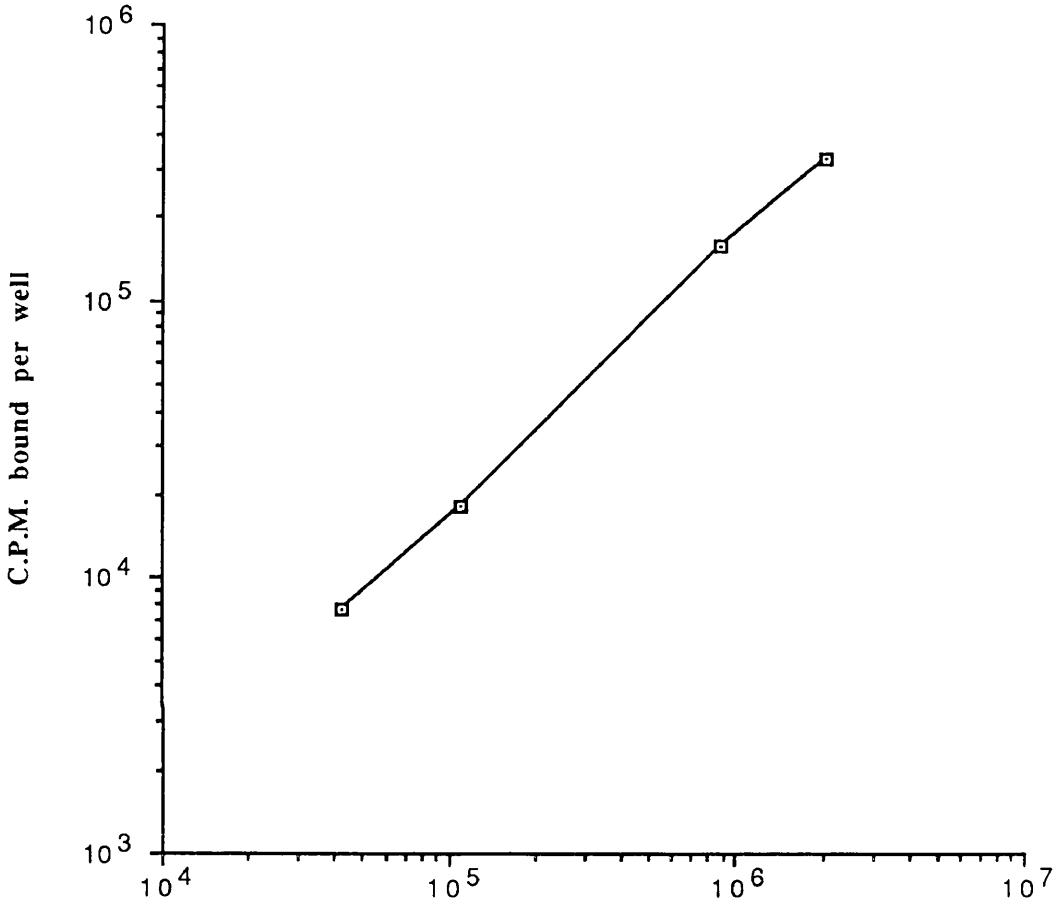
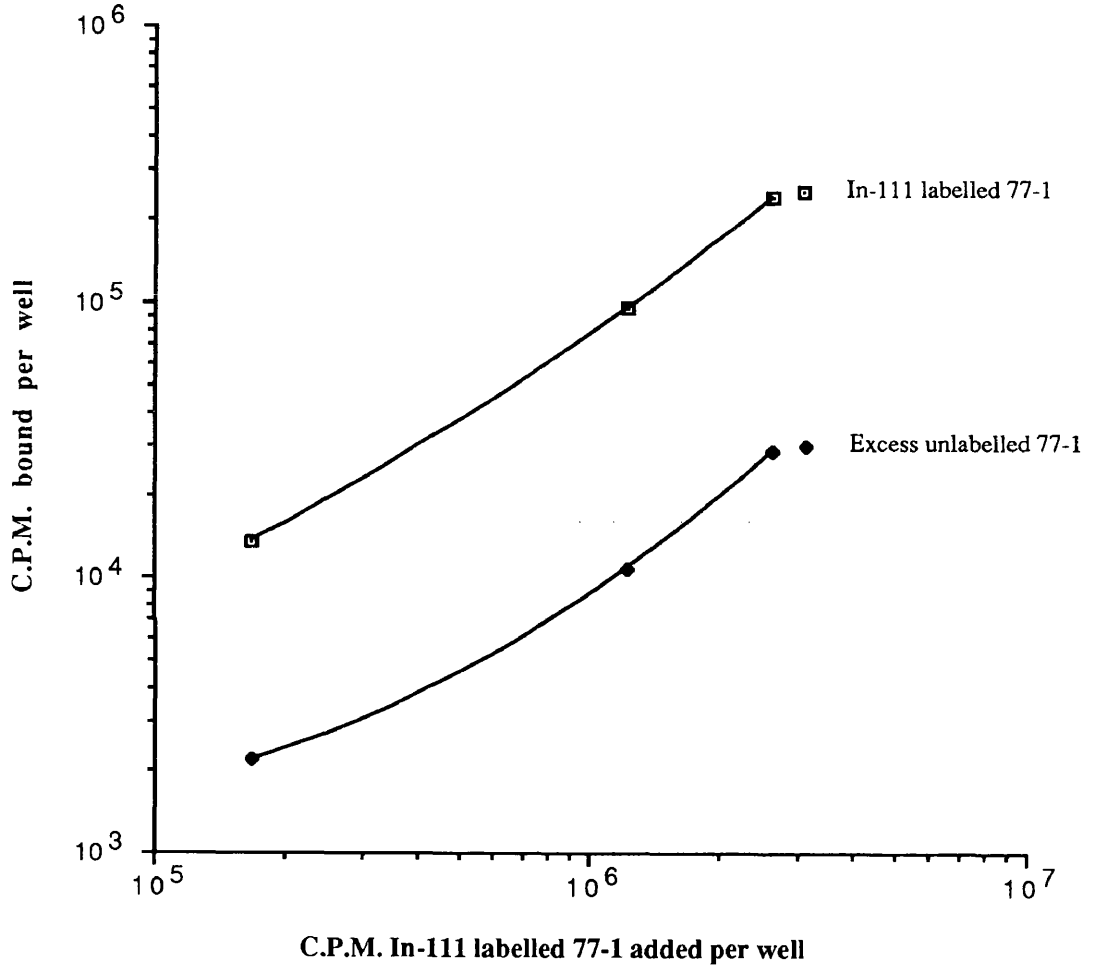


Figure 37 The binding of In-111 labelled 77-1 with MCF7 cells and the effect of the addition of excess unlabelled 77-1



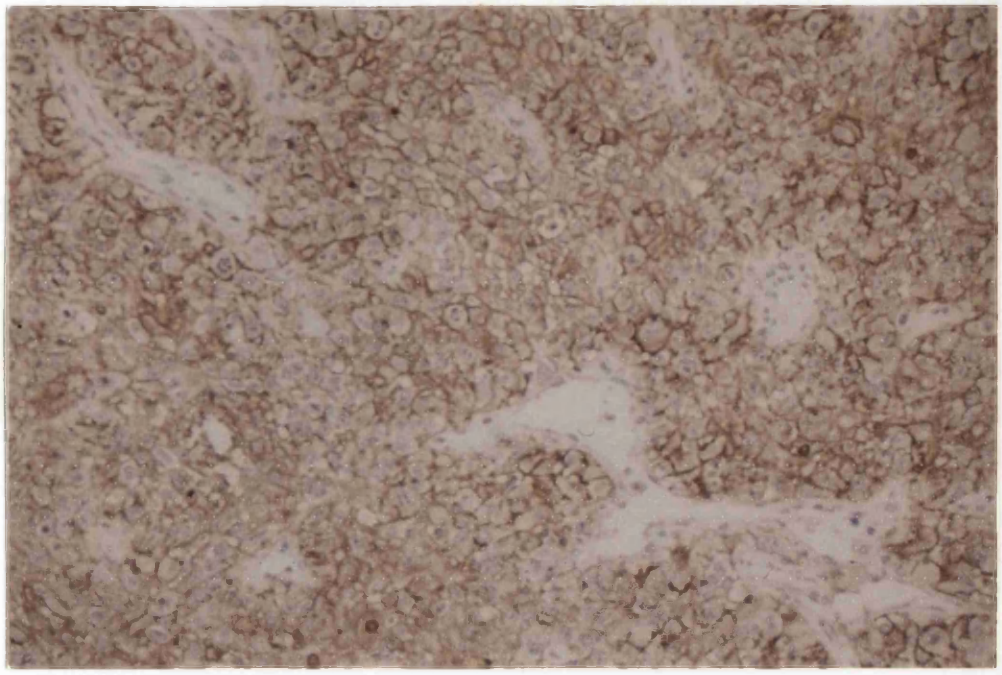


Figure 38 **Immunoperoxidase staining of the xenograft XK1 with 77-1 showing membrane reactivity x 120**

i ^{125}Iodine labelled 77-1 localisation

Two to three weeks after subcutaneous implantation, the xenografts reached 200-300 mg and were ready for the antibody localisation study. Cystic changes soon developed in the centres of the tumours from the third week onwards. The grafts usually took on both the implantation sites. The failure rate of graft implantation was 5-10% of the total.

The mean tumour to blood ratios with the iodine-125 labelled 77-1 on XK1 xenografts (four animals / group / time point) at 24, 48 and 72 hours were 1.37 $\pm$ 0.095, 1.875 $\pm$ 0.525 and 2.375 $\pm$ 0.350 respectively (Table 10). The corresponding results for the radiolabelled control antibody 48-1 were 0.405 $\pm$ 0.087, 0.49 $\pm$ 0.064 and 0.53 $\pm$ 0.061 respectively (Table 11). Statistical analysis by means of the Student's unpaired 't' test showed a significantly higher uptake of the radiolabelled 77-1 to XK1, with 'p' values of less than 0.002 for all time points (Appendix 1). The results indicated that iodine-125 labelled 77-1 localised specifically to the tumour.

With regards to other tissues, in the groups of animals administered iodine-125 labelled 77-1, the various normal tissue to blood ratios were all below 0.4 at 24 and 48 hours and below 0.6 at 72 hours (Table 10). Statistical analysis by means of the paired 't' test showed that the uptake of the radiolabelled 77-1 was higher in the tumours than all normal tissues at all time points, with 'p' values of less than 0.012 (Appendix 1).

In the animals injected with control antibody, the tumour uptake of the iodine-125 labelled 48-1 at 24 hours was higher in the tumour compared with some tissues, but not in others (lungs, colon, kidneys, spleen, pancreas and fat). The uptake of 48-1 by the spleen at 24 hours was much higher than all other tissues (spleen/blood ratio of 1.812 $\pm$ 1.043). However, at 48 and 72 hours the

radioactivity in the spleen was much lower. At 48 and 72 hours the tumour to blood ratios of 48-1 remained about the same as were the radiolabels in other tissues, which remained lower than that of the tumour (compare Tables 10 and 11).

The relative localisations of iodine-125 labelled 77-1 and 48-1 to XK1 and other tissues at 24, 48 and 72 hours are shown in Figures 38, 39 and 40 respectively. They show that while the tumour to blood ratios in animals injected with ^{125}I-48-1 remained about the same with time, the tumour to blood ratios in the groups of animals injected with ^{125}I-77-1 continued to increase with time, achieving a tumour radiolabel uptake significantly higher than any other tissues.

ii ^{111}Indium labelled 77-1 localisation

In the groups of animals administered indium-111 labelled 77-1, the mean tumour to blood ratios at 24 and 48 hours were 3.418 $\pm$ 0.970 and 7.140 $\pm$ 3.628 respectively (Table 12). The corresponding results for the radiolabelled control 48-1 were 0.873 $\pm$ 0.137 and 1.255 $\pm$ 0.372 respectively (Table 13). The tumour uptake of indium-111 labelled 77-1 was significantly higher than the control antibody 48-1. Statistical analysis of the results with the unpaired 't' test gave 'p' values of 0.002 and 0.018 for the two time points, indicating that specific localisation of indium-111 labelled 77-1 to the tumour had occurred.

With regards to indium-111 labelled 77-1 uptake by other organs at 24 hours, no significant difference was observed between the tumour and the following tissues : muscle, kidneys, spleen, pancreas and fat. However, at 48 hours the uptake by the tumours was significantly higher than all organs, except the kidney and fat.

In the control groups of animals the uptake of indium-111 labelled 48-1

was not significantly different to the muscle, kidneys, spleen pancreas and fat at 24 hours. By 48 hours the radiolabels in most organs fell below that of the tumours with the exception of the kidneys (Table 12 and 13).

The relative localisations of indium-111 labelled 77-1 and 48-1 to XK1 and other tissues at 24 and 48 hours are shown in Figures 41 and 42 respectively. They show that, similar to the iodine-125 localisation study, the tumour to blood ratios in the groups of animals injected with indium-111 labelled 48-1 did not increase with time to the same extent as those animals injected with indium-111 labelled 77-1, which achieved a significantly higher uptake in the tumours at all time points.

Table 10 **The mean tumour / organ to blood ratios of iodine-125 labelled 77-1 on XK1 at 24 / 48 and 72 hours**

	24 hours	*48 hours*	*72 hours*
Tumour	1.37±0.095	1.875±0.525	2.375±0.35
Skin	0.218±0.189	0.39±0.078	0.373±0.18
Muscle	0.145±0.105	0.12±0.029	0.228±0.185
Lung	0.25±0.109	0.12±0.029	0.295±0.104
Heart	0.287±0.025	0.293±0.075	0.252±0.039
SI	0.16±0.05	0.147±0.021	0.148±0.088
Colon	0.11±0.065	0.14±0.023	0.129±0.086
Bone	0.123±0.084	0.138±0.017	0.18±0.047
Brain	0.025±0.01	0.023±0.005	0.03±0.00
Kidney	0.305±0.067	0.302±0.015	0.32±0.059
Liver	0.26±0.085	0.275±0.026	0.465±0.271
Spleen	0.343±0.181	0.325±0.128	0.425±0.204
Pancreas	0.225±0.205	0.197±0.041	0.168±0.031
Fat	0.055±0.11	0.363±0.374	0.215±0.093

Table 11 **The mean tumour / organ to blood ratios of iodine-125 labelled 48-1 on XK1 at 24 / 48 and 72 hours**

	24 hours	*48 hours*	*72 hours*
Tumour	0.405±0.087	0.49±0.064	0.53±0.061
Skin	0.42±0.09	0.303±0.046	0.32±0.014
Muscle	0.14±0.038	0.11±0.018	0.148±0.041
Lung	0.34±0.071	0.37±0.071	0.4±0.048
Heart	0.22±0.022	0.228±0.025	0.22±0.014
SI	0.128±0.085	0.192±0.059	0.157±0.029
Colon	0.198±0.13	0.14±0.022	0.13±0.026
Bone	0.11±0.029	0.127±0.046	0.14±0.00
Brain	0.04±0.027	0.04±0.014	0.11±0.16
Kidney	0.243±0.142	0.337±0.034	0.325±0.017
Liver	0.045±0.044	0.245±0.031	0.265±0.042
Spleen	1.812 ±1.043	0.293±0.158	0.227±0.055
Pancreas	0.465±0.364	0.198±0.036	0.157±0.035
Fat	0.407±0.118	0.265±0.142	0.135±0.033

Figure 39 Relative localisation of I-125 labelled 77-1 and 48-1 to xenograft XK1 and tissues at 24 hours

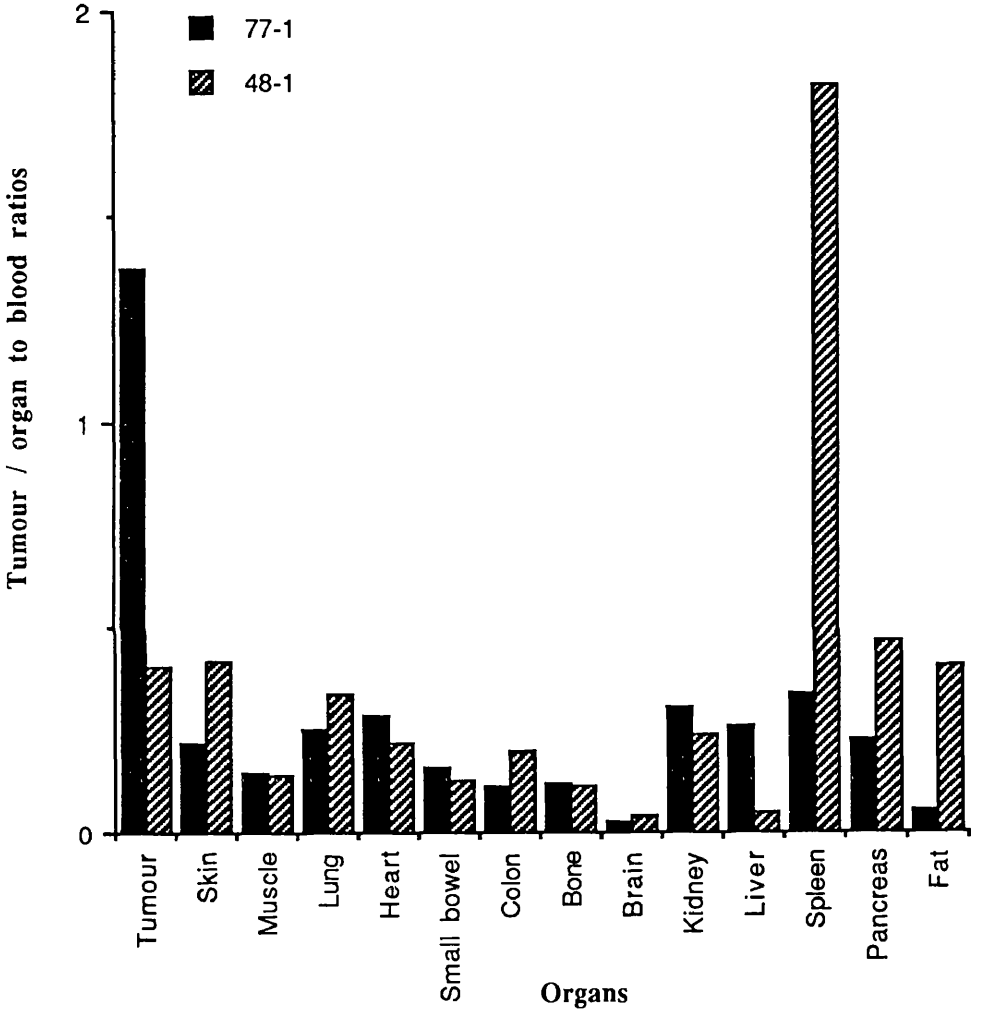


Figure 40 Relative localisation of I-125 labelled 77-1 and 48-1 to xenograft XK1 and tissues at 48 hours

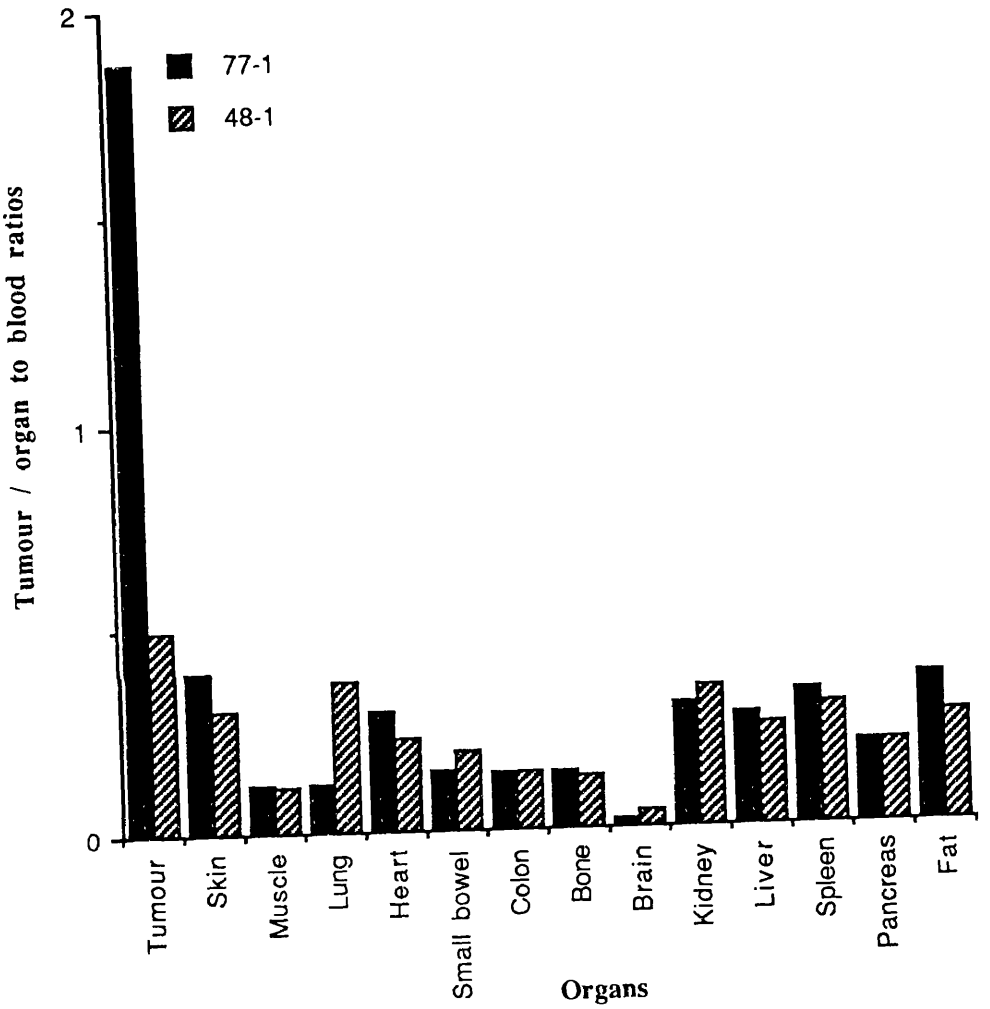


Figure 41 Relative localisation of I-125 labelled 77-1 and 48-1 to xenograft XK1 and tissues at 72 hours

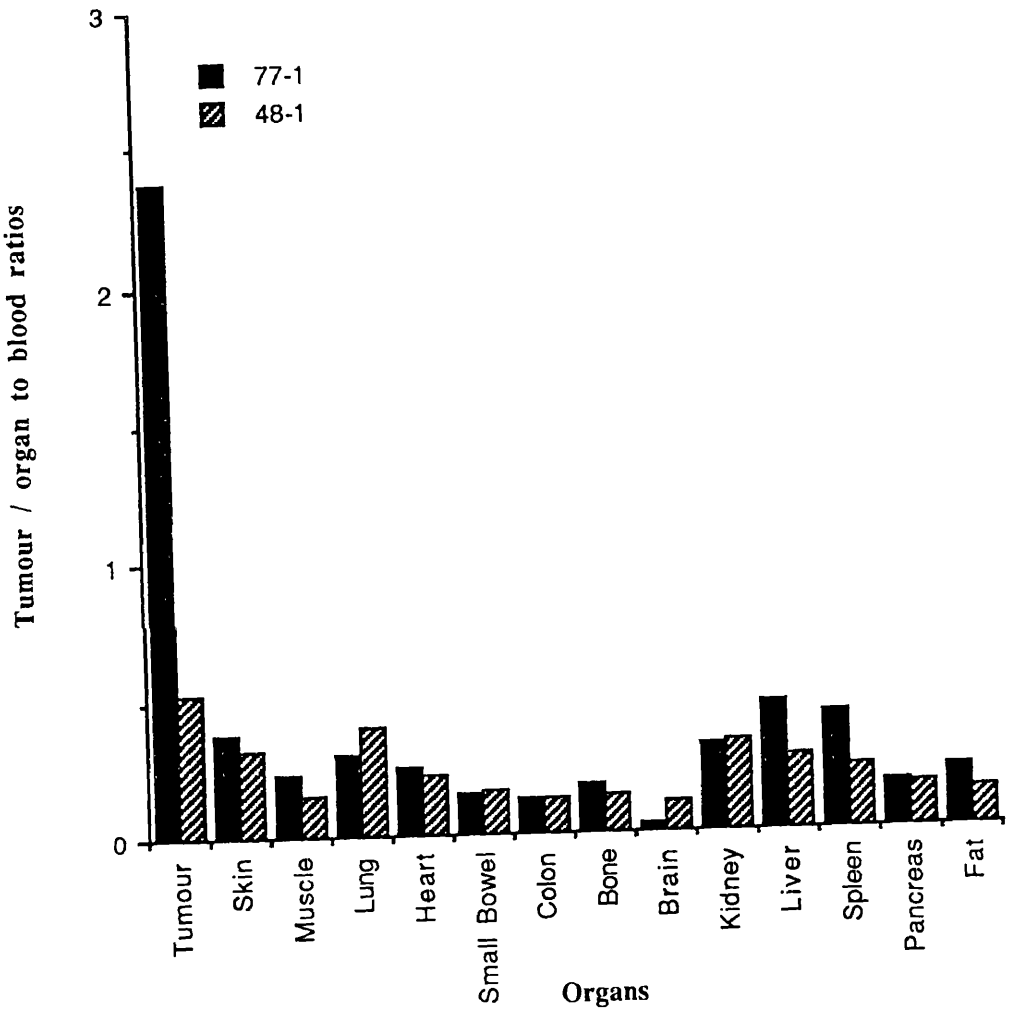


Table 12 The mean tumour / organ to blood ratios of indium-111 labelled 77-1 ON XK1 at 24 and 48 hours

	24 hours	*48 hours*
Tumour	3.418±0.97	7.14±3.628
Skin	0.603±0.178	0.962±0.196
Muscle	1.105±1.045	0.525±0.214
Lung	0.538±0.095	0.683±0.067
Heart	0.325±0.194	0.435±0.088
SI	0.655±0.222	0.765±0.199
Colon	0.748±0.34	0.752±0.543
Bone	0.465±0.185	0.507±0.117
Brain	0.045±0.01	0.06±0.00
Kidney	1.567±0.515	2.395±0.429
Liver	1.070±0.488	1.535±0.519
Spleen	1.117±0.972	1.793±1.161
Pancreas	2.013±2.193	1.117±0.403
Fat	1.478±0.986	1.35±1.049

Table 13 The mean tumour / organ to blood ratios of indium-111 labelled 48-1 on XK1 at 24 and 48 hours

	24 hours	*48 hours*
Tumour	0.873±0.137	1.255±0.372
Skin	0.545±0.148	0.732±0.147
Muscle	0.145±0.006	0.275±0.211
Lung	0.425±0.12	0.543±0.095
Heart	0.25±0.079	0.34±0.048
SI	0.325±0.103	0.435±0.117
Colon	0.27±0.078	0.398±0.059
Bone	0.238±0.06	0.295±0.1
Brain	0.035±0.01	0.05±0.029
Kidney	1.775±0.465	2.37±0.51
Liver	0.473±0.071	0.743±0.365
Spleen	0.55±0.145	0.92±0.469
Pancreas	0.515±0.108	0.615±0.289
Fat	0.532±0.222	0.848±0.244

Figure 42 Relative localisation of In-111 labelled 77-1 and 48-1 to xenograft XK1 and tissues at 24 hours

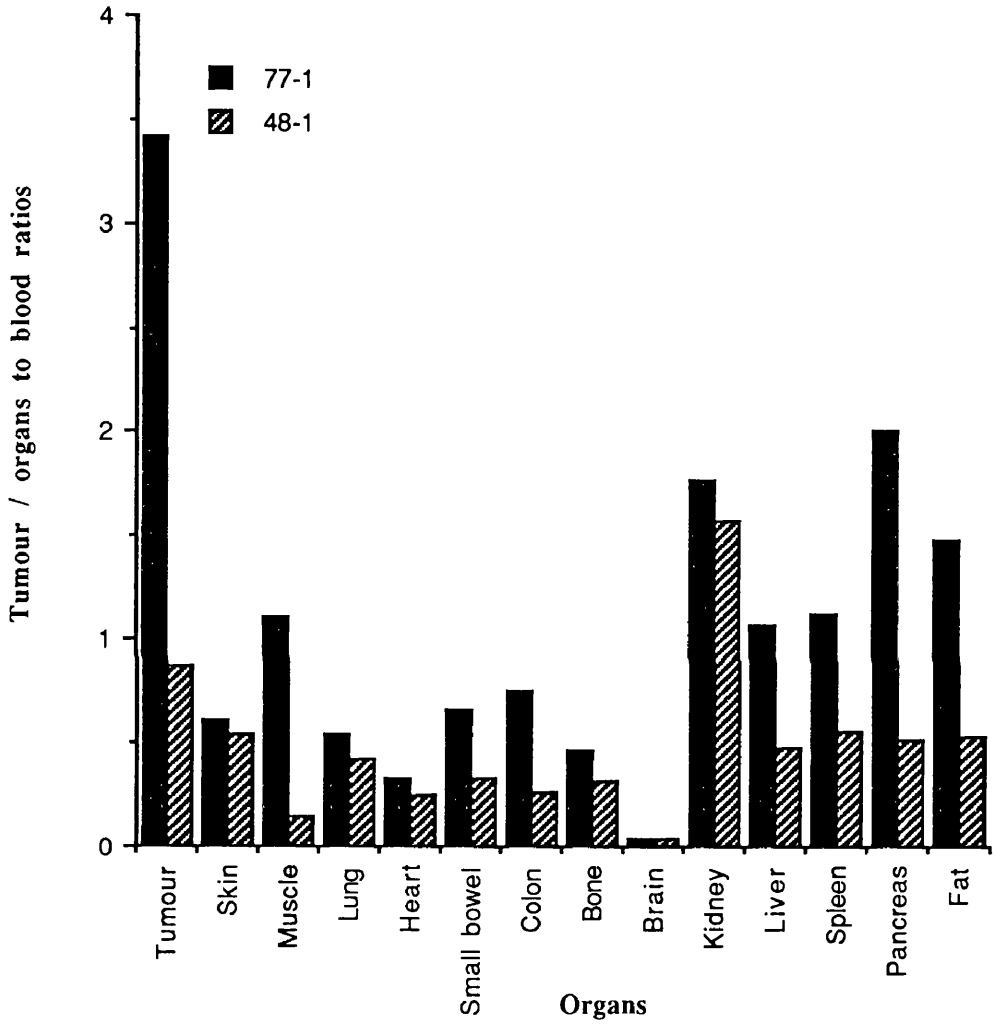
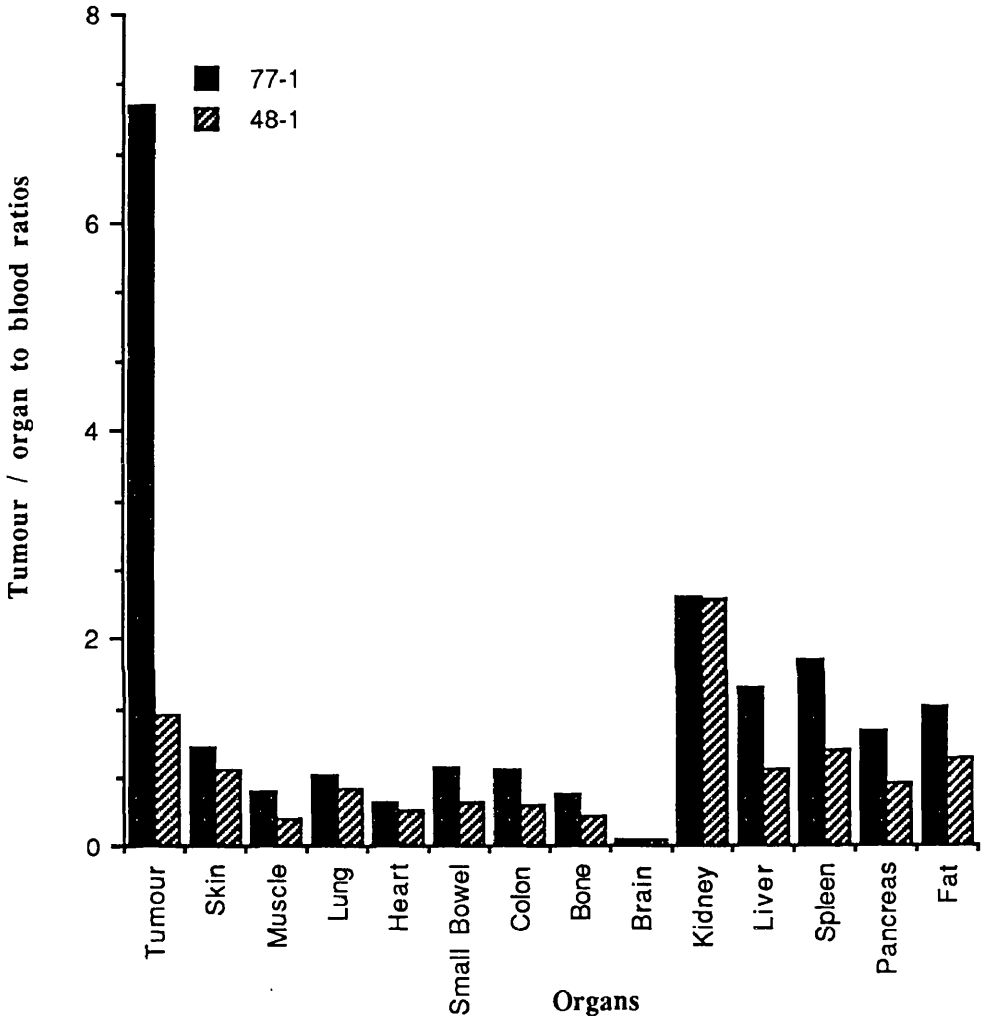


Figure 43 Relative localisation of In-111 labelled 77-1 and 48-1 to xenograft XK1 and tissues at 48 hours



5.4 DISCUSSION

Monoclonal antibodies produced as ascites in the mouse were used in this study as the amount of reagent obtained was much greater than using the tissue culture method. The concentration of antibody secreted in tissue culture is about 10 μg/ml compared with 0.2-1 mg/ml in ascites. An advantage of antibody production in tissue culture is a purer preparation, as antibodies produced as ascites may contain other mouse immunoglobulins. However, the extraction of antibodies with the Protein A-Sepharose from ascitic fluid was satisfactory as shown by the *in vitro* and *in vivo* immunoreactivity of the radiolabelled antibodies.

In the preparation of the monoclonal antibody 77-1 for the immunoscintigraphy of colorectal cancer a cell binding asssay after radiolabelling with indium-111 and iodine-125 was necessary to determine if the immunoreactivity of the antibody was damaged by the labelling process. For iodination, the chloramine T and the iodogen methods were also compared. The labelling efficiency of the iodogen method was better than the chloramine T method, although there was no difference in the immunoreactivity of the radiodinated antibody.

The breast carcinoma cell line MCF7 was used in the cell binding assay to test the activity of the radiolabelled antibody because the surface antigen was recognised by the monoclonal antibody 77-1. None of the colorectal cancer cell lines tested reacted with this antibody. MCF7 was originally used to test the immunoreactivity of the monoclonal antibody M8 (Rainsbury, 1984). As the monoclonal antibodies M8 and 77-1 both recognise epitopes of epithelial membrane antigen (EMA) (McIlhinney et al., 1985; Summerhayes et al., 1985), it was not surprising that they both reacted with MCF7. In the testing of the

immunoreactivity of radiolabelled antibodies, antigens extracted from cells (Colcher et al., 1984) or tumour, such as CEA (Halpern et al., 1983) have been used in other studies. Used in this context, a colon cancer cell line would not have any advantage over MCF7 in evaluating the immunoreactivity of the radiolabelled 77-1 as the reactivity of 77-1 with colorectal cancer *in vitro* had already been demonstrated by immunocytochemistry (Chapter 4). Its failure to react with cultured colonic tumour cells may reflect changes in antigen expression caused by *in vitro* culture.

The cell binding assay showed that the iodine-125 labelled 77-1 achieved a higher binding than the indium-111 labelled 77-1 with the results of 18% and 8% respectively. This indicated that the iodination process had a less adverse effect on the immunoreactivity of 77-1 than the indium labelling procedure. The reaction between the indium-111 labelled 77-1 and MCF7 was, however, specific as this was confirmed by the competitive cell binding assay when excess unlabelled 77-1 depressed the binding to 0.8%.

Xenograft models are used to provide comparative information on the localisation and binding characteristics of different antibodies. Xenografts are more homogeneous than the corresponding tumours in man because of a process of selection in serial transplantation. Furthermore, their growth pattern and blood supply are highly artificial compared to the autochthonous situation. The main factor in the choice of a xenograft for antibody localisation study is the expression of an antigen recognised by the antibody and not the tissue type. XK1, a xenograft originated from a hypernephroma was used for this purpose as immunocytochemistry demonstrated its reaction with the monoclonal antibody 77-1. The colorectal cancer xenografts tested were, like the cell lines *in vivo*, unreactive with the antibody. It is possible that a colorectal xenograft reactive with

77-1 could be found. However, such a xenograft would also express similar antigen as XK1, and would therefore not produce additional information about the immunoreactivity of the radiolabelled 77-1. In the selection of cell lines and xenografts for investigating the cytotoxic effects of an IgG2a monoclonal antibody L6, cell lines originated from different tissues have been used. The choice was determined by the expression of the target antigens as in the present study, rather than as being of a specific tissue type (Hellstrom et al., 1986).

The tumours were implanted subcutaneously in these studies, and the radiolabelled antibodies injected intraperitoneally. The studies by Rainsbury (1984) indicated that the uptake of antibodies by xenografts was not influenced by their implantation sites or by either the intravenous or intraperitoneal route of antibody administration. The tumour to blood ratios of the iodine-125 labelled 77-1 obtained at 24, 48 and 72 hours were similar to those achieved by M8 (Rainsbury, 1984). A similar ratio was obtained by the monoclonal antibody 791T/36 labelled with 131-iodine which gave a tumour to blood ratio of 2.6 at day three (Pimm & Baldwin, 1985). The uptake of the iodine-125 labelled 77-1 by the tumours was higher than all other tissues at all time points and continued to increase with time. In contrast, the uptake of the radioiodinated 48-1 by the tumours remained about the same with time, and they were significantly lower than the corresponding tumour to blood ratios achieved by ^{125}I labelled 77-1. The results showed that specific localisation of ^{125}I labelled 77-1 had occurred in the tumours, and confirmed that the iodinated 77-1 retained its immunoreactivity *in vivo*.

In the indium-111 labelled antibody localisation study, the uptake of the indium-111 labelled 77-1 by the tumours at 48 hours was significantly higher than all organs except the kidneys and fat, indicating that any non-specifc antibody localisation to other tissues at 24 hours was temporary. Furthermore, unlike the

localisation of 77-1 to the various tissues, the tumour to blood ratio continued to increase with time. The uptake of indium-111 labelled 77-1 by the kidneys might be a reflection of renal excretion of the antibody, as there was also a high radiolabel uptake by the kidneys in the animals injected with the control antibody. The high radioactivity in the fat at 48 hours was distorted by a disproportionately high reading obtained from one of the four animals in the group. The fat to blood ratio in this mouse was 2.92 whereas the other three were between 0.75 to 0.93 (Appendix 1). In the control study, the localisation of indium-111 labelled 48-1 to the tumours was significantly lower than those achieved by indium-111 labelled 77-1 at both 24 and 48 hours. The results indicated that the radiolabelled 77-1 localised to the tumours specifically, and that the indium-111 labelled 77-1 retained its immunoreactivity *in vivo*.

The tumour to blood ratio was used to determine the localisation of the radiolabelled 77-1 to the xenograft in this study. Two other measurements are commonly used in antibody localisation study. One is the localisation index. In this method two different radioisotopes, usually iodine-131 and iodine-125, are used to label the test and the control antibodies so that their localisation to the tumour can be measured simultaneously rather than separately as in the present study. The tumour to blood ratio of the test antibody is then expressed as a ratio to that of the tumour to blood ratio of the control antibody (Moshakis et al., 1981c). A ratio of one or less indicates that no specific localisation has occurred. The advantages of this method is that it eliminates variation between individual animals and groups and it is particularly relevant if only a limited measurements can be taken in a small number of subjects as explained by Pressman et al. (1957) when they originally developed the paired-labelled radioisotope localisation method. However, in Pressman's earlier antibody localisation studies, as in the present study, two

groups of animals were used. One group of animals was injected with the specific radiolabelled antisera and the other group with a non-specific immunoglobulin. Specific localisation was demonstrated by the higher uptake of the radiolabelled antisera in the Wagner osteogenic sarcoma and Murphy lymphosarcoma of rats when compared to the the animals injected with the non-specific immunoglobulins (Pressman and Korngold, 1953; Korngold and Pressman, 1954). A similar higher localisation of radiolabelled 77-1 to XK1 was found in this study when compared with the non-specific radiolabelled 48-1, confirming the specific localisation of the radiolabelled 77-1 to the xenografts. Another measurement of antibody localisation to tumour is the percentage of the injected dose uptake per gram of tissue. This measurement is more useful for calculating the optimal dose of an antibody for delivery to a tumour such as in therapy then for testing the immunoreactivity of a radiolabelled antibody, as the amount of antibody localize to a tumour varies with the quantities of the antibody administered (Pimm & Baldwin, 1985 ; Rogers et al., 1986).

The localisation results obtained with the iodine-125 and indium-111 labelled 77-1 were not due to a non-specific pooling of blood in the tumour. This was evident from the tumour to blood ratios obtained with the control antibody 48-1 which were statistically significantly lower than that achieved by 77-1. Moreover, the tumour to blood ratio of the radiolabelled 77-1 increased with time, while that of 48-1 remained about the same. The increase in the tumour to blood ratios shown by both the iodine-125 and indium-111 labelled 77-1 was due to the clearance of antibody from the circulation, while the antibody concentration in the tumour remained essentially unchanged because of the specific binding with the tumour. The tumour to blood ratios therefore demonstrated the specific localisation to the xenograft by the radiolabelled 77-1.

In conclusion, the cell binding assay and the animal localisation studies confirmed that both the iodine-125 and indium-111 labelled 77-1 retained their immunoreactivity *in vitro* and *in vivo*, and were thus suitable for human studies.

CHAPTER 6

IMMUNOSCINTIGRAPHY OF COLORECTAL CANCER WITH MONOCLONAL ANTIBODIES

6.1 INTRODUCTION

The prime objective of this thesis for reasons already explained was to identify some more monoclonal antibodies suitable for the radiolocalisation of colorectal cancer. By screening antibodies raised mainly to breast and bladder cancers (Chapter 4), two monoclonals, both reactive with the epithelial membrane antigen (EMA) - M8 and 77-1, were selected for this purpose. The indium-111 labelled monoclonal antibody M8 has already been used in the imaging of breast cancer but it has not been used for the radiolocalisation of colorectal cancer. No side effects were reported from the use of this radiopharmaceutical (Rainsbury, 1985). Radiolabelled 77-1 has not, however, been used before clinically. The normal tissue distribution as well as their reaction to colorectal cancer (Chapter 4) of M8 and 77-1 are similar as shown by immunocytochemistry (Foster et al., 1984a; Summerhayes et al., 1985). The monoclonal antibody 2.94.2 derived from the same fusion as 77-1 has comparable reactions with normal tissues to 77-1 (Summerhayes et al., 1985), and has been used for the immunoscintigraphy of breast cancer without any untoward effects (Ward, 1987). Furthermore, both the iodine-125 and indium-111 labelled 77-1 have been shown to retain immunoreactivity in vitro and in vivo as demonstrated by cell binding assays and the xenograft localisation studies (Chapter 5).

At present, several monoclonal antibodies have been used in the immunoscintigraphy of colorectal cancer. For example, an anti-CEA monclonal antibody C46 detected 83% of primary and metastatic cancers (Armitage et al., 1986). Another anti-CEA monoclonal Mab 23 detected 50% of colorectal and pancreatic cancers (Mach et al., 1981). A monoclonal raised against colorectal cancer-associated antigen localised to 31 of 52 (60%) of patients with primary and

secondary colorectal cancer (Mach et al., 1983) and another monoclonal YPC2/12.1 to 13 of 16 (81%) patients (Smedley et al., 1983). A monoclonal antibody 791T/36T produced against an osteogenic carcinoma cell line detected 13 out of 24 primary colorectal cancers (54%) and 21 out of 26 metastatic sites (85%) (Armitage et al., 1985). Another antibody raised against a breast cancer metastasis (B72.3) detected about 75% of colorectal cancers (Colcher et al., 1987). In general the tumour detection rate of monoclonal antibodies is in the region of 50-80%.

In most of these studies iodine-131 labelled antibodies were used. indium-111 labelled monoclonal antibody was first used in the imaging of breast cancer with M8, although this radionuclide has been used to label polyclonal antiserum (Fairweather et al., 1983). Indium-111 has also been used with the monoclonal antibodies 791T/36 and C46 (Armitage, 1986) for the imaging of colorectal cancer. Iodine-131 has a high energy emission, undergoes urinary excretion and uptake by the thyroid, and has a relatively long half life of eight days. In contrast, indium-111 has a half life of 2.8 days and two peaks of gamma energy are better detected by the gamma camera commonly used (Armitage, 1986). The images generated by indium-111 labelled antibodies are better than the corresponding iodine-131 labelled antibodies (Fairweather et al., 1983; Armitage, 1986). A disadvantage of indium-111 labelled antibodies is the high uptake of radiolabel by the liver, preventing proper detection of liver metastases.

In this study indium-111 labelled M8 and 77-1 were used for immunoscintigraphy of colorectal cancer patients. A skin test with the antibody-DTPA conjugate was performed to exclude an allergic reaction before the intravenous administration of radiolabelled antibodies to patients. A specimen of the tumour along with specimens of normal colon, epiploic fat from the resected specimens, small samples of skin, subcutaneous fat and muscle from the wound

edge were collected at operation for measurement of radiolabel uptake. Initially scanning was performed daily for five days to determine the optimal scanning time, and this was correlated with blood and urine clearance of radioactivity in a few patients. Tumours were tested for reactivity with M8 and 77-1 by immunocytochemistry to determine if antibody staining correlated with the imaging result. In patients who did not undergo surgery, colorectal cancer specimens obtained either from biopsies or previous operations were used instead.

Several methods have been used to demonstrate the localisation of antibody to a tumour in man. In general the confirmation of localisation has been provided by external scanning and measurement of antibody radioactivity in surgical specimens (Rainsbury, 1984). A commonly used method is the measurement of radioisotope uptake by the tumour expressed as a ratio to blood (tumour/blood ratio).

The tumour to blood and the tumour to normal colon ratios were used in this study to demonstrate the localisation of radiolabelled antibodies to tumours. The tumour to blood ratio was also compared to the similar ratios of few other tissue samples collected at operation. The findings were compared with the other investigations such as barium enemas or computed tomography (CT scan) and operative findings.

6.2 MATERIALS AND METHODS

6.2.1 Patients

The approval of the ethical committee of University College Hospital was obtained for the clinical study. Colorectal cancer patients under the care of the Department of Gastrointestinal Surgery at University College Hospital were invited to take part. A fully informed verbal and written consent was obtained. A full history was taken from the patients and clinical examination carried out. The diagnosis of colorectal cancer was established by one or a combination of the following procedures: a) proctosigmoidoscopy/colonoscopy with positive biopsies, b) double contrast barium enema, c) computed tomography (CT), d) cytological findings e.g. malignant ascites, and further confirmed at operation and by histological examination of the resected specimens in those who had undergone surgery. Some patients did not undergo surgery and the diagnosis was confirmed by different imaging modalities or endoscopy. Patients with significant renal (blood urea > 12 mmol/l) or hepatic damage (bilirubin > 30 mmol/l), a history of atopy (asthma, eczema or drug reaction), or had a positive skin test to the monoclonals before antibody administration were excluded.

A total of 37 patients (21 in the M8 and 16 in the 77-1 groups) aged between 46 and 92 years were recruited to the study. Five patients (three in the M8 group and two in the 77-1 group) were found to have other bowel diseases although a provisional clinical diagnosis of colorectal cancer had been made. Two patients were not scanned at the appropriate time and were excluded. Thirty patients with colorectal cancer thus entered the study. Sixteen patients were given M8 (of whom three had recurrent tumours) and 14 patients were given 77-1 (of whom two had recurrent diseases).

6.2.2. Methods

i Preparation of radiolabelled monoclonal antibodies

The same procedures as described in 5.2.1 were used. These involved the use of a Protein A-Sepharose affinity column to purify the antibodies which were then conjugated to DTPA. The conjugated antibodies were purified by gel filtration. The antibody-DTPA conjugates were sterilised by ultrafiltration using a 0.22 μm filter (Millipore) in a laminar flow hood. A sample was then sent for pyrogenicity testing (Safepharm, Derby). These preparations were kept at 4°C until use and were stable for up to 6 months in storage.

The labelling of antibody-DTPA conjugates with indium-111 was performed under aseptic conditions in a radiopharmceutical preparation unit and all procedures were carried out in a laminar flow hood. The antibodies were labelled on the same day of administration.

A liquid chromatography column used for separating indium-111 labelled antibodies from free indium-111 was prepared as follows: 2.5 gm of Sephadex G25 (Pharmacia) was swelled in 20 ml of 0.1M glycine in water (pH 3.5) and the gel sterilised by autoclaving. Two ml of human serum albumin (1% w/v) was added to the G25 gel and the mixture was loaded into a 10 x 200 mm column to which a three-way tap was attached. To ensure proper coating of the gel with human serum albumin the fluid was emptied from the column and the procedure repeated three times. The fluid was finally drained from the column to the same level as the gel. The immunoreactivity of the radiolabelled antibodies was tested in a cell binding assay using MCF 7 cells (5.3.2).

Between 0.7-1 mg of antibody-DTPA conjugate was added to 148 MBq (4mCi) of indium-111 chloride (Amersham) and left to label for 20 minutes at

room temperature. The mixture was loaded into the G25 column prepared above and eluted with normal saline solution. Ten one ml aliquots were collected from the column. The antibody fraction was the first to emerge and was identified by counting the radioactivity of the aliquots in the gamma counter. It was usually collected between tubes 4 - 6, or a combination of 2 consecutive fractions in these positions. The free ^{111}indium emerged towards the end of the collection. The radiolabelled antibody was sterilised by passage through a 0.22 μm filter (Millipore). The amount of antibody with the required specific activity (1-2 mCi/mg) was added to normal saline to give a final volume of 10 ml. The specific activity injected to the patients was 70-80 MBq/mg.

ii Administration of radiolabelled antibody and scanning

After ensuring that renal and hepatic function tests were normal, a skin test was performed 20 minutes before the intravenous injection of the labelled antibody. Antibody-DTPA conjugate (10μg/0.2ml) was injected intradermally into the forearm. A 2 cm diameter circle was drawn around the injection site and inspected later for signs of reaction. A positive result with induration and erythema greater than 1 cm precluded the administration of antibody. A set of observations consisting of temperature, pulse, blood pressure and respiratory rate was taken as a base line. Observations were then made four hourly after a slow bolus intravenous injection of antibody into the antecubital vein, and the patients were kept in hospital during the scanning period. In the later part of the study, after the safety of the intravenous administration of the radiolabelled antibodies had been established, observations were made before and after the injection of antibody, and scanning was then performed as an outpatient procedure. Further observations were taken when the patients attended for scanning.

Immunoscintigraphs of patients were obtained with a Siemens LFOV gamma camera with a 280 KeV medium energy parallel hole collimator and information stored on the Nodecrest Micas 1000 computer. Initially, four patients had scans taken at 24 hours and then daily for up to 5 days. The remaining patients were scanned at days 3 to 5 which were determined as the optimal scanning time. Scans of the anterior abdomen, anterior and posterior pelvis and a sitting-down view with the camera placed under the patient were taken. The scans were reported by a nuclear medicine specialist who had no information about the patients.

iii Collection of specimens and examination of tissues and blood

Surgery was usually performed within 6 days of the radiolabelled antibody injection with the exception of three patients in the 77-1 group whose operations were postponed and no specimens were collected. Samples of the tumour, normal colon at the resection edge, and appendix epiploica were obtained from the resected specimen. From the abdominal incision edge small sections of muscle, subcutaneous fat and skin were taken. A venous blood sample was collected at the time of the operation. All specimens were put into scintillation tubes. They were weighed and the radioactivity was counted in a Packard Multi-Prias autogamma counter. The uptake of antibody by the tumour and tissues was expressed as a ratio to blood and comparisons made by the paired Student's 't' test. Surgical specimens were not available in another seven patients (two in the M8 group and five in the 77-1 group) for measurement of radiolabel uptake as the cancers were not resected. Six of these patients underwent laser therapy and the remaining one a bypass procedure. However, paraffin-embedded biopsy specimens from these patients were available for immunocytochemistry.

Five patients had recurrent tumours (three in the M8 group and two in the 77-1 group). Biopsies were obtained from two of these patients for radiolabel uptake measurement.

Formal-saline fixed paraffin sections of resected specimens of colorectal cancer were checked for reactivity with M8 or 77-1 using the indirect immunoperoxidase technique described in 3.2.3.iv.a. In patients with recurrent disease and who did not undergo surgery, paraffin-embedded histological sections from the original operations were used.

The optimal scanning time is related to the background radioactivity, so that a better image is obtained when the non-specific binding of radiolabel to normal tissue and radioactivity in the circulation decreases. Blood specimens were taken from seven patients (four from M8 group and three from 77-1 group) and urine was also collected from the four patients in the M8 group for antibody clearance measurements to determine if optimal imaging correlated with the clearance of the radiolabelled antibodies from the circulation. Ten ml of blood was collected from the patients five minutes after the administration of radiolabelled antibodies. The blood was centrifugated to pellet the cells, and two ml of plasma was counted for radioactivity which was taken as the standard to be compared against later samples. The radioactivity in the circulation, taken at 24-hour intervals for up to six days, were similarly measured and expressed as a percentage of the standard. The radioactivity on the cell pellets was also counted to determine if significant radiolabel was present in the cells.

The urinary excretion of radiolabelled M8 in four patients was measured over four days. An aliquot of radiolabelled M8 was weighed and diluted into one litre of N saline. The radioactivity of a two ml fraction of this solution was counted, and this was used to calculate the activity of the dose of radiolabel (which

was also weighed) administered to the patient. The urines were collected over 24-hour periods. The activity from two ml of the urine collected from each 24-hour period was counted and the total activity in the urine was calculated from this result. This 24-hour activity in the urine was expressed as a percentage of the injected dose estimated above.

The blood urea and electrolytes and hepatic function of all patients were checked at weekly intervals for two weeks.

6.3 RESULTS

6.3.1 Imaging with indium-111 labelled monoclonal antibody M8

A total of 21 patients took part in this study. Two patients were excluded due to indequate scanning. One of these patients was scanned up to the third day following antibody injection and underwent surgery on the fourth day. The tumour to blood ratio at operation was 3.5 and the tumour to normal colon ratio was 4.2 suggesting that a positive scan might be obtained if scanning had continued. The tumour from this patient reacted with M8 on staining. The second patient had disseminated abdominal disease with ascites and was scanned for one day only. Three patients had other bowel conditions (diverticular disease, proctitis and a rectal villous adenoma) and will be considered separately.

The actual number of colorectal cancer patients included was therefore 16, of whom nine were males and seven females. The mean age was 69 years (range 50-84 years). Primary tumours were present in 13 patients and three of these had liver metastases. Specimens of tumours and tissues were obtained from 11 of the patients with primary cancers, as two patients with rectal cancers were not fit for surgery and underwent laser therapy instead. No tumour was obtained from these patients for antibody estimation, although biopsies taken previously were available for staining with M8.

Recurrent disease was present in the remaining three patients, two of whom had hepatic metastases. Tumour specimen was obtained from one of these patients as a biopsy. No patients developed any allergic reaction to the antibody and no deterioration in renal or hepatic functions was detected up to two weeks following antibody administration. No hepatic metastases were detected due to a high uptake of radiolabel by the liver.

Considered all patients as a whole, a total of 21 tumour sites (13 primary tumours, 5 liver metastases and 3 recurrent tumours) were present in these 16 patients and antibody scans were positive in 13 (62%) sites (11 primary and 2 recurrent cancers). If hepatic metastases were excluded, radiolabelled M8 detected 13 out of 16 tumours or 81%.

i Primary colorectal cancer (13 patients)

If the liver metastases were considered separately, positive scans were obtained from the 11 (85%) primary tumours of these 13 patients (six rectosigmoid, four rectal, two caecal and one hepatic flexure cancers). The hepatic metastases in three of patients were not detected due to a high uptake of radiolabel by the liver. However, taking liver metastases into consideration a total of 16 tumour sites were present and 11 (69%) of these were detected by M8. Figures 44 and 45 show positive scans of rectal cancers with indium-111 labelled M8.

All scan-positive tumours had positive immunocytochemistry with M8. The immunoperoxidase staining reaction between M8 and a tumour which scanned positively is shown in Figure 46. The two patients with rectal cancers and who underwent laser therapy had negative scans; one tumour stained negatively but the other stained positively with M8. The range of the sizes of tumour detected was 2 x 2 to 7 x 7 cm with a mean tumour to blood ratio of 5.3 $\pm$ S.D. 3.7 and a range of 1.9 to 14.3. The mean tumour to normal colon ratio was 2.5 $\pm$ S.D. 1.2 with a range of 1.2 to 5.5. The tissue to blood ratios (mean $\pm$ S.D.) from the other tissues were less than the tumour to blood ratios: normal colon 2.14 $\pm$ 1.58, epiploic fat 1.17 $\pm$ 0.90, subcutaneous fat 1.1 $\pm$ 1.57, muscle 0.75 $\pm$ 0.39 and skin 2.12 $\pm$ 2.45. The tumour / tissue to blood ratios are shown in Figure 47. Statistical analysis using the paired Student 't' test showed that the uptake by the tumours

were significant greater than other tissues. The 'p' values between tumours and normal colon, epiploic fat, subcutaneous fat, muscle and skin were 0.004, 0.004, 0.013, 0.004 and 0.000 respectively, indicating that specific localisation of M8 had taken place in the tumours.

ii Recurrent cancer (three patients)

Three recurrent cancers were present in these three patients. In addition two patients also had liver metastases, giving a total of five tumour sites. The scans of two recurrent tumours were positive (40%). If hepatic metastases were excluded, this would give a detection rate of two out of three tumour sites (67%). Specimens were obtained from one of these patients. The tumour to blood ratio was 1.27 and the tumour to normal colon ratio was 1.4. Both these tumours reacted with M8 on staining. The patient who had a negative scan had a recurrent sigmoid cancer, and the tumour obtained from the original operation did not react with M8.

Specimens were obtained from all 16 patients, although specimens from two patients with recurrent cancers were from the original operations. Fourteen tumours stained positively with M8, 13 of which had positive scans. Two tumours which stained negatively with M8 also failed to scan with the radiolabelled M8.

iii Other conditions - (three patients excluded from study)

One patient with sigmoid diverticulitis presented with a stricture of the sigmoid colon which was clinically and radiologically more suggestive of a carcinoma than diverticular disease. This patient underwent scanning with the radiolabelled M8 pre-operatively. A positive scan was obtained. However, diverticulitis was found in the resected colon. Another patient who presented with

rectal bleeding and an irregular rectal mucosa on barium enema was scanned before histological confirmation. This patient had a positive scan with M8. Biopsy showed that this patient had severe proctocolitis. A patient with a large rectal tumour with the appearance of a carcinoma was scanned with the radiolabelled M8. A negative scan was obtained. The tumour proved to be an adenoma on histology and it did not react with M8 on immunocytochemistry.

iv Blood and urine clearance of M8

The blood radioactivity in four patients was estimated. The mean level, expressed as a percentage of the blood activity measured at five minutes after radiolabelled antibody injection, dropped below 26% in the first day, to 20% at day 2, 10% at day 3, 7.5% at day 4 and progressively to below 5% by day 6 (Figure 48). There was no significant radioactivity in the cells separated from the plasma, indicating that the radiolabel in the blood was contained in the plasma which was used for radioactivity measurement. The urine excretion measured in the same four patients showed that a mean of 20% of injected dose was recovered in the urine at day 1, 12.5% at day 2, 10% at day 3 and 6% at day 4 (Figure 49).

6.3.2 Imaging with indium-111 labelled monoclonal antibody 77-1

A total of 16 patients were scanned with indium-111 labelled 77-1. Two of these patients with other conditions were excluded and will be considered separately. The mean age of these patients was 73 with a range of 52 to 92 years. No adverse reaction was observed from these patients as a result of antibody administration. The renal and liver function tests were not affected within two weeks after the study.

Fourteen patients (seven males and seven females) had colorectal cancer, two of whom had recurrent disease. Three of the 12 patients with primary tumours had liver metastases. A total of 17 tumour sites (12 primary tumours, 3 liver metastases and 2 recurrent tumours) were present in these 14 patients. The indium-111 labelled 77-1 detected 10 of the tumour sites (59%). If hepatic metastases were excluded from consideration, the radiolabelled 77-1 detected 10 out of 14 tumours or 71%.

i Primary colorectal cancer (12 patients)

When primary colorectal cancers were considered separately, the scans were positive in eight (67%) of these 12 patients. High hepatic uptake of the radiolabel prevented the detection of three liver metastases. However, taking hepatic metastases into consideration, the radiolabelled 77-1 detected eight out of 15 tumour sites (53%). The smallest tumour detected was 3 x 2.5 cm. and the range was up to 10 x 10 cm. Figures 50 and 51 show the rectal cancers scanned positively with indium-111 labelled 77-1. Tissues were obtained from four patients for antibody uptake measurement. The mean tumour to blood ratios was 3.6 $\pm$ S.D. 1.43 (range 1.6 - 5.07) and the mean tumour to normal colon ratio was 2.0 $\pm$ S.D 0.81 (1.0 - 3.3). The mean normal colon, subcutaneous fat, muscle and skin to blood ratios (mean $\pm$ S.D.) were 2.26 $\pm$ 0.635, 0.63 $\pm$ 0.23, 0.52 $\pm$ 0.19 and 1.80 $\pm$ 0.92 respectively; statistical analysis using the paired Student's 't' test showed that the tumour to blood ratios were significantly higher than that of the other tissues, with 'p' values of 0.016, 0.002, 0.002 and 0.022 respectively. The tumour / tissue to blood ratios are shown in Figure 52.

All eight patients with positive scans had tumours which stained positively with 77-1. However, three patients with sigmoid cancers which stained

positively with 77-1 had negative scans. The tumour to blood ratios from two of these patients were 1.6 and 2.43, and the tumour to normal colon ratio was 1 for the former patient, but this ratio was not available for the latter. The remaining one patient who had a negative scan also had a tumour which did not react with 77-1 on immunocytochemistry.

ii Recurrent cancer (two patients)

Two patients with recurrent rectal cancers had positive scans (100%). The tumour to blood ratio from one of these patients was 4.84. Immunocytochemistry of the tumour was positive in one patient and tissue was not available for staining and uptake measurement in the other.

iii Other conditions - (two patients excluded from study)

A patient who underwent a transverse colectomy for carcinoma two years earlier was found to have a marked elevation of blood CEA levels on routine follow up. All other investigations for recurrent cancer were negative. Immunoscintigraphy with radiolabelled 77-1 was negative. A second look laparotomy was performed, but no recurrent tumour was found.

Another patient presented with an entero-vaginal fistula. She had undergone a pelvic exenteration for a recurrent rectal carcinoma six months earlier and received post-operative radiotherapy. A CT scan showed that there was a thickened and irregular vaginal wall with a soft tissue mass lying in front of the fornix which involved loops of ileum. The 77-1 scan in this patient was positive. At laparotomy a mass of friable tissue was found in the pelvis adherent to the small bowel. Histological specimen showed inflammatory tissue with no evidence of tumour.

iv Blood clearance of radiolabelled 77.1

The radioactivity level in the blood was measured in 3 patients. This was expressed a percentage of activity 5 minutes after radiolabelled antibody injection. There was no significant radioactivity in the cells pelleted from the blood, indicating that all radiolabel was present in the plasma which was used for radioactivity measurement. The radioactivity dropped to below 37% at 24 hours, 35% at 48 hours and to 20% by 72 hours (Figure 53).

Table 14 Primary colorectal cancers
Details of indium-111 labelled M8 uptake, staining and scanning results

Patient	Age/sex	Site	Grade	Size(cm)	T:B ratio	T:NC ratio	Staining	Scan
1.	50/F	caecum	Mod	5x5	4.3	3.1	+ve	+ve
2.	52/F	sigmoid	Well	5x5	5.7	3.3	+ve	+ve
3.	71/F	R/S	Well	5x5	7.7	3.2	+ve	+ve
4.	53/M	R/S	Poor	4x4	8.1	5.5	+ve	+ve
5.	69/M	rectum	Well	7x7	14.3	2.2	+ve	+ve
6.	84/M	caecum	Mod	3x3	3.6	1.9	+ve	+ve
7.	77/M	rectum	Mod	NA	NA	NA	+ve	-ve
8.	83/M	rectum (L)	NA	NA	NA	NA	-ve	-ve
9.	71/F	R/S	Well	2x2	3.3	1.45	+ve	+ve
10.	82/F	R/S (L)	NA	NA	1.9	1.2	+ve	+ve
11.	74/M	rectum	Mod	NA	4.98	2.04	+ve	+ve
12.	72/F	hepatic flexure	Mod	3x8	1.26	2.0	+ve	+ve
13.	63/M	R/S (L)	Poor	3x3	2.70	2.37	+ve	+ve

Table 15 Recurrent colorectal cancers

Details of indium-111 labelled M8 uptake, staining and scanning results

Patient	Age/sex	Site	Grade	Size(cm)	T:B ratio	T:NC ratio	Staining	Scan
14.	63/M	sigmoid (L)	Mod	NA	NA	NA	-ve	-ve
15.	71/F	rectum (L)	Well	NA	NA	NA	+ve	+ve
16.	62/F	rectum	Well	NA	1.27	1.4	+ve	+ve

Well = well differentiated

Mod = moderately differentiated

Poor = poorly differentiated

R/S = rectal sigmoid

(L) = liver metastases

NA = not available

Table 16 **Indium-111 labelled M8**
Tumour /Tissue to blood ratios from 13 patients with primary colorectal cancer

Patients	Tumour	N colon	Colon fat	S fat	Muscle	Skin	Staining	Scan
1	4.3	1.4	0.24	0.52	0.77	1.7	+ve	+ve
2	5.76	1.74	2.05	4.58	0.86	1.84	+ve	+ve
3	7.74	2.41	1.81	0.71	1.63	3.50	+ve	+ve
4	8.2	1.5	0.6	0.5	1.0	2.0	+ve	+ve
5	14.3	6.62	2.7	0.2	0.8	8.5	+ve	+ve
6	3.2	1.9	2.0	3.3	0.8	NA	+ve	+ve
7	NA	NA	NA	NA	NA	NA	+ve	-ve
8	NA	NA	NA	NA	NA	NA	-ve	-ve
9	3.3	2.2	1.08	0.04	0.54	1.63	+ve	+ve
10	1.9	1.6	0.65	0.27	0.37	0.81	+ve	+ve
11	4.98	2.4	NA	NA	NA	NA	+ve	+ve
12	1.26	0.63	0.35	0.31	0.35	0.32	+ve	+ve
13	2.7	1.13	0.23	0.07	0.38	0.93	+ve	+ve

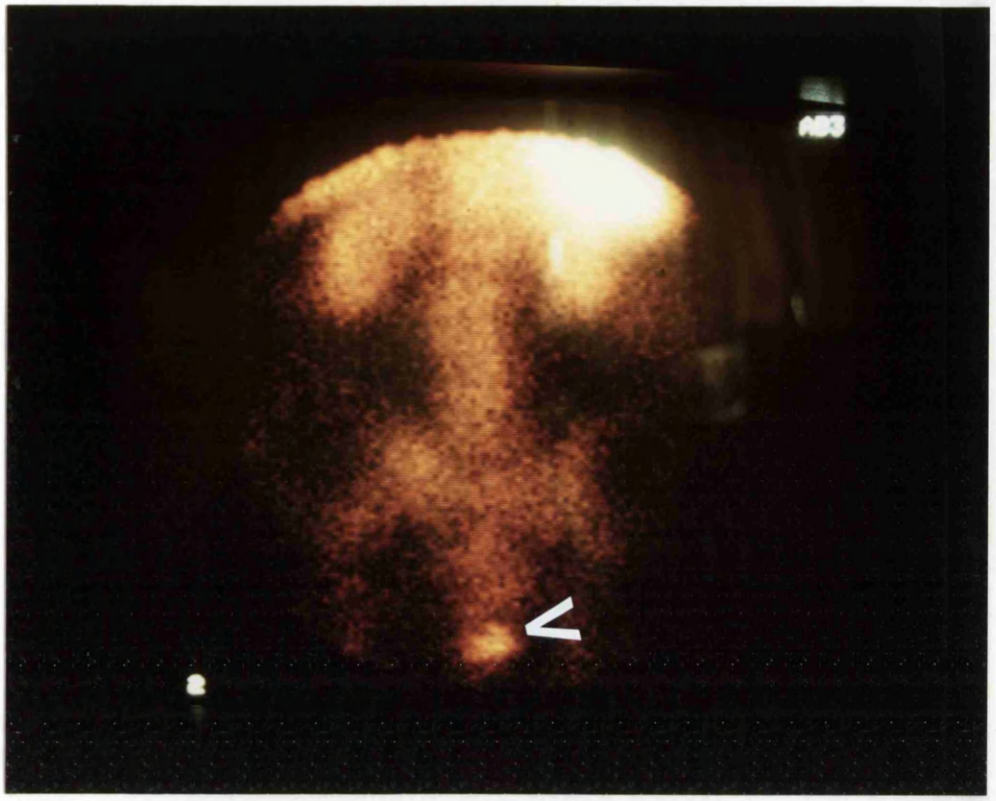


Figure 44 A posterior scan of a rectal cancer (arrow) detected by indium-111 labelled M8. Background activity is present in the bone, kidneys and liver (most intense activity)

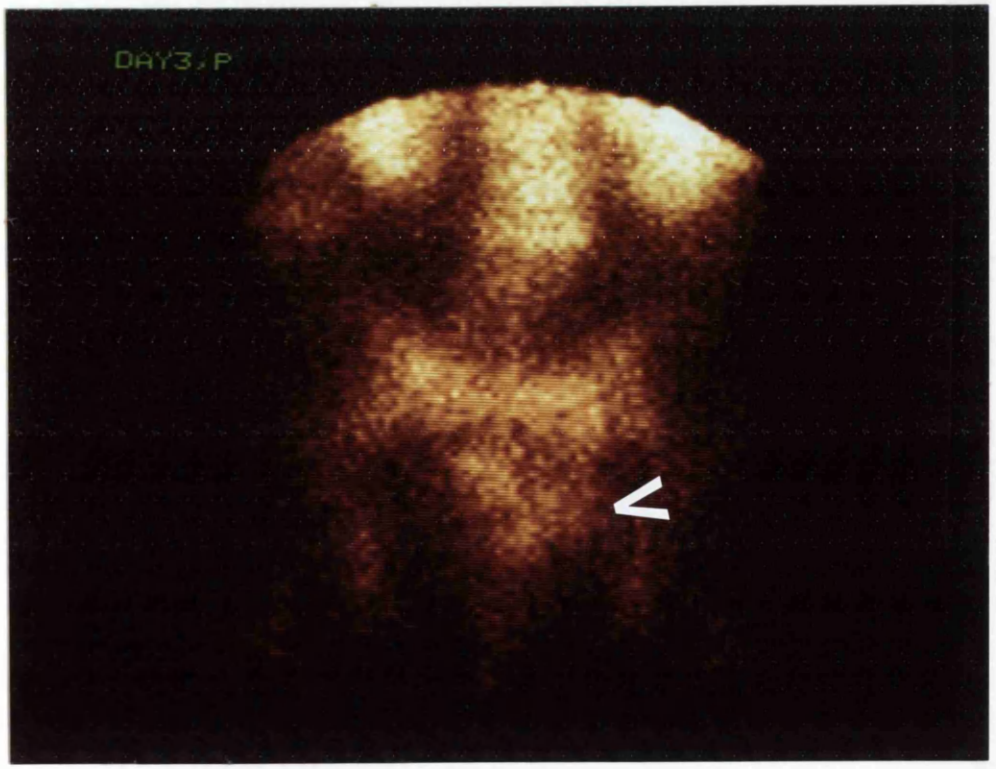


Figure 45 Another rectal cancer (arrow) scanned positively with indium-111 labelled M8

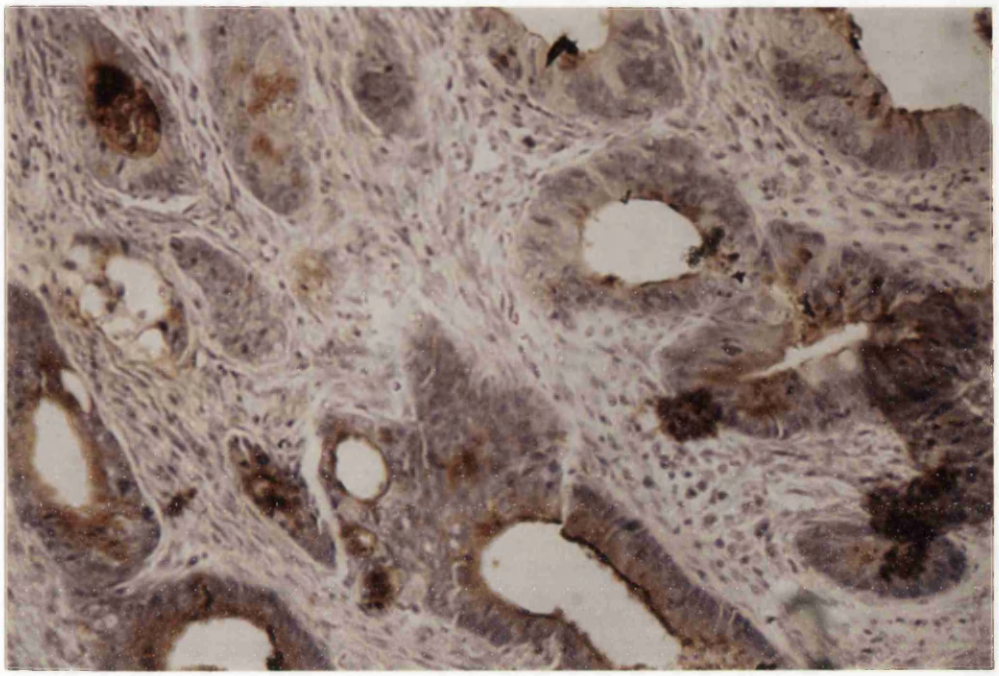


Figure 46 **Immunoperoxidase staining of the rectal cancer in a patient with a positive scan with indium-111 labelled M8 x 150**

Figure 47 Uptake of In-111 labelled M8 in tumour and tissues expressed as a ratio to blood

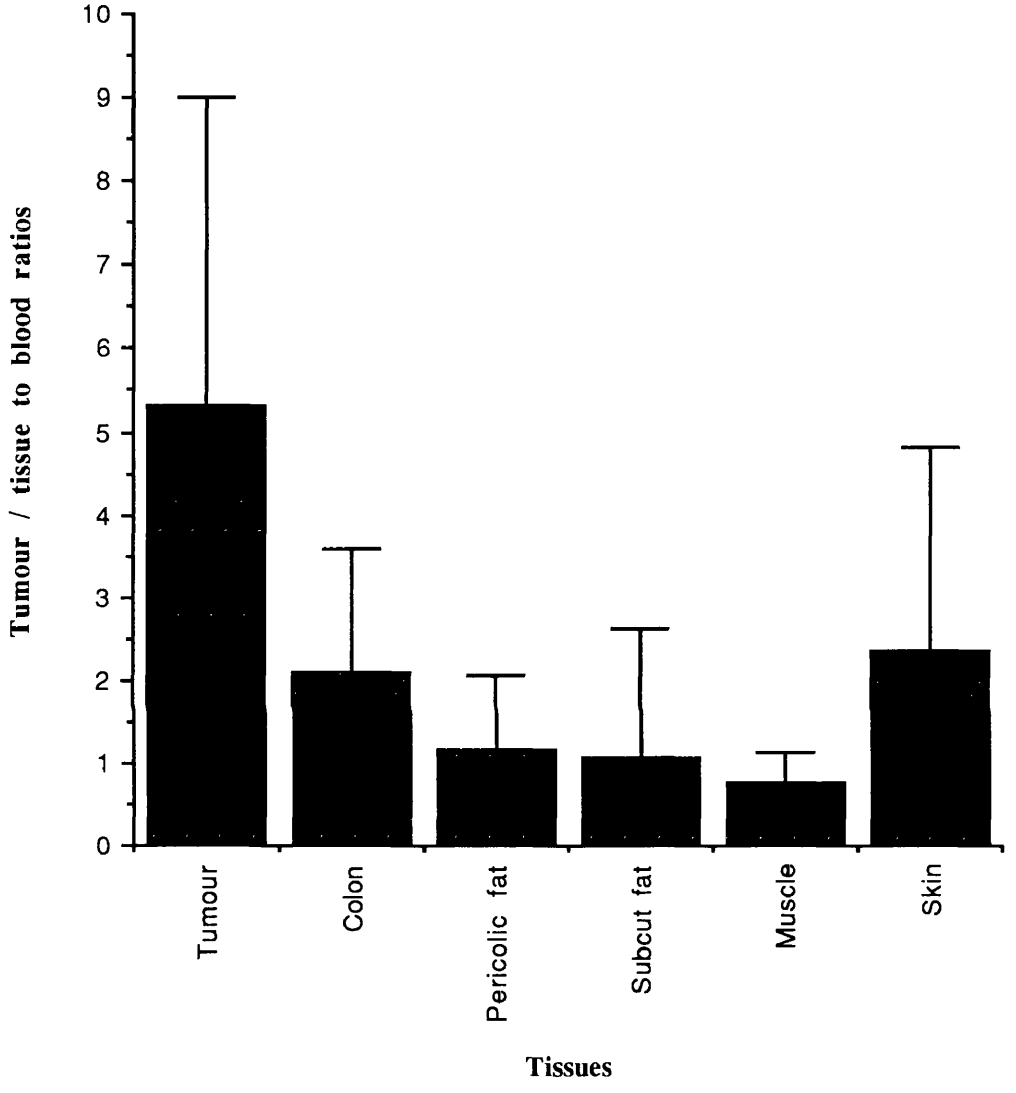


Figure 48 Mean blood activity of In-111 labelled M8

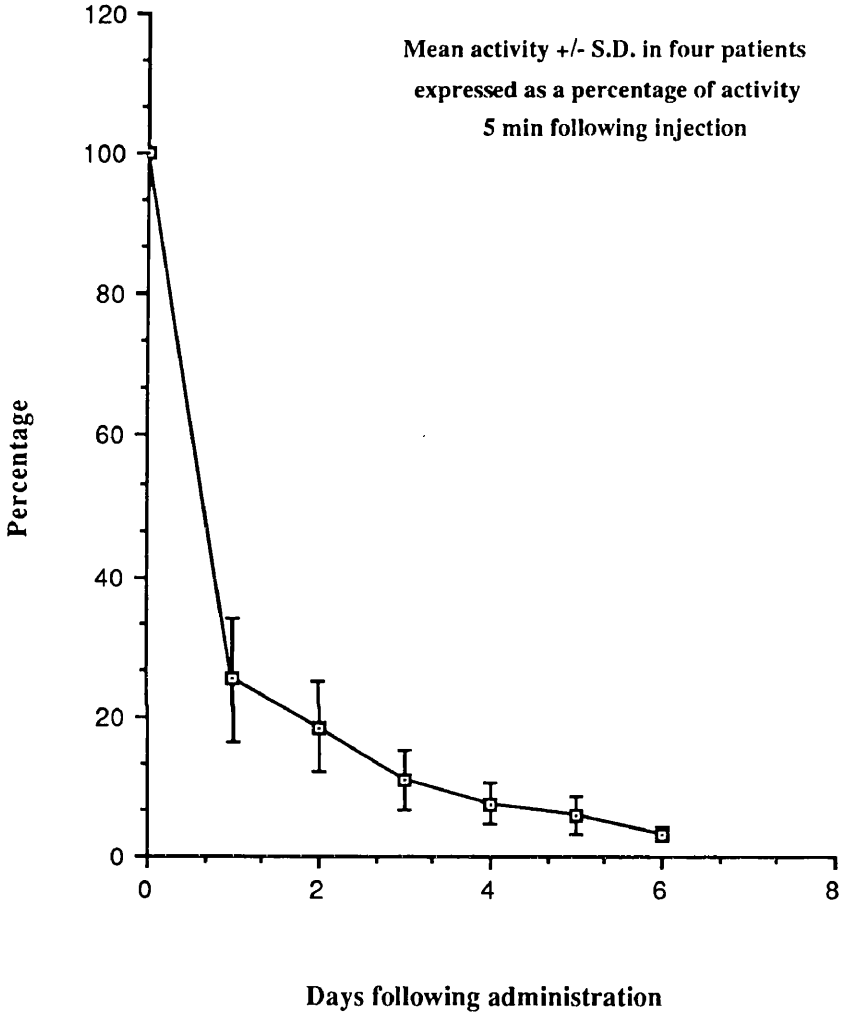


Figure 49 Urinary excretion of In-111 labelled M8

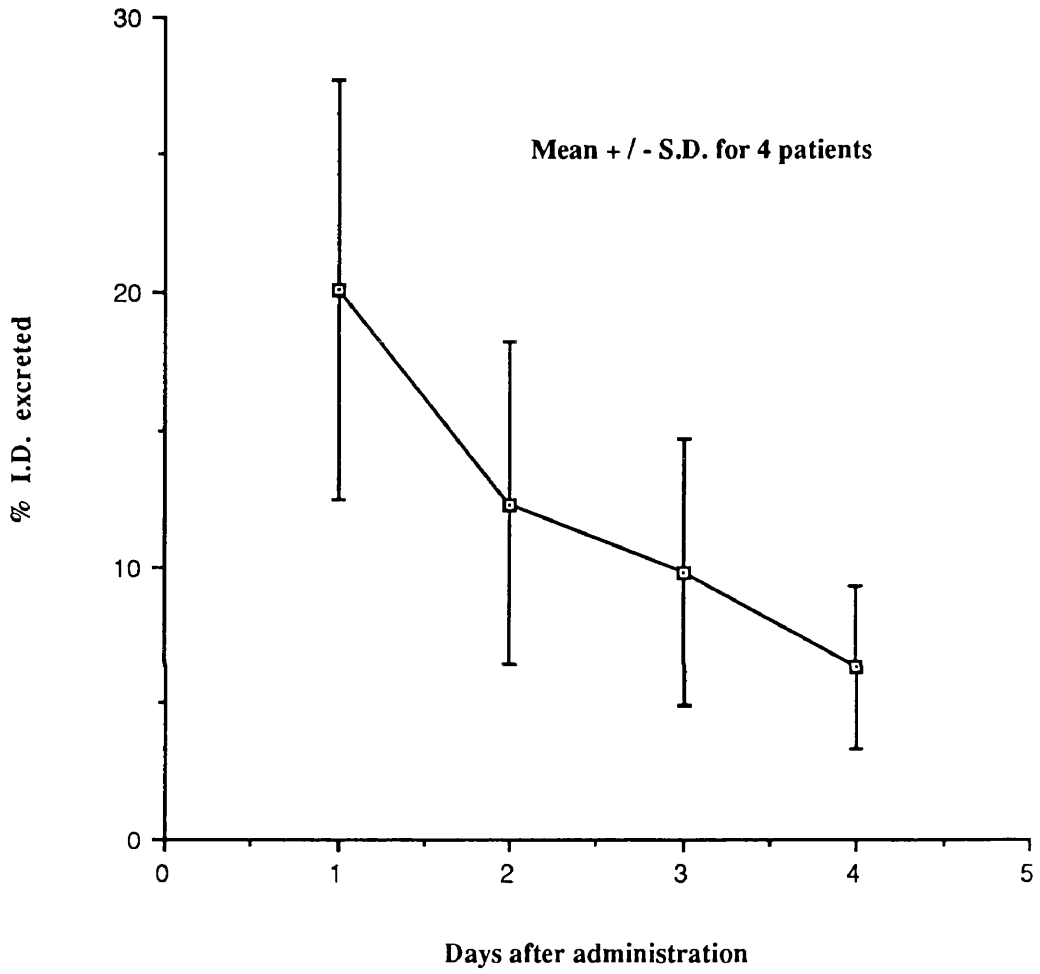


Table 17 Primary colorectal cancers

Details of indium-111 labelled 77-1 uptake, staining and scanning results

Patient	Age/sex	Site	Grade	Size(cm)	T:B ratio	T:NC ratio	Staining	Scan
1.	72/F	sigmoid	Mod	5x2.5	1.6	1	+ve	-ve
2.	79/F	rectum	Well	4x3	2.4	1.9	+ve	+ve
3.	59/M	rectum	Mod	NA	NA	NA	+ve	+ve
4.	69/M	sigmoid	Mod	3x2.5	4.0	3.26	+ve	+ve
5.	75/F	sigmoid	Mod	3x3	NA	NA	+ve	-ve
6.	52/M	rectum	Poor/mod	5x4	NA	NA	+ve	+ve
7.	66/F	caecum	Mod	3.5x3.5	NA	NA	-ve	-ve
8.	92/F	caecum (L)	Mod	NA	NA	NA	+ve	+ve
9.	85/M	sigmoid	NA	NA	2.43	NA	+ve	-ve
10.	70/M	sigmoid (L)	Mod	10x10	4.94	1.95	+ve	+ve
11.	77/F	rectum (L)	Mod	NA	5.07	1	+ve	+ve
12.	74/M	rectum	Mod	NA	NA	NA	+ve	+ve

Table 18 **Recurrent colorectal cancers**
Details of indium-111 labelled 77-1 uptake, staining and scanning results

Patient	Age/sex	Site	Grade	Size(cm)	T:B ratio	T:NC ratio	Staining	Scan
13.	66/M	sigmoid	Well	5x5	4.84	2.45	+ve	+ve
14.	84/F	rectum	Mod	NA	NA	NA	NA	+ve

Well = well differentiated

Mod = moderately differentiated

Poor = poorly differentiated

(L) = liver metastases

NA = not available

Table 19 Indium-111 labelled 77-1

Tumour /Tissue to blood ratios from 4 patients with primary colorectal cancer and 1 patient with recurrent cancer

Patients	Tumour	N colon	S fat	Muscle	Skin	Staining	Scan
1	1.66	1.67	0.67	0.45	1.8	+ve	-ve
2	2.44	1.26	0.27	0.45	0.81	+ve	+ve
4	4.03	3.22	0.82	0.45	3.60	+ve	+ve
10	4.94	2.53	0.61	0.40	1.9	+ve	+ve
13	4.84	2.45	0.55	0.50	0.91	+ve	+ve

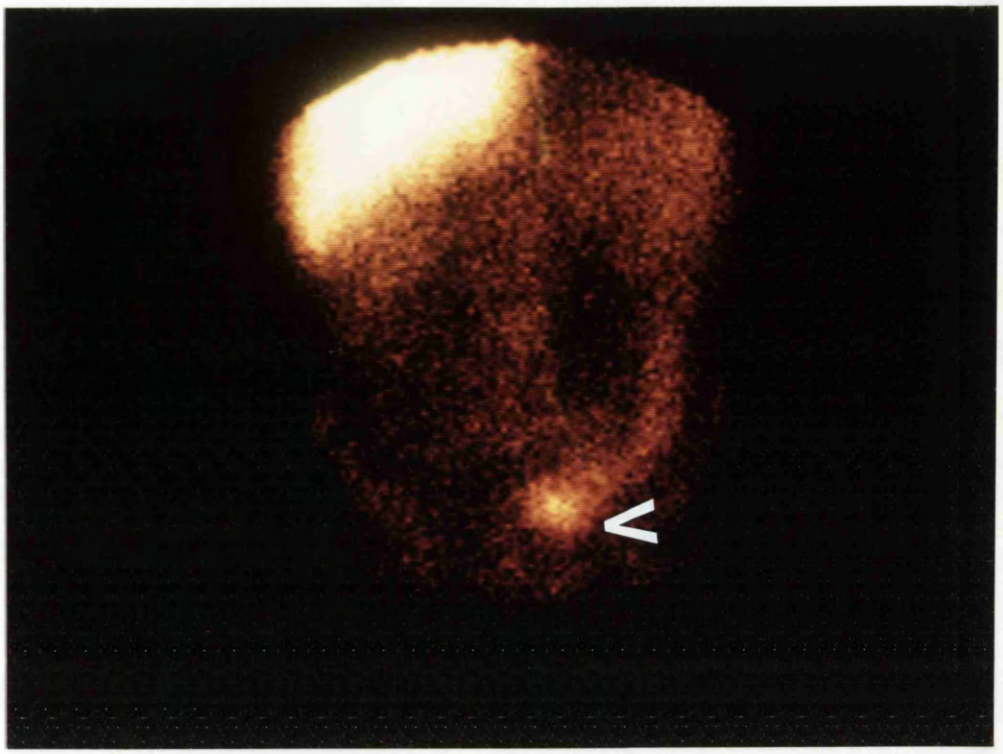


Figure 50 An anterior view of a rectal cancer (arrow) scanned positively with indium-111 labelled 77-1. Note high background activity in the liver and to a lesser extent in the descending colon

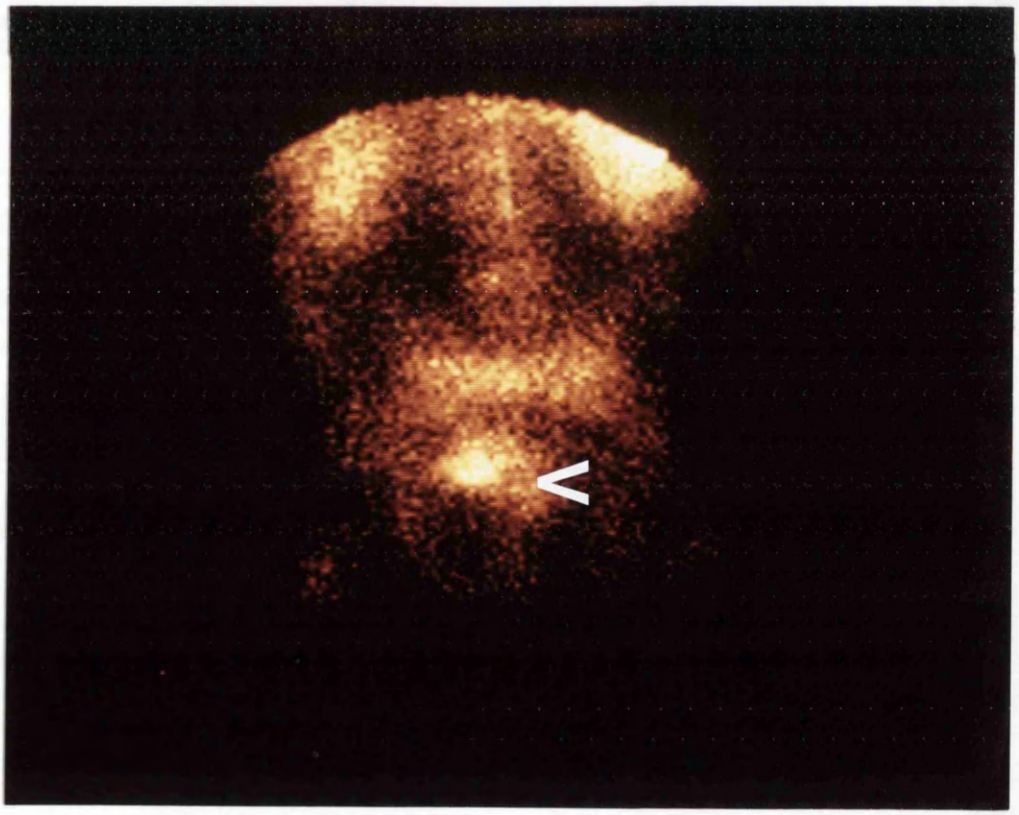


Figure 51 A posterior view of another rectal cancer (arrow) scanned positively with indium-111 labelled 77-1

Figure 52 Uptake of In-111 labelled 77-1 in tumour and tissues expressed as a ratio to blood

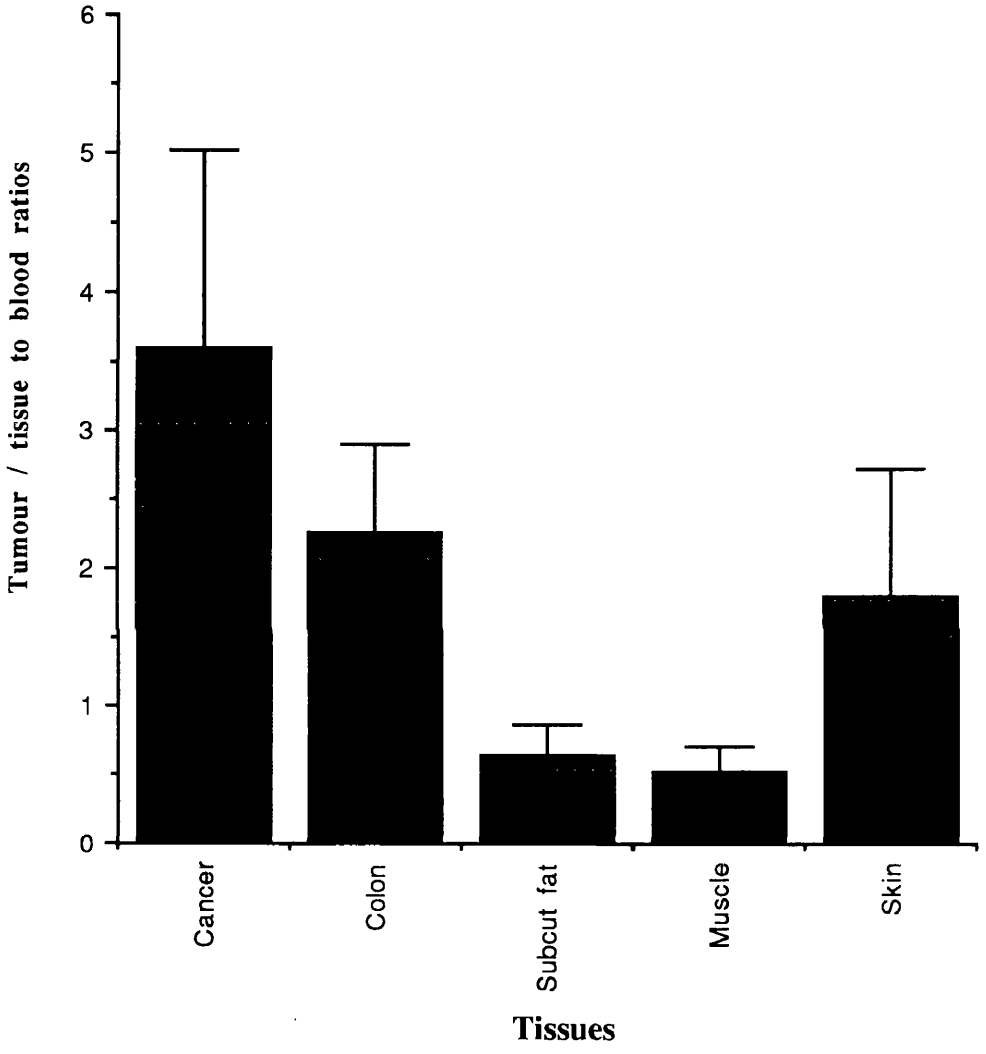
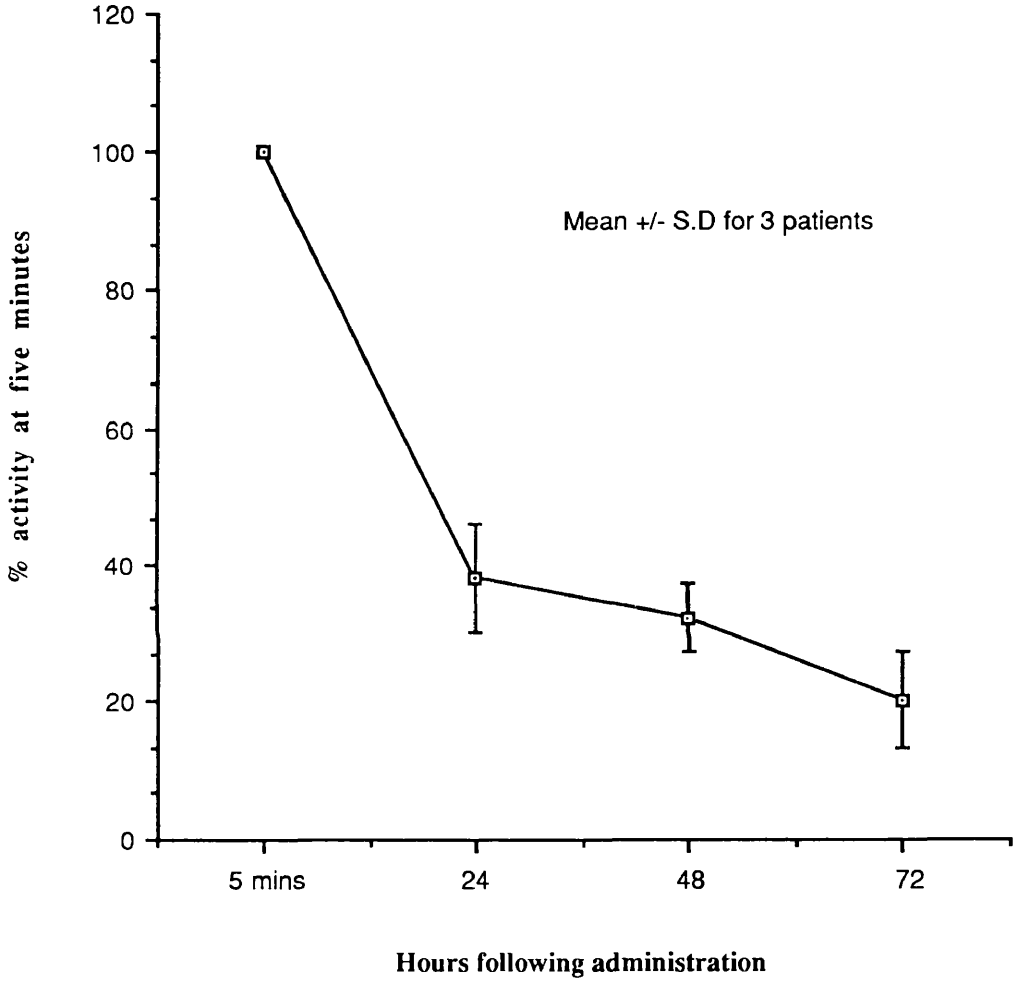


Figure 53 Mean blood activity of In-111 labelled 77-1



6.4 DISCUSSION

The monoclonal antibodies M8 and 77-1 were chosen for the immunoscintigraphy of colorectal cancer because of their preferential reaction on immunocytochemistry with the tumour compared with normal colon and polyps (Chapter 4), and their retention of immunoreactivity following radiolabelling (Chapter 5). This investigation is the first detailed study to use anti-EMA monoclonal antibodies in the immunoscintigraphy of colorectal cancer. The monoclonal antibody M8 has already been used without any side-effects in the imaging of breast cancer (Rainsbury, 1984) and would present no problem in its application to colorectal cancer. However, there has been one report of allergic reactions following monoclonal antibody administration to patients (Dillman et al., 1984). It was found that the anti-CEA antibody also reacted with the white blood cells because of the reaction with NCA, the non-specific cross reacting antigen. Therefore the safety in the administration of the monoclonal antibody 77-1 had to be considered. The monoclonal antibody 2-94-2 which was raised from the same fusion as 77-1 and had similar tissue distribution, had been used for the immunoscintigraphy of breast cancer without any side-effects (Ward, 1987). There was no reaction of 77-1 with the bone marrow or any reaction with the white blood cells on immunocytochemistry (Chapter 4). It was therefore expected that 77-1 would have the same physiological behaviour as 2-94-2 when administered to patients. A skin test was essential before the administration of either M8 or 77-1 to the patients to detect any allergic response to mouse immunoglobulin before antibody injection. No patients developed an allergic response to the skin test and liver, renal and haematological functions showed no adverse reaction following antibody administration. Thus no clinical

complications have resulted from antibody administration.

In the immunoscintigraphy of breast cancer, indium-111 labelled M8 has been found to be useful only in bony secondaries but not the primary cancers (Rainsbury, 1984). This may represent a difference in the circulation to the bone marrow and the primary tumour and therefore the access of antibody to the tumour. The interval between antibody injection and scanning may be relevant. The breast cancer patients were scanned once or twice between 5 and 96 hours (Rainsbury, 1984), but an optimal scanning time had not been determined. When M8 was first used for immunoscintigraphy of colorectal cancer, scanning was performed daily. It became clear that the best images were obtained at 3 to 5 days after the administration of either radiolabelled M8 or 77-1. Therefore to avoid unnecessary scanning the imaging was commenced at day 3 and ended at day 5. A similar optimal scanning time has also been reported by others (Armitage et al., 1984). This is explained by the clearance of antibody in the blood pool but the continuing retention of antibody in the tumour, thereby increasing the tumour to blood or tumour to non-tumour ratios.

The blood radioactivity of M8 dropped to below 30% and that of 77.1 to below 40% in the first 24 hours. The reduction of radioactivity from the blood was less rapid after this and fell to 10% by day 3 for M8 and to 20% for 77-1. From this time onwards the clearance was less rapid as shown by M8 which fell to 5% by day 6. This bi-phasic pattern of clearance of M8 and 77-1 from the circulation is similar to the indium-111 labelled monoclonal antibody C46 (Armitage et al., 1986) which has a comparable rate of radioactivity clearance as M8. The rapid fall of radioactivity in the blood in the first 24 hours probably corresponds to the shift of radiolabels from the vascular to the extra-vascular compartment, and the slower rate of fall thereafter probably corresponds to the

clearance and excretion of the antibodies (Armitage et al., 1985). This pattern of clearance explains why better scanning images are obtained after three days, as most background radioactivity is cleared from the circulation. In the first 24 hours 20% of the radioactivity in M8 patients was recovered in the urine. About 10% of the activity was excreted over each of the next three 24-hour periods. This pattern of excretion reflected the clearance from the blood.

The scans as reported by an independent observer correlated with the operative findings of the tumours. Indium-111 labelled M8 detected 11 out of 13 primary colorectal cancers (85%), but none of the three liver metastases. It localised to two out of three recurrent cancers except the two hepatic metastases. When these tumours are considered together, M8 detected 13 out of 21 cancer sites (62%). Broadly similar results were obtained with indium-111 labelled 77-1. The scans were positive in 8 out of 12 patients (67%), but the hepatic metastases in three of these patients were not detected. Both the patients with recurrent tumours had positive scans. Considered as a whole, a total of 10 out of 17 tumour sites were demonstrated by 77-1 (59%). However, if hepatic metastases were excluded, M8 detected 13 out of 16 cancers or 81% and 77-1 detected 10 out of 14 tumours or 71%. Failure of the detection of hepatic metastases is due to the use of indium-111 as a radiolabelling agent, and the consequent high uptake of radiolabel by the liver which obscured any specific antibody localisation. It was estimated by Rainsbury (1984) that 35-40 per cent of injected dose of the indium-111 labelled M8 accumulated in the liver within 24 hours of injection. There was no mention of the detection of hepatic metastases when radiolabelled M8 was use in the localisation of breast cancer (Rainsbury,1984).

The accumulation of radioactivity in the liver is probably due to the high uptake by the reticuloendothelial system (RES) of the indium-111 released

by the DTPA linker (Goldenberg, 1988). This problem is particularly relevant to colorectal cancer as hepatic secondaries are important causes of treatment failure, and their detection in the staging or planning of treatment is essential. New and more stable conjugates are being prepared to overcome this problem (Esteban et al., 1987). Alternatively, iodine-131 labelled antibodies can be used to detect liver metastases by means of a computer-assisted subtraction of background radioactivity. The background reagent can be ^{99m}Tc human serum albumin and ^{99m}Tc pertechnetate, or iodine-123 labelled irrelevant immunoglobulin. However, iodine-131 labelled antibodies can be deiodinated rapidly by iodinases present in tissues, resulting in the accumulation of free iodine in the thyroid, gut and rapid urine excretion (Goldenberg, 1988). Another approach to reducing the background radioactivity is the use of a second antibody which reacts with the non-targeted first antibody to form complexes which are then removed by the RES in the liver and spleen. When iodine-131 labelled primary antibodies are used, the complexes are removed rapidly from the blood and most tissues, including the liver, thus enabling a high early tumour to non-tumour ratio to be achieved without necessitating a subtraction technique (Sharkey et al., 1984; Goldenberg et al., 1987). The efficiency of tumour detection may be increased by the use of a probe at operation, so that smaller lesions with a lower radioactivity may be located (Aitken et al., 1984), although this may have a limited role in patients who undergo CEA-guided second look laparotomy.

The results obtained here with indium-111 labelled M8 and 77-1 are similar to other monoclonals used for the immunoscintigraphy of colorectal cancer. The monoclonal antibody 17-1A labelled with iodine-131 gave a diagnostic sensitivity of 54% (Mach et al., 1983), and a sensitivity of 66% was achieved by the iodine-131 labelled monoclonal antibody 19-9 (Chatal et al.,

1984). Chatal et al. (1985) used three monoclonal antibodies labelled with iodine-131 for the imaging of colorectal cancer in 25 patients with suspected recurrent colorectal cancer. The antibodies included an anti-CEA monoclonal antibody 202, monoclonal antibody 17-1A and 19-9. Twenty two patients received a mixture of antibody 202 and $F(ab')_2$ fragments and 3 patients received a mixture of $F(ab')_2$ fragments of 17-1A and 19-9. Immunoscintigraphy was positive in 13 out of 18 recurrent sites (72%). Armitage et al. (1984) using iodine-131 labelled monoclonal antibody 791T/36 demonstrated 26 out of 38 colorectal cancer (68%) on immunoscintigraphy. Begent et al. (1986) using a goat anti-CEA or a mouse monoclonal anti-CEA antibody labelled with iodine-131 reported that in 31 patients, 94% of tumour sites were detected by using a numerical analysis in the areas of radioactive uptake. An anti-CEA monoclonal antibody labelled with indium-111 detected all seven primary colorectal carcinomas, and eight of 11 metastatic tumours (Armitage et al., 1986). Positive liver images were considered to be those where a filling defect on the blood pool view at 20 minutes had filled in at 72 hours.

The ranges of the tumour to normal colon ratios for both indium-111 labelled M8 (2.5) and 77-1 (2.0) are similar to those reported by others in the antibody localisation of colorectal cancer. For examples, the ratio for monoclonal antibody 791T/36 was 2.8 (Armitage, 1986); monoclonal antibody 17-1 was 3.0 (Mach et al., 1983); monoclonal antibodies 19-1 and 17-1 were 2.6-3.0 (Chatal et al., 1984), and monoclonal YPC 2/12.1 was 1.3 (Finan et al., 1985). However, an anti-CEA antibody C46 achieved a tumour : non-tumour ratio of 5.8 : 1 (Armitage et al., 1986). The tumour to blood ratios achieved by radiolabelled M8 and 77-1 were higher than similar ratios achieved by all other tissues sampled on statistical analysis, confirming that specific localisation to the cancers by both

antibodies had occurred. A more objective and direct but more difficult to perform measurement is autoradiography to demonstrate the presence of antibody in the tumour. This technique has been applied by Rainsbury (1984) in two patients to confirm the localisation of the iodine-125 labelled M8 to breast cancer.

The antibody uptake of M8 and 77-1 by the colorectal cancers as measured by the tumour to blood ratio and the tumour to normal colon ratio was not significantly different. However, a better tumour uptake of 77-1 might be achieved if radioiodinated 77-1 was used, as iodine-125 labelled 77-1 had a binding of 18% with MCF 7 compared with 8 to 12% achieved by indium labelled 77-1 (5.3.2).

In general, the results of immunoscintigraphy have shown that regardless of antibody, antibody form, or imaging process used, the sensitivity of tumour detection is generally about 80-90% with iodine-131 labelled antibodies and 50-60% for indium-111 labelled antibodies. The lower detection rate for the latter is probably due to a high uptake by the liver, preventing the identification of tumour. However, the quality of scans obtained with indium-111 labelled antibodies is better than the corresponding iodine-131 labelled antibodies (Goldenberg, 1988). The use of F(ab') fragment may improve imaging by reducing the background due to a more rapid clearance of the fragments. Mach et al. (1980b) have shown that F(ab') fragment gave a better result for immunoscintigraphy than the corresponding intact antibody. However, Armitage et al. (1985) found that this did not occur with the monoclonal antibody 739/36T. Compared with intact immunoglobulins, antibody fragments may show a decrease affinity and binding to tumour and may therefore be at a disadvantage when they are applied in therapy (Goldenberg, 1988).

In the present study positive scans were obtained with patients with

inflammatory conditions of the bowel such as diverticular disease, inflammatory tissue after surgery or proctocolitis. This phenomenon has been observed with other monoclonal antibodies. For example, the monoclonal antibody 739/36T gave a positive reaction in a patient with a bone abscess (Armitage et al.,1985) and a patient with colitis. Similarly, Chatal et al. (1985) reported that a patient with diverticular disease gave a positive scan with the monoclonal antibody 17.1. The mechanism of this localisation is not clear but may be related to the changes in the blood and lymphatic circulation during the inflammatory process. Awareness of this potential false positive result is important in the interpretation of immunoscintigraphy findings.

There was good correlation between immunocytochemistry and imaging, as scan positive tumours also had positive staining with the antibodies, and tumour staining negatively with the antibodies also had negative scans. Thus two colorectal cancer patients and a patient with a rectal polyp had negative scans and the tumours did not react with M8. Similarly, a negative scan with 77-1 was found in a patient whose tumour did not react with 77-1 and in another who had a negative second-look laparotomy. However, some tumours reacting with the antibodies on staining can produce negative scans. It is not clear if this is due to an insufficient uptake of antibody by the tumours or whether another mechanism is involved. In the one instance of a negative scan in a tumour which reacted with M8, no tumour sample was available for radiolabel analysis. This patient underwent laser therapy for a rectal cancer, and the effect of laser on antibody localisation is unknown. In the three such occurrences with 77-1, the tumour to normal colon ratio was 1.0 in one patient, and tumours were not available in the other two patients for radioactivity measurement. It appears, therefore, that the possibility of false-negative scans is greater than false-positive scans.

The correlation between positive staining of M8 and imaging has practical importance. One of the main applications of immunoscintigraphy is in the detection of recurrent colorectal cancer at a stage when further curative surgery is possible. To optimise the detection rate of recurrent tumours, immunocytochemistry can be performed before immunoscintigraphy by testing a monoclonal antibody against the original primary cancer. However, it has to be taken into account that some recurrent tumours may not express the same antigens as the primary tumours (Chatal et al., 1985). Perhaps the correlation between positive immunocytochemistry and immunoscintigraphy is more significant in the pre-selection of patients for targeting therapy (Larson and Carrasquillo, 1987).

The present results show that both M8 and 77.1 can localise to colorectal cancer. However, the number of patients in this study with recurrent diseases is small, and a more detailed evaluation is necessary to determine their effectiveness in the detection of recurrent cancers. Both M8 and 77-1 have anti-EMA properties, and the results obtained suggested that other anti-epithelial membrane antigen antibodies for example, ICR2, a rat anti-EMA monoclonal antibody (Davidson et al, 1989) should be assessed for the immunoscintigraphy of colorectal cancer. EMA is expressed by a large number of carcinomas so that these antibodies would not only be suitable for colorectal cancer but also other tumours. M8 has already demonstrated its suitability for breast cancer and ovarian cancer imaging. The monoclonal antibodies HMFGM 1 and HMFGM 2 which were raised against similar antigen (human milk fat globule membrane) have been used for immunoscintigraphy of different carcinomas, and detected two out of four colorectal cancer (Epenetos et al., 1982). It is possible that these antibodies all recognise similar epitopes as human mucins are very immunogenic in the mouse.

mouse.

Although EMA is expressed by a large number of epithelial tissues, this has not precluded its imaging of tumours. One explanation is the relatively higher expression of EMA by tumour cells so that a higher density of antibody would localise to the tumour compared with the normal tissues. Another explanation is the relative accessibility of the antibody to the carcinoma and the normal tissues. It is likely that normal tissues with their intact basement membrane would not allow the antibody to penetrate into the lumen where EMA is expressed.

The use of a cocktail of antibodies each recognising different epitopes should improve the number of positive scans. Chatal et al. (1985) showed that by using a cocktail of monoclonal antibodies the number of positive scans can be increased. Munz et al. (1986) have demonstrated that the antibodies cocktail is more effective if different antibodies recognise different antigens rather than different epitopes of the same antigen. Anti-epithelial membrane antigen monoclonal antibodies may, therefore, be used in combination with other antibodies recognising other colorectal antigens, for example CEA, for this purpose.

CHAPTER 7

CONCLUSIONS AND FUTURE PROSPECTS

7.1 MONOCLONAL ANTIBODIES AND THE PROGNOSIS OF COLORECTAL CANCER

The prognosis of colorectal cancer has not significantly improved in the past few decades and the overall survival remains about 20-50% at 5 years (1.1). Much effort has been made in many areas in an attempt to change the outlook of colorectal cancer. The value of various radical surgical techniques, or the prevention of tumour cell dissemination by the no-touch technique (1.2) remain to be proven. At a more fundamental level, some progress has been made in the investigation into the mechanism of malignant transformation and the genetic changes responsible for this development (Bodmer et al., 1987; Solomon et al., 1987), and the agents which may be involved in the promotion of carcinogenesis, such as bile acids (Hill, 1985). These have provided some basis for the possible prevention of colorectal cancer in the future and the identification of individuals or families at high risk.

Until an effective means for preventing this cancer becomes a reality, the quest for better theraupeutic results must continue. In this respect, the detection of early carcinomas by faecal occult blood screening in the population (Clark et al., 1987) has enabled some patients to get the maximal benefit of surgery by being detected early. For patients with more advanced disease, there is an urgent need for better adjuvant therapies. Current adjuvant chemotherapy, radiotherapy and immunotherapy have not increased the survival of this group of patients. A major difficulty in chemotherapy is the lack of effective cytotoxic agents. Chemicals such as 5-FU or FUDR, which have been administered via different routes to colorectal cancer patients, can only be expected to achieve a response rate of 20%, without any prolongation in life. Although these results are disappointing, the search for

more effective adjuvant therapies must continue if the prognosis of colorectal cancer is to improve. In the evaluation of such therapies, comparison must be made between properly controlled groups. The staging of colorectal cancer is therefore important as it forms the basis for the stratification of patients in a clinical trial, and the assessment of the results of treatment. There have been some advances in staging techniques in recent years, with imaging such as nuclear magnetic resonance (NMR), computed tomography (CT scan), ultrasound or radioisotope scans providing information about the extent of tumour load and its dissemination. However, there is still a need for better tumour staging or detection methods.

The advent of the monoclonal antibody technique has brought new prospects for the management of colorectal cancer. Monoclonal antibodies may be applied for the diagnosis and staging of the primary tumours, for the detection of recurrent diseases, and for adjuvant therapy by acting as carriers for cytotoxic agents for delivery to the tumour. However, whether such potentials can be achieved depend on a number of factors, one of which is the specificity of monoclonal antibodies to colorectal cancer. To date no tumour-specific monoclonal antibodies have been produced to any human solid tumours, including colorectal cancer. Nevertheless, some monoclonal antibodies with a selective affinity for colorectal cancer have been produced, and have been applied for the radioimmunolocalisation of this carcinoma (1.9). The results so far have been encouraging and confirm the value of continuing to develope monoclonal antibodies for the management of colorectal cancer. However, several problems have arisen in the application of monoclonal antibodies, some of which relate to the antibodies themselves. Apart from the need of antibodies with better specificity for colorectal cancer, there is the human anti-mouse antibody response (HAMA), and

the phenomenon of tumour heterogeneity (1.9). All these factors point to the need for more antibodies with a greater affinity for colorectal cancer which initiated this study.

The aims of this thesis were therefore fourfold. First, to generate and evaluate monoclonal antibodies raised from colorectal cancer tissue; second, to screen existing antibodies to other tissues for their affinity to colorectal cancer; third, to perform *in vitro* and *in vivo* testing of the immunoreactivity of the radiolabelled antibodies; and fourth, to perform a clinical localisation study with selected antibodies in colorectal cancer patients.

7.2 OBSERVATIONS ON THE SEARCH FOR NEW MONOCLONAL ANTIBODIES TO COLORECTAL CANCER

7.2.1 Production of new monoclonal antibodies

In the generation of new colorectal cancer-reactive monoclonal antibodies, an immunogen consisting of a crude membrane extract of colorectal cancer from different patients was used in this study, because immunogen prepared from a pool of different tumours had not been used in colorectal cancer previously, and it offered some potential advantages (see 3.1). Characterisation of the five monoclonal antibodies selected in this study by immunocytochemistry showed, however, that they were not specific to colorectal cancer. They reacted with normal colon as well as colorectal cancer, and with other epithelial cells. The reaction of antibody UC-ICR 12.1 with polymorphs suggests that it may belong to the Class 1 anti-CEA antibodies (Reynoso et al., 1985 - see 1.9.i). The reaction

of UC-ICR 20.3 with red blood cells and blood vessels and the reaction of the monoclonal antibodies generated with normal colon are similar to many other monoclonal antibodies raised to colorectal cancer. The current methods of tumour immunogen preparation, such as cell lines or various forms of tumour extracts, have not, in general, produced monoclonal antibodies with significantly different colorectal cancer specificity. Further production of such antibodies using similar immunogens does not, therefore, seem worthwhile particularly as the effort involved is considerable. The production of potentially more tumour-specific monoclonal antibodies is discussed in 7.4. However, UC-ICR 41.2 and 41.6 appear promising, as they reacted with 94% of different colorectal cancers, and had a restricted normal tissue distribution. The reaction with normal colon would not necessary limit their application to colorectal cancer, as such reactivity is a feature common to number of monoclonal antibodies used in the immunoscintigraphy of colorectal carcinoma. The reaction of UC-ICR 41.2 and 41.6 with 90% of colorectal polyps, although with a small percentage of cells in each tumour, may be an advantage, as detection and removal of such polyps may prevent their progression to carcinoma. Further assessment with tnese two monoclonal antibodies, particular in their reaction with circulating cells or antigens (see 7.3.1), would be worthwhile to determine their suitability for clinical application. It would also be appropriate to investigate the cross-reactivity of UC-ICR 41.2 and 41.6 with other carcinomas.

It is a common practice by most investigators not to use antibodies reacting with a large number of normal tissues for tumour localisation. However, it is possible that antibodies with good utility for imaging or targeting may have been rejected with this criterion. The presence of a tumour associated antigen on normal tissue does not necessarily cause a high uptake of labelled antibody by that tissue.

The uptake of indium-111 labelled anti-CEA monoclonal antibody by normal colon, for example, is no greater than the uptake of an anti-melanoma antibody by the colon (Halpern & Dillman, 1987). It is not clear why a normal tissue that expresses an antigen reactive with an antibody does not acquire a large amount of that antibody. This may be related to antigen density, accessibility or most probably, to unknown factors (Halpern & Dillman, 1987). The experience with anti-CEA antibodies suggests that investigation into the use of antibodies which react with normal tissues is justified (Halpern & Dillman, 1987).

More than 100 monoclonal antibodies to various human carcinomas have now been reported in detail (Schlom & Weeks, 1985). These antibodies reveal several basic principles regarding tumour associated antigens (TAA) (Schlom, 1986). These are: a) A given tumour associated antigen is usually expressed by more than one type of carcinoma. A monoclonal antibody generated against a breast cancer is likely to react with other carcinomas such as colorectal or lung cancer or vice versa; b) Not all tumours within a given tumour type will express a given tumour associated antigen; c) not all cells in a given tumour (primary or secondary) will express the tumour associated antigen, as a result of the phenomenon of tumour heterogeneity; d) there is not only antigenic heterogeneity among carcinoma cell populations but also a temporal modulation of tumour antigens; e) many tumour associated antigens are stable components of the cell membrane, so that antibodies binding to these antigens on the cell surface are not internalised, capped or shed; f) tumour associated antigens may be 'private' or 'public'. The more public an antigen the more likely that it will be expressed by other carcinomas and normal cell. The more private antigens are not expressed or expressed weakly by normal adult cells. However, a smaller percentage of tumours or cells within a given tumour express the private antigen.

7.2.2 Screening for colorectal cancer-reactive antibodies

Because tumour-selective monoclonal antibodies were not obtained directly by tumour membrane imunization, other monoclonal antibodies were screened in this study. In the screening of such antibodies for cross-reactivity with colorectal cancer, immunoperoxidase staining identified three antibodies: M8, 77-1 and 8-30-3. These antibodies react with epitopes of epithelial membrane antigen (EMA) and they react with a wide variety of epithelial cells. However, they show selectivity for colorectal cancer when compared with normal colon and polyps. This preferential reaction with cancer places them in a favourable position for imaging colorectal cancer.

Epithelial membrane antigen (EMA) is a large and heavily glycosylated membrane protein, first described by Heyderman et al. (1979). It has a wide distribution in normal and malignant epithelial cells (Sloane & Ormerod, 1981), and is useful in determining the epithelial nature of malignant cells. In a survey of various tumours, Pinkus and Kurtin (1985) have found that EMA is expressed by various adenocarcinomas (breast, lung, colon, stomach, pancreas, gallbladder, prostate, endocrine glands, ovary, kidney, thyroid). It is also expressed by squamous and transitional cell carcinomas, small cell anaplastic carcinomas and mesotheliomas. Malignant lymphomas of the Hodgkin's and non-Hodgkin' types, except for the true histiocytic lymphomas and occasional T-cell lymphomas are nonreactive with EMA. Endocrine tumours, including carcinoid tumours, medullary carcinoma of the thyroid, adrenocortical carcinomas and pheochromocytomas, germ cell tumours and a variety of soft tissue tumours are generally non-EMA expressing. The same is true for malignant melanoma. Our results here confirm that EMA is expressed by more cells of colorectal cancer than normal colon, and the staining intensity is also stronger with the cancer.

It is interesting that adenomatous polyps only express EMA at a lower frequency than colorectal cancer but more than normal colon. It is generally accepted that colorectal cancer develop from polyps in the so called adenoma-carcinoma sequence (Morson & Day, 1981). The progressive expression of EMA in the polyps tend to support this theory. The risk factors for a malignant change in polyps are size - if they are bigger than 2 cm.; if they are dysplastic and if they have more villous change (Morson, 1978). It would be interesting to see if those polyps which express EMA show a greater potential for malignant change.

The nature of the antigens recognised by many tumour-reactive monoclonal antibodies is unknown. The considerable effort required to identify them means that only those antigens with a proven interest have been fully characterised. There is thus a confusion in the terminology of antigens recognised by monoclonal antibodies when full identification has not been carried out.

This is a continuing problem as new monoclonal antibodies are being produced all the time (Fink & Clarke, 1984). Thus monoclonal antibodies produced in different laboratories which recognise similar antigens may be called by different names. There are similarities between Ca1 antibody and HMFG-1 and HMFG-2 monoclonal antibodies which were raised against human milk fat globule membrane. HMFG-1 recognises two membrane glycoproteins with molecular weights between 300 and 400kD mol wt while Ca1 recognises similar glycoproteins of 350 and 390kD (Woodhouse et al., 1985). M8 has a similar properties to the monoclonal antibodies HMFGM 1 and HMFGM 2, which were raised against human milk fat globule (Taylor-Papadimitriou et al., 1981). It is probable that all these antibodies are reacting with various epitopes of EMA.

7.3 OBSERVATIONS ON THE IMMUNOSCINTIGRAPHY OF COLORECTAL CANCER

7.3.1 Xenograft localisation

A xenograft localisation study was performed for the radiolabelled monoclonal antibody 77-1. The result confirmed the localisation of both the indium-111 and the iodine-125 labelled antibody to the target xenograft. However, xenograft localisation cannot be used to predict the localisation in patients (Halpern & Dillman, 1987; Goldenberg, 1988). Three monoclonal antibodies YPC 2/12.1 (Finan et al., 1986), and two anti-CEA antibodies 065 and 326 (Dillman et al., 1984) showed good localisation to xenografts, yet failed to localise to tumour in clinical studies. Finan et al. (1986) stressed the importance of careful clinical study and pointed out that animal localisation study might be misleading.

In the case of the anti-CEA antibodies, the reaction of some of them with an antigen present on the human granulocytes and erythrocytes was not detected by the xenograft model. Administration of the indium-111 labelled antibodies resulted in the absorption of the antibodies by the blood cells and free CEA and their clearance by the reticulo-endothelial system. The monoclonal antibody YPC 2/12.1 also reacts with polymorphonuclear leucocytes in the peripheral blood and with cells of the granulocyte series in the bone marrow (Smedley et al., 1983). Another anti-CEA monoclonal antibody 202 also cross-react with granulocytes and myelocytes in the bone marrow and was not used further (Mach et al., 1985). An important study before the application of 77-1 was therefore the immmunocytochemical staining of the cells of the human bone marrow which proved negative. It can be argued that in the preparation of an appropriate monoclonal antibody for clinical localisation, xenograft localisation

may be omitted. In the preparation of the monoclonal antibody 2-94-2 for the immunoscintigraphy of breast cancer, no xenograft localisation study was performed (Ward, 1987), and the radiolabelled antibody did localise successfully to breast cancer. However, a positive result on an appropriate xenograft, while not guaranteeing human tumour localisation, does provide useful information on antibody-tumour reactions and confirms the *in vivo* immunoreactivity of the radiolabelled antibodies.

7.3.2 Immunoscintigraphy

The monoclonal antibodies M8 and 77-1, which react with epitopes of EMA, were chosen for the immunoscintigraphy of colorectal cancer as they showed a greater reaction with the carcinoma than normal colon. This study is the first detailed assessment of the use of anti-EMA monoclonal antibodies in the immunoscintigraphy of colorectal cancer. Previous study using similar type of antibodies, the monoclonal antibody HMFGM 1, has shown that the scans were positive in two out of four colorectal cancer patients (Epenetos et al., 1982b). The expression of EMA by other normal tissues would not seem to be a hindrance to its use for immunoscintigraphy due to their expression in relatively low concentration and at sites not readily accessible to circulating antibodies (Halpern & Dillman, 1987). The immunoscintigraphy results of M8 and 77-1 confirmed that colorectal cancer reactive antibodies suitable for imaging could be selected by means of immunocytochemistry. The scanning results with M8 and 77.1 are similar to most other antibodies (19.1, 739/36T, B72.3, and Mab 23) being in the region of 65% (6.3). Most of these antibodies react with other tumours and normal tissues (3.4). It is possible that M8 and 77-1 may also be used for the immunoscintigraphy of other carcinomas. The overall result of imaging of various tumours are in the

region of 60-80%, for example, malignant melanoma, breast cancer, colorectal cancer, head and neck cancer, ovarian cancer and malignant lymphoma (Goldenberg et al., 1987).

This finding has shown that other anti-EMA antibodies may be used for the immunoscintigraphy of colorectal cancer. Like anti-CEA antibodies, it is likely that other anti-EMA antibodies, by reacting with different epitopes of the antigen with different distributions, concentrations or accessibilities, may have different overall reactivity with colorectal cancer and normal colon. In the evaluation of new anti-EMA monoclonal antibodies for the immunolocalisation of colorectal cancer, a comparison of the reactivity between anti-EMA and anti-CEA antibodies with colorectal cancer and normal colon would be of interest. There has been report of raised EMA level in the circulation of breast and colon cancer patients (Hendrick et al., 1986). EMA , like CEA, may act as a marker for monitoring the progress of colorectal cancer patients, and a further study into the value of circulating EMA in the follow-up of colorectal cancer patients would be worthwhile. The presence of free antigen in the circulation does not necessarily compromise localisation, as seen with, for example, anti-CEA antibodies. It may, under some circumstances, actually improve results as circulating antigen-antibody complexes may be cleared faster than free radiolabelled antibody, and reduces background radioactivity. The administration of unlabelled anti-EMA antibody before the injection of radiolabelled antibody may thus help to achieve better imaging results.

The reactivity of new anti-EMA antibodies with colorectal cancer may be checked by immunocytochemistry, and the retention of their immunoreactivity following radiolabelling confirmed by a cell binding assay. Provided that they do not react with circulating cells they may be used clinically without a xenograft localisation study. The monoclonal antibody ICR 2 is a new high affinity

anti-EMA monoclonal antibody raised against EMA, purified from human milk fat globule membrane, produced at the Institute of Cancer Research (C. Dean, personal communication). An assessment of ICR 2 in the immunoscintigraphy of colorectal cancer would be appropriate as a result of the finding with M8 and 77-1.

In common with the results reported by others, the experience in the production and immunoscintigraphy of colorectal cancer in this study has revealed several factors which limit the effectiveness in the clinical application of monoclonal antibodies. Some are antibody related, others radioisotope related and some reflect limitations in imaging techniques.

7.4 DEVELOPMENTS IN THE CLINICAL APPLICATION OF MONOCLONAL ANTIBODIES

7.4.1 Improved tumour specificity of monoclonal antibodies

Various methods have been used to produce monoclonal antibodies with greater tumour specificity. However, as discussed in 7.2.1, the generation of monoclonal antibodies using current immunogen preparations is unlikely to yield antibodies with greatly different specificities. Many of the monoclonal antibodies raised to carcinomas or human milk fat globule membranes have now been shown to react with epitopes present on large molecular weight glycoproteins, and that many of the antibodies react with the same group of molecules which appear to be mucins, including EMA (Burchell et al., 1987).

Burchell et al., (1987) examined whether mucin found in normal differentiated cells and those expressed by tumours contained the same core protein by raising monoclonal antibodies to the milk mucin core protein. The immunogen

was prepared by stripping the carbohydrate component of the human skimmed milk by treatment with hydrogen fluoride. These antibodies were used to study the processing of mucins and to isolate the gene coding for the core protein. One of these antibodies SM3 reacts with 91% of breast carcinomas but show little or no reaction with benign breast tumours or normal, resting or lactating breast. It appears that this monoclonal antibody reacts with an epitope that is normally masked by oligosaccharide in normal cells but which is exposed in cancer cells.

It is not yet clear how many genes code for the different mucins, and whether more than one of these genes is expressed by a single type. Taylor-Papadimitriou (1989) has obtained partial cDNA clones for the human mammary mucin. This has enabled the prediction of part of the protein sequence of the core protein and to look for its expression in other cell types. The gene shows a polymorphism which is due to the presence of varying numbers of a 60 bp tandem repeat element. The mucin shows cross reaction with primate DNA but not with rodent, explaining the strong immunogenicity of mammary mucins in the rodent.

A synthetic peptide from the gene probe has reacted with antibodies HMFG-1, HMFG-2 and SM-3, each of which bind to a different epitope. The identification of epitopes which are preferentially expressed on carcinomas should allow us to produce more tumour specific monoclonal antibodies and to study the differences in the processing of mucins seen in carcinomas.

A second generation of monoclonal antibodies may also have improved specificity. The antigen recognised by the monoclonal antibody B72.3 which is a high molecular weight glycoprotein, with properties of mucin, termed TAG-72 (tumor-associated glycoprotein). Purified TAG-72 has been used as an immunogen to produce B72.3 second generation monoclonal antibodies (Muraro et

al., 1989). Xenograft studies showed that these antibodies were better than B72.3 in their localisation to tumours (Colcher et al.,1988). There are, however, some disadvantages in this approach, for example, the low immunogenicity of purified proteins, and the difficulty involved in the purification of some antigens. Some antigen proteins may be unknown, or the antibodies may not immunoprecipitate them.

7.4.2 Computer modelling of antigen-antibody reaction

It is now possible to explore the molecular basis of antibody affinity and specificity by combining the techniques of molecular modelling using computer graphics, energy calculations and protein engineering using site directed mutagenesis. Webster et al. (1989) have shown that they were able to specifically engineer antibodies with altered affinities from native protein. The topographical surfaces of antibodies can be altered by a small number of changes, resulting in effects on antibody binding, ranging from complete abolition to increased affinity for antigen. Once the rules of antibody-antigen interactions are established, high affinity specific antibodies can then be designed.

7.4.3 Suppression of Human anti-mouse antibody (HAMA) Response

The administration of mouse monoclonal antibodies to human has resulted in a human anti-mouse antibody response (HAMA). This response may be directed at the immunoglobulins in general or at the idiotypic regions (Shawler etal., 1985; Jaffers et al., 1986). Thus Chatenoud et al. (1989) studied 75 patients who underwent renal transplants and received OKT3, an anti-T cell monoclonal antibody for immunosuppression. OKT3 was given consecutively for 14-30 days.

It was found that human anti-OKT3 antibody response consisted of both IgG and IgM and was against both the OKT3 isotypic and idiotypic determinants. Moreover, this response was shown to be oligoclonal. Only the IiI anti-idiotypic component neutralises the therapeutic effects of monoclonal antibodies used for immunoscintigraphy. When antibodies were administered at doses of about 1 mg, 15 patients showed significant anti-idiotypic anti-mouse titres 10 to 20 days following even one antibody injection.

The use of monoclonals which recognise different antigens or epitopes of the same cancer, in rotation, for example, anti-CEA and anti-EMA monoclonal antibodies is one way of avoiding the HAMA response, if multiple doses have to be administered, as anti-idiotypic antibodies to one will not block the others.

The HAMA response can be suppressed by giving the patients an immunosuppressant such as cyclosporin A which is a potent inhibitor of the humoral immune response (Borel et al., 1976). Using ^{131}I labelled antibody for therapy of CEA producing tumours, Ledermann et al. (1989) have shown that cyclosporin A can delay and reduce the intensity of human anti-mouse antibody response so that up to 4 times as many doses of antibody therapy can be given.

The HAMA response can also be abrogated by induced tolerance. The coupling of an optimal number of monomethoxypolyethylene glycol (mPEG) chains (M. Wt. 3,000-6,000) results in tolerogenic derivatives. Sehon (1989) injected human myeloma monoclonal immunoglobulin conjugated with mPEG into inbred mice followed 7 days later with injection with immunogenic heated aggregated human monoclonal immunoglobulin (Ha-HIgG). This resulted in a marked reduction of murine response (-90%) of IgG, IgM and IgA antibodies to HIgG. The suppression was dose-dependent and the state of tolerance could be transferred with the spleen cells of immunosuppressed mice to syngenic recipients,

as well as by freeze thaw extracts of these cells. The possible mechanism underlying immunosuppression by mPEG conjugates may involve the activation of suppressor T cells. In the therapy context, mPEG conjugates should be injected before injection of monoclonal antibodies.

7.4.4 Human monoclonal antibodies

Human monoclonal antibodies should reduce the problem of anti-antibody responses as occurred with mouse antibodies, although they are not devoid of problems in this regard. They should reduce the induction of antibodies in general and be less likely to be compartmentalized in normal tissues and therefore reduce the non-specific background activity during immunoscintigraphy (Halpern & Dillman, 1987). However, human monoclonal antibodies with useful specificities are still very difficult to produce. At present, there are only a few human monoclonals available due to technical difficulties which include unstable hybrids, low hybrid yield, the dependent on a natural process of immunisation in the human and low antibody production by the hybrid (O'Hare and Yiu, 1987). So far, there are only three reported human monoclonals to colorectal cancer (Haspel et al., 1985; Borup-Christensen et al., 1986; Yiu et al., 1987; O'Hare et al., 1989). In the study by the author (Yiu et al., 1987; O'Hare et al., 1989) lymph node lymphocytes from 19 colorectal cancer patients and 6 patients with inflammatory bowel diseases were used to generate human monoclonal antibodies to examine the immune response to these diseases. A total of 2000 hybrids were generated. Twelve of these secreted antibodies reactive to colorectal cancer cell membrane and cell lines on screening. Further characterisation of these antibodies is underway. Advances in *in-vitro* immunisation and the application of cell sorter to screen hybrids may overcome some of the difficulties outlined (O'Hare and Yiu,

1987). Human monoclonal antibodies have recently been used successfully in the immunoscintography of colorectal cancer. McCabe et al. (1989) reported that 20 out of 24 patients with colorectal cancer were detected with ^{131}I labelled human monoclonals 16-88 or 28A32 with lesions as small as 1.5cm detected. Urticaria was seen in two patients. There was no reaction to 16-88 in any patients. Injection of up to 200 mg of antibody consecutively for up to 4 weeks did not show any toxicity. Two patients had positive skin tests, indicating an antibody response to 28A32. Those patients received 28A32 only showed a four-fold increase in anti-28A32 titre over the 2 month observation period. It is not yet clear whether this is an anti-idiotypic or anti-allotypic response. An IgM human monoclonal antibody L55 produced by a B-lymphoblastoid cell line and which reacts with the ganglioside GM2 of malignant melanoma has been used in the therapy of this tumour by direct intralesional injection. This resulted in the regression of the tumours in two patients (Irie et al., 1989).

7.4.5 Chimaeric monoclonal antibodies

An alternative to the production of human monoclonal antibodies is to humanise desirable mouse monoclonal antibodies by forming chimaeric antibodies using recombinant DNA techniques. Details of the procedure were reported by Morrison et al. (1984) and Jones et al. (1986). A chimaeric antibody has been produced using these techniques from a mouse antibody B6.2. This was achieved by coupling cDNA clones from B6.2 - producing hybridoma cells coding for the variable regions which dictate antibody specificity, to human genomic gamma 4 and kappa constant region genes. The chimaeric immunoglobulin genes were placed under the control of a strong viral promotor, and transfected into COS-1 cells. The antibodies so formed were correctly synthesised and retained the binding

characteristics of the parent B6.2 antibody. The reactivity of the chimaeric antibody was similar to the original antibody (Sahagan et al., 1986). Another mouse monoclonal antibody B72.3, reactive with colorectal cancer has also been converted to a 'human' form using genetic engineering. The localisation of the chimaeric antibody in nude mice was equivalent to that of the parent B72.3 (Bodmer, 1988). This technology can be applied to other rodent antibodies.

7.4.6 Improved access of antibody to tumour

i Use of Fab fragments

The variable or idiotypic regions which form the antibody-antigen binding sites are contained in the $F(ab')_2$ regions of the antibodies. The constant or Fc fragments of the antibodies do not take part in the antibody-antigen reaction but do contain non-specific binding domains (so called Fc receptor region) which results in immune complex formation. By removing the Fc fragments from the antibody by enzyme cleavage, the Fab fragments will be cleared from the circulation faster than the intact antibodies by eliminating receptor binding to cells in the liver, lung and spleen (Mach et al., 1980; Carrasquillo et al., 1984; Wahl et al., 1983). This will tend reduce the background of the radiolabelled antibodies and thus a better image may be obtained.

However, for therapeutic administration the faster clearance of $F(ab')_2$ fragments is a disadvantage (Ballou et al., 1985). Moreover, antibody fragments are not easy to prepare, and many $F(ab')_2$ fragments are too unstable to be used clinically (Halpern & Dillman, 1987). The Fc moeity of the antibody may also prolong its residence in tumour through immune complex formation. One

theoretical solution to this problem in the use of $F(ab')_2$ fragment is to inject another Fab fragment directed at the first $F(ab')_2$ so as to induce immune lattice formation (Rogers, 1986). Another possibility is to increase the molecular weight of $F(ab')_2$ fragments, so that they remain in the vascular compartment for a longer period and enhance their uptake by the tumours (Halpern & Dillman, 1987), but without the binding to liver, lung and spleen seen with intact antibody.

ii Bi-specific antibody

Another approach to enhance tumour uptake is the use of bi-specific ('hybrid') antibody $F(ab')_2$ fragments (Boss et al., 1985; Brennan et al., 1985). One arm of the fragment reacts with the tumour antigen, and the other with a synthetic peptide which can be radiolabelled (Rogers, 1986).

The development of bi-specific antibodies (Boss et al., 1985; Brennan et al., 1985) with ability to bind tumour antigen and another site directed at a T-cell receptor as suggested by Staerz et al. (1985), or a synthetically constructed radioactive source may be effective for therapy. This prospect is encouraged by the production of monoclonal antibodies to metal chelates (Reardan et al., 1985). The antibody would be administered in the unlabelled state and localises to the tumour. A radiolabelled molecule reactive with the metal chelate binding site is then administered. The localisation of this radiolabelled molecule to the bi-specific antibody will enable the tumour to be detected by scanning.

iii Antigen enhancement

To maximise the reactivity of antibodies with tumours, one possible solution is to increase antigen expression by the tumours. This has been possible

to some extent in vitro by the use of drugs such as theophylline, interferon, and other compounds. Further research efforts in this area may result in an enhanced antigen expression by tumours in vivo (Halpern & Dillman, 1987).

iv Antibody for future radioimmunodetection or therapy

Taking the above problems and concepts into considerations, Halpern and Dillman (1987) postulated that antibody for future immunoscintigraphy or targeting will have the following characteristics:

1. It will be an altered fragment, rather than an intact molecule or fragment.

2. It will be small, perhaps 60,000 molecular weight, yet remain in the vascular compartment for a comparatively long period of time, then be eliminated via the kidney.

3. It will be of human origin or a murine molecule altered to mask its immunogenic properties.

4. It will be ^{111}In- or ^{99m}Tc-labelled or bifunctionally chelated to another metal ion.

5. It will target a cell surface antigen, but one that does not circulate.

6. It will be used in combination with derivatives of other monoclonal antibodies that target other antigens on the cell.

7. It would be a hapten-type device that follows the administration of a "bifunctional monoclonal antibody".

8. Whatever the final molecule, it will be administered to a patient who has been "prepped" with an antigen-enhancing substance or one that "unmasks" a gene and allows a repressed marker to be expressed by the tumour cell to which the monoclonal antibody was developed.

9. It may be a monoclonal antibody that attaches to a white cell, which then chemotactically seeks the tumour that has been previously induced to produce a substance that the white cell recognises.

7.4.7 New radioisotopes and chelates for immunoscintigraphy

i Problems with iodine and indium

Iodination has been the standard for radiolabelling proteins for many years. The tendency of radioiodinated antibodies to de-halogenate *in vivo* is a major problem. This leads to urinary excretion and free iodide accumulation in the thyroid and gastric mucosa (Begent, 1985). Dehalogenation may be related to the iodination methods (Halpern & Dillman, 1987). For example, the Bolton-Hunter reaction seems to be the most unstable of all the iodination procedures (Stern et al., 1982). However, the problems associated with iodination are subtle and difficult to determine *in vitro* (Halpern & Dillman, 1987). *In vitro* radioimmunoreactivity cannot predict the *in vivo* stability of the labelled antibody which can be removed rapidly from the circulation. Another danger is overlabelling of antibodies which may lead to loss of immunoreactivity, with the radiopharmaceutical leaving the vascular compartment quickly with rapid dehalogenation. An increased uptake by the liver and spleen may result due to a change in bio-distribution. The uniqueness of each monoclonal antibody also makes it difficult to standardise iodination method, as using the same procedure, some iodinated antibodies will dehalogenate at a rapid rate, whereas others will remain stable (Halpern & Dillman, 1987).

A new antibody iodination technique has been described by Wilbur et al. (1986). The iodine molecule is not added to the antibody directly but to added groups. This process is said to reduce dehalogenation. However, the general

effectiveness of this method remains to be seen.

Indium-111 labelled antibodies have the advantages of better images not requiring subtraction. However, the high uptake by the liver associated with this radioisotope remains a problem, particularly with colorectal cancer in which liver metastases are main sites of secondaries.

ii New chelating agents

As the chelating agent DTPA appears to play a large part in the liver uptake of labelled antibodies, new chelaters have been under development. The acyclic chelates commonly used (EDTA, DTPA-based) have the problem of metal dissociation. Macrocyclic ligands would be better as acid-promoted dissociation would be slower. New methods have been developed for the selective attachment of these macrocycles to the thiol residues on an antibody for ^{99m}Tc and ^{64}Cu (Eaton et al., 1989).

Other bifunctional ligands are also being developed. Maecke et al. (1989) synthesied macrocycles for antibody labelling with radionuclides. They found that monofunctional macrocycles were suitable for labelling with Cu^{2+} and $Co^{2+}(Co^{3+})$. The complexes are very stable even in acidic environment. New ligands designed for $^{111}In^{3+}$ and $^{90}Y^{3+}$ have been developed using 1,4,8,11-tetraacyclotetradecane-N,N'N''-triacetic acid (14-aneN_4triac) as the starting material. The secondary amine was alkylated to produce bifunctional compounds.

Gansow et al. (1989) reported that polyazamacrocyclic polymethylenecarboxylate has the best thermodynamic stability constants for ^{90}Y, ^{203}Pb, ^{212}Bi which are suitable for imaging and therapy. These complexes have been synthesised for conjugation to monoclonal antibodies.

It is too early to determine which of these new techniques will overcome the uptake of radiolabelled antibodies by the liver. However, it can be expected that these developments will eventually lead to an improvement in the detection of metastases in the liver. In the future, imaging will probably be performed using only metal chelates, as the advantages of the isotopes that can be chelated over isotopes of iodines are such that their general use is almost inevitable (Halpern & Dillman, 1987). In the preparation of the new anti-EMA monoclonal antibody ICR 2 for the immunoscintigraphy of colorectal cancer, tissue culture of hepatocytes may be used to evaluate the uptake of different chelating agents, before assessment with animal studies.

iii Other radioisotopes

At present radioiodines and indium-111 are the radionuclides most widely used for antibody labelling. To overcome the problems described above, however, other isotopes are being tested clinically.

^{99m}Tc was used to label the monoclonal antibody NR-M1-05, NR-LU-10 and NR-Ce-01 which recognised antigen expressed by melanoma, lung and colon cancers. The preformed chelate approach was used with a diamide dimercaptide (N_2S_2) ligand. Tumours were detected by 6-20 hours in 200 patients with these cancers. Increased uptake was only seen in the thyroid (cross-reactivity) and ocassionally in areas of osteoarthritis positive on bone scan, and infrequently in inflammatory lesions. However, excretion of the label via the renal and the hepatobiliary routes complicated interpretation of images (Abrams et al., 1989).

Using bifunctional chelating agent Halpern and Dillman et al. (1987) have also been able to prepare ^{90}Y labelled antibodies which have the same in vivo properties of endogenously labelled antibodies. These antibody preparations are

under assessment.

7.4.8 Problems with imaging

Nuclear medicine technique in general is dependent on two factors: the absolute concentration of radioactive substance in the target, and its concentration in the background tissues. A low concentration of radiolabelled antibody in the tumour will result in low quality imaging unless the scanning is extended over a long period and the background is low. However, a high background will not permit a good image irrespective of the amount of radiolabelled protein in the tumour (Halpern & Dillman, 1987). Compartmentalization of the radiolabel is therefore important. The background and compartmentalization of a monoclonal antibody is related to several factors. With regards to indium-111 labelled antibodies the most important factors are the particular antibody and the absolute mass of the antibody administered. It is advantageous to maintain a high level of radiolabelled antibody in the blood to improve the tumour detection rate (Halpern & Dillman, 1987). This mass effect has not been well documented with iodinated antibodies.

The absolute concentration of radiolabelled antibodies in tumour is another problem in radioimaging. Mach et al. (1980) and Carrasquillo et al. (1983) reported localised ^{131}I-labelled antibody as about 0.001% of the injected dose per gram of tumour.There are several reasons for the low radiolabelled protein uptake, which include poor blood flow to the tumour and poor antibody extraction in each pass through the tumour capillary bed (Halpern & Dillman, 1987). The use of fragments actually reduces the amount of radiolabel in the tumour, principally because the smaller fragments are excreted by the kidneys more quickly than the increased extraction by the tumour (Halpern & Dillman, 1987).

The optimal amount of radiolabelled antibody in the tumour for good imaging is dependent on the background activity, the amount of antibody that can be administered safely, and the physical characteristics of the radionuclides, for example, the photon energy, photon abundance, collimation required, physical half-life, and the imaging equipment available.

i Computerized tomographies : SPECT, PET

Static photoscanning is limited in part by the radioiodine release through dehalogenation causing a high background radioactivity. Axial transverse tomoscintigraphy developed by Kuhl and Edwards (1963) may solve some of these problems. This technique is also called single photon emission computerized tomography (SPECT) or ECT, corresponds to the application of the tomographic technique used in transmission CT scanning. Mathematical techniques similar to those used in positron and x-ray tomographies enable the reconstruction of transverse sections as well as frontal, sagital, or oblique sections of patients. ECT improves the sensitivity and specificity of tumour detection using ^{131}I-labelled anti-CEA antibody Mab23 (Mach et al., 1985). However, numerous non-specific radioactive spots, some as intense as the tumour, were present, but the three dimensional localisation of radioactive spots by ECT helped to resolve the true tumour uptake from non-specific 'hot spots' (Mach et al., 1985).

Positron Emission Tomography (PET) is a computed tomography that provides information about the physiological and biochemical processes in addition to anatomical data. Its major difference from other forms of computed tomography such as CT scan and NMR is the requirement of a positron emitting compound that traces these processes (Hawkins & Phelps, 1989). All three techniques employ the same computer-based image generation method.

PET is being applied to detect tumours using ^{124}I labelled monoclonal antibody at the Royal Marsden Hospital, Sutton (Babich, Personal communication). It is hoped that this technique would generate superior images to those obtained with conventional gamma scans.

ii Intra-operative probe

External imaging technique suffers from the reduced ability to detect deep seated lesions as the sensitivity varies inversely with the square of the distance from the source. The sensitivity of radioimmunodetection can be improved by the use of a hand-held gamma-detecting probe (GDP) for use at operation. The influence of the inverse square law is minimised as the probe is held over the lesion; any residual tumour can be identified and objective interpretation of gamma count is possible (Sickle-Santanello et al., 1987). Preliminary study in 6 patients with primary colorectal cancer showed that tumour detection was achieved in 89% of cases. In 31 patients with recurrent cancer, tumour was diagnosed in 82.4% of the cases. False positive results were obtained in a patient with Crohn's disease, adenomatous polyp and a patient with a partially obstructed small bowel.

iii Second antibody for imaging

One of the problems with immunoscintigraphy is high background. A number of methods have been used to reduce the background. The use of more specific antibodies has already been discussed. Another approach is the use of a second antibody directed against the first antibody to accelerate its clearance from the circulation. Begent et al. (1982) used a liposomally entrapped second antibody (LESA) to reduce the blood levels of anti-CEA antibody. An enhanced clearance of

anti-CEA antibodies was observed in patients with gastric cancer 24 hours after the intravenous administration of LESA. However, it was found later that the liposome entrapment was not necessary and the second antibody worked just as well. In a study involving 5 patients with colorectal cancer, a second antibody directed against an anti-CEA antibody was used. The clearance of radioactivity was accelerated and the tumour imaging was enhanced. There was no significant toxicity with the use of the second antibody (Begent et al., 1987).

7.4.9 New methods of antibody localisation

The avidin-biotin system used in the sandwich technique in immunocytochemistry has also been applied to tumour localisation in vivo. In this method, the strong affinity between avidin and biotin is used as a link for antibodies and radionuclides: the avidin is conjugated to the antibodies and the biotin to the radionuclides. The antibody conjugate is injected first to allow time for antibody conjugate localisation to the tumour at the same time to permit clearence of unbound conjugate to reduce the background. This is now followed by the injection of the radiolabelled biotin which will localise to the avidin-antibody conjugate and allow immunoscintigraphy to be performed. By substituting stronger radioisotopes, therapy can also be performed. This technique has the advantage of magnification and reduced background. Hnatowich et al.(1989) tested this system by conjugating the HMFG-1 antibody to streptavidin and injected it intravenously into nude mice. This was followed at 1,4 and 7 days later with injection of ^{111}In labelled biotin. At 2 hours, the radioactivity levels in normal tissues were lower than the conventional method of antibody localisation and the tumour to normal tisue uptake was also increased by 4 times. The only exception was the kidneys which probably excrete the product.

Paganelli et al. (1989) used a reverse procedure to Hnatowich by conjugating the biotin to the antibody and the radionuclide to the streptavidin in a study using a colorectal cancer xenograft. They reported that this two step approach increased the percentage of radioactivity uptake by the tumour (24% v 6%) and improved the target to non-target ratio by an index greater than 6.5.

7.4.10 Targeting of monoclonal antibodies

An important goal in the application of monoclonal antibodies to colorectal cancer is the targeted therapy of metastatic tumours. This may be achieved by 'arming' monoclonal antibodies with cytotoxic agents. Much of the investigation is still in the laboratory stage. However, clinical trials of antibody-targeting have been started in leukaemia, ovarian tumours and leptomeningeal tumours with some success.

i Chemotherapeutic agents

The monoclonal antibody 791T/36 has been conjugated with cytotoxic drug methotrexate either directly or by a human serum albumin bridging agent. The retention of immunoreactivity of the conjugates was tested by their competitive binding with unlabelled antibody and target cells, measured by flow cytometry. The retention of drug cytotoxicity was tested by colony inhibition assays. Xenograft study showed that the antibody-methotrexate conjugate had a greater therapeutic effect than methotrexate given alone (Baldwin et al., 1987).

ii Prodrug targeting

Another approach developed by Bagshawe et al. (1989) to improve the selectivity and effectiveness of antibody targeting is to use a two-stage technique in

which an activator for a cytotoxic precursor is delivered first to the tumour using monoclonal antibody as a carrier; the precursor of a cytotoxic agent is then administered after clearance of the antibody-conjugated first component from the circulation. A local cytotoxic effect results when the toxic component is activated by the first. In a xenograft study, a bacterial enzyme carboxypeptidase G2 (CPG2) was conjugated with $F(ab')_2$ fragment of a monoclonal antibody reacting with the beta subunit of human chorionic gonadotrophin and injected into nude mice bearing human choriocarcinoma expressing this antigen. After localisation of the antibody conjugate to the tumour and its clearance from the circulation, para-N-bis (2-chloroethyl) aminobenzylglutamic acid was administered. A benzoic acid mustard was released when the glutamic acid moeity of this substance was removed by CPG2, and this suppressed the growth of the tumour significantly (Bagshawe et al., 1989).

iii Toxins

A potential disadvantage of drug targeting is the possibility of low antigen density and therefore the difficulty in accumulating an effective dose in the tumour. Immunotoxins have shown great potency in killing cancer cells in the in-vitro conditions. However, in animal studies three problems have been encountered. 1. The ricin A-chain immunotoxins are cleared from the circulation rapidly because of the oligosaccharides present on the toxin moiety which is recognised by liver cells (both parenchymal and non- parenchymal). 2. The disulphide cross-linking agents available commercially are not suitable for forming stable A-chain immunotoxins in the in vivo environment. 3. The contamination of free antibody which competes for target antigens.

The antitumour activity of ricin A-chain immunotoxins is greatly

improved by deglycosylating the A chain to prevent liver uptake. A better cross linker (SMPT) is also now available to form immunotoxin. Free antibody can be removed by Blue Sepharose chromatography. In an experimental study all but 0.001% of murine T-cell leukaemia cells were killed by a single injection of immunotoxins. Clinical study is now awaited (Thorpe, 1989). However, solid tumours such as colorectal cancer are likely to pose more problems. Monoclonal antibodies have recently been raised to a dimethylhydrazine induced rat colorectal cancer model for immunotoxin targeting study (Baker et al., 1989).

iv Radioisotopes as therapeutic agents

The choice of a suitable radioisotope is essential to achieve a balance between toxicity to haematopoietic system and normal tissues and the destruction of tumour. The nuclide must decay with the emission of high LET (linear energy transfer) radiation and to do so with an appropriate physical half-life so that the radiation does not penetrate and damage normal tissue beyond the target (Hnatowich et al., 1989). Both ^{131}I and ^{90}Y partially fulfil this requirement. The half-life of ^{131}I at 8 days is probably too long for therapy, that of ^{90}Y at 64 hours is better, but the logistics of labelling is made difficult. In the decay of ^{90}Y only beta rays are emitted, so that adjacent tissues are not involved. The strong gamma rays emitted by ^{131}I may not be desirable therapeutically but they can be used for external detection and for dosimetry calculations.

A radioimmunotherapy of human colon carcinoma implanted in athymic mice had been performed using the monoclonal antibody B72.3 labelled with ^{131}I (Estaban et al., 1987). Injection of a dose of 300 uCi or 500 uCi resulted in a marked inhibition of growth of the xenograft. The control tumours weighed 2.7 to 3.7 more than that of the treated ones and extensive necrosis of the latter was

demonstrated. The lower radiation dose caused bone marrow supression of approximately 50% of the cells. However, the higher dose caused bone marrow aplasia and destruction of the spleens. This was lethal in 2 out of 10 animals. Even with the higher radiation regime, the tumour cells at the periphery continued to grow. It was possible that some cells escape due tumour heterogeneity or the cells in these areas had a higher mitotic rate and were not affected by the single radiation attack. However, these difficulties have to be overcome for radioimmunotherapy to be useful.

A study using ^{131}I labelled antibodies (HMFG1, HMFG2, AUA1, and H17E2) have been reported in which 29 patients with chemoresistant ovarian cancer were treated (Hnatowich et al., 1989). In a phase I/II trial up to 30 mg were given intraperitoneally to each patient with an escalating dose of ^{131}I from 20 to 157 mCi. No response was obtained from 8 patients with bulky tumours (tumour nodules greater than 2 cm). For tumour smaller than 2 cm, response was obtained in 2 patients who had tumours measured a few mm in size, one completely and the other had a 50% reduction in size. It is estimated that a dose of 150 mCi ^{131}I labelled antibody given intraperitoneally will deliver 300-400 rads to the bone marrow, and a dose of 120 mCi will result in bone marrow toxicity. A phase I clinical trial using ^{90}Y labelled antibodies is now in progress at Hammersmith Hospital (Hnatowich et al., 1988).

In a pilot study ^{131}I labelled antibody (11-40 mCi) was injected intrathecally to five patients with leptomeningeal tumours in whom conventional therapy has failed. The therapy was well tolerated with mininal acute toxicity. Four of the five patients achieved an objective response to treatment sustaining between 7 months to 2 years, and no chronic toxicity has been observed 1 and 2 years after therapy (Lashford et al., 1988).

A calculation of dosimetry by Vaughen et al. (1986) has shown that radioimmunotherapy is not likely to be feasible due to the small amount of radiation that can be delivered to the tumour by bound activity. However, this estimation may be over pessimistic.

For radioimmunotherapy, accurate dosimetry is necessary in the calculation of required radiation doses. Such measurements may be taken by 3-dimentional (SPET) gamma camera imaging, and the optimal therapeutic condition can then be selected. Available data suggests that multiple dose therapy may be necessary (Begent, 1989).

The bone marrow is the limiting factor for the systemic administration of radiolabelled antibodies. Bone marrow rescue technique may be used to overcome this problem, and mathematical models may be used to estimate radiation doses (Wheldon, 1989).

iv Combined radiotherapy

Another approach is a combination of subtoxic radioimmunotherapy supplemented by regional external radiotherapy to obtain a tumoricidal dose at the tumour site.

The binding of monoclonal antibody to tumour may also be enhanced by a single dose radiation. In a study of human melanoma xenograft transplanted into nude mice, enhanced binding of ^{111}In-labelled antibody anti-p97^{a} was observed following a single dose radiation of 10 Gray. Water bath hyperthermia to 41^{o}C for 45 minutes also enhanced antibody binding but less consistently (Stickney et al., 1987).

7.4.11 Overall view

Monoclonal antibodies have made a significant impact in the histological classification of various tumours. The ability of radiolabelled antibodies to detect various tumours has also been confirmed by many studies.

The role of monoclonal antibody in the immunoscintigraphy of colorectal cancer may, however, not be better than other imaging techniques such as CT scan or NMR. Nevertheless, there were instances where radioimmunodetection has led to successful second-look laparotomies. Perhaps the significance of immunoscintigraphy is the demonstration of tumour localisation by the antibody which paves the way for tumour-targeting.

Although many problems in antibody-targeted therapy remain to be solved, the steady advances made in the production of more specific monoclonal antibodies, the new methods for antibody conjugation to radioisotopes and cytotoxic agents are encouraging. There is ground for optimism that antibody-directed tumour therapy may play a part, at least in conjunction, with other methods of treatment (Rogers, 1986). In the opinion of the investigator, this optimism also applies to colorectal cancer.

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APPENDIX 1

Chapter 3

LU1 fusion.

A total of 14 wells were positive on intial screening with ELISA. A total of 212 clones were picked from these wells. ELISA was positive on 12 of these clones. However, there were no positive results on immunocytochemistry.

LU1

ELISA positive agar well (Well number)	No. of clones picked from each agar well	ELISA + ve clones	Immuno-peroxidase +ve clones
1	22	3	nil
3	23		
7	12	2	nil
13	15	1	nil
14	9	2	nil
18	8	1	nil
19	16	1	nil
23	7		
25	18	1	nil
27	18		
28	19		
29	14		
31	20	1	nil
32	11		

Chapter 3

LU2 fusion

A total of 15 wells were positive on intial screening with ELISA. A total of 61 clones were picked from these wells. ELISA was positive on 3 of these clones. However, there were no positive results on immunocytochemistry.

LU2

ELISA positive agar well (Well number)	No. of clones picked from each agar well	ELISA + ve clones	Immuno-peroxidase +ve clones
1	3		
3	3		
4	3		
11	4		
17	6	1	
19	6	2	
21	1		
22	5		
37	2		
40	2		
41	2		
42	12		
43	2		
44	3		

Chapter 3

LU3 fusion

A total of 28 wells were positive on intial screening with ELISA. A total of 127 clones were picked from these wells. ELISA was positive on 52 of these clones. However, there were 8 positive results on immunocytochemistry. Two were reactive with normal colon, 2 reacted patchily with the sections 2 were weak and 1 stained connective tissue and the other blood vessel.

LU 3

ELISA positive agar well (Well number)	**No. of clones picked from each agar well**	**ELISA + ve clones**	**Immuno-peroxidase +ve clones**
3	4	3	nil
6	9	3	nil
8	6		
10	1	1	nil
11	3	1	nil
14	1	1	nil
15	1		weak/mod
16	10	6	nil
17	2	1	nil
19	3	2	nil
20	6	2	nil
23	2		
24	5	5	.1 one patch .2 normal
25	5		
27	2	1	nil
29	5	3	nil
32			

Chapter 3

LU 3

ELISA positive agar well (Well number)	No. of clones picked from each agar well	ELISA + ve clones	Immuno-peroxidase +ve clones
33	3	1	nil
34	2		
36	6	1	
37	5	1	.5 one patch
38			
40	4	3	nil
41	13	7	nil
42	6	3	nil
43	6	1	nil
46	9	1	nil
48	7	4	.2 weak .6 bv .7 conn tiss

Chapter 3

LU4 fusion

A total of 29 wells were positive on intial screening with ELISA. A total of 113 clones were picked from these wells. ELISA was positive on 36 of these clones. However, only 1 antibody stained faintly with normal colon.

LU 4

ELISA positive agar well (Well number)	**No. of clones picked from each agar well**	**ELISA + ve clones**	**Immuno-peroxidase +ve clones**
3	5	3	nil
5	9	1	nil
6	11	4	nil
7	12	3	nil
8			
9	15	5	nil
10	11	5	nil
12			
14	5	3	nil
15	4		
17	7	2	nil
18	3		nil
20			
21			
22			
24			
25			
28			

Chapter 3

LU 4

ELISA positive agar well (Well number)	No. of clones picked from each agar well	ELISA + ve clones	Immuno-peroxidase +ve clones
29	1		
30	2	1	nil
33	4		nil
34	5	1	faint n colon
35	7	3	nil
39			
41	7	4	nil
42	5	1	nil
43			
44			
46			

Chapter 3

LU5 fusion

A total of 6 wells were positive on intial screening with ELISA. A total of 6 clones were picked from these wells. ELISA was positive on 2 of these clones. No immunocytochemistry was positive.

LU 5

ELISA positive agar well (Well number)	**No. of clones picked from each agar well**	**ELISA + ve clones**	**Immuno-peroxidase +ve clones**
5			
6			
9	2	2	nil
23			
31			
43	4		

Chapter 3

LU6 fusion

A total of 31 wells were positive on intial screening with ELISA. A total of 102 clones were picked from these wells. ELISA was positive on 47 of these clones. However, only 2 antibodies stained faintly with normal colon.

LU 6

ELISA positive agar well (Well number)	**No. of clones picked from each agar well**	**ELISA + ve clones**	**Immuno-peroxidase +ve clones**
1	7	2	nil
2	8	5	nil
4			
7			
10			
11			
12	4	1	
13	1		
14	3	2	nil
15	4	1	nil
18	4	3	faint n colon
19	5	3	nil
20	4	1	
22	4	4	nil
24	6	4	weak n colon
27			
30			
31	1	1	nil

Chapter 3

LU 6

ELISA positive agar well (Well number)	No. of clones picked from each agar well	ELISA + ve clones	Immuno-peroxidase +ve clones
32	5	2	nil
33	3	3	nil
34	5	1	nil
36	2	2	nil
37	5		
38	5	1	nil
39	7	3	nil
40	7	1	nil
42			
44	4	3	nil
46	1	1	nil
47	3	3	nil

Chapter 3

LU7 fusion

A total of 15 wells were positive on intial screening with ELISA. A total of 56 clones were picked from these wells. ELISA was positive on 23 of these clones. Immunocytochemistry was positive on 17 of these clones. They stained both the normal colon and the cancer.

LU 7

ELISA positive agar well (Well number)	**No. of clones picked from each agar well**	**ELISA + ve clones**	**Immuno-peroxidase +ve clones**
3	4	4	.4
12	4		
17	4	4	.1;.3
21	6	2	.1;.6
24	4	3	.1; .2;.3;.4M
27	6	1	.6
28	4	2	nil
29	3	1	nil
30			
34	3	1	nil
35	5	1	normal colon
36	4	1	nil
42	4	1	.4
47	5	3	.1; .2; .5
49	1	1	.4

Chapter 3

LU8 fusion

A total of 12 wells were positive on intial screening with ELISA. A total of 55 clones were picked from these wells. ELISA was positive on 16 of these clones. Immunocytochemistry was positive on 12 of these clones. They stained both the normal colon and the cancer.

LU 8

ELISA positive agar well (Well number)	**No. of clones picked from each agar well**	**ELISA + ve clones**	**Immuno-peroxidase +ve clones**
2	1	1	nil
4	2	1	nil
7	6	3	.1;.6
12	3	3	.1; .2; .3
17	6		
20	8	4	.3;.5
22	10		
28	4		
31	3	2	.2;.3
37			
40	10	2	.1; .3; .10M
45	2		

Chapter 3

LU10 fusion

A total of 28 wells were positive on intial screening with ELISA. A total of 109 clones were picked from these wells. ELISA was positive on 38 of these clones. 14 antibodies stained tumour and normal colon. 1 antibody stained the stroma.

LU 10

ELISA positive agar well (Well number)	**No. of clones picked from each agar well**	**ELISA + ve clones**	**Immuno-peroxidase +ve clones**
1	4	2	stroma
4	9	1	
5	8	5	nil
7	3	2	nil
8	2		
9	7	1	.7
10	2		
11	4		
12	4	2	
14			
17	7	2	
18	5	2	
23	6	2	.3;.6 normal
24	1	1	
25			
26			
29	2	1	nil
31	3	2	nil

Chapter 3

LU 10

ELISA positive agar well (Well number)	No. of clones picked from each agar well	ELISA + ve clones	Immuno-peroxidase +ve clones
36	4	2	.3
37	3	1	
38	4	3	.2;.3 .4(weak)
40	10	1	nil
41	6	4	.1;.2;.4;.5;.6
42	4	3	nil
44	4	3	nil
45	4		
46			
48	3	1	.2

Chapter 4

Reaction of monoclonal antibodies raised against bladder cancer secondaries to colorectal cancer (CRC)

CRC		Monoclonal antibody designation				
		M8	**77-1**	**8-30-3**	**48-1**	**3-48-2**
1	83/4222	+	+	+	+	+60%
2	4618/82A	+30%	+10%	+10%	+40%	+40%
3	4430/82B	+few%	+30%	+10%	+40%	+50%
4	1566/82B	+50%	+few%	+80%	+	+
5	279/84B	+70%	+	+	+90%	+
6	225/84A	+	+	+	+40%	+50%
7	2034/82B	+	+20%	+	+	+
8	2594/82A	+50%	+	+80%	+50%	+
9	83/4220	+20%	+80%	+50%	+90%	+
10	84/417	+few%	+50%	+10%	+	+
11	84/172	+few%	+	+few%	+	+
12	83/507	+	+	+	+	+
13	83/505	+40%	+	+	+	+
14	83/508	+30%	+10%	+	+	+
15	7602/82 2A	-	+/-	-	+	+
16	1461/82A	+	+	+	+	+
17	4122/82B	+20%	+	+50%	-	+60%

Polyps		Monoclonal antibody designation				
		M8	77.1	8.30.3	48.1	3.48.2
1	2753/82	-	-	-	+	+
2	4584/82	-	-	-	+	+
3	4589/82	-	-	-	+	+
4	4645/82	-	-	-	+20%	+
5	4730/82	-	+few%	-	+	+
6	4793/82	-	-	+50%	+60%	+
7	4870/82	-	-	+25%	+60%	+
8	4889/82	+50%	-	+few%	+20%	+10%
9	5009/82	-	-	+few%	+30%	+
10	5085/82	-	+few%	+80%	+70%	+
11	5200/82	+few%	-	+45%	+	+
12	5359/82	-	-	-	+	+

Chapter 4

Reaction of M8, 77.1, 8.30.3, 48-4 and 3-48-2 on liver metastases

	Liver metastases	Monoclonal antibody designation				
		M8	77.1	8.30.3	48-1	3-48-2
1	857/82	+	+few%	+few%	+	+
2	3234/82	+	+	+	+	+
3	3483/82	+	+	+	+	+
4	4106/82	+	+	+	+	+40%
5	5079/82	+	+ few%	+20%	+	+
6	6443/82	+	+	+	+	+
7	6653/82	+	+few%	+few%	+	+

Chapter 4

Reaction of M8, 77-1, 8-30-3, 48-1 and 3-48-2 on lymph node metastases

Lymph nodes metastases		Monoclonal antibody designation				
		M8	**77.1**	**8.30.3**	**48-1**	**3-48-2**
1	225/84F	+	-	+	+	-
2	5106/82C	-	-	-	+/-	-
3	5261/82D	-	+few%	-	+50%	+
4	5840/82E	+20%	+	+	+	+
5	7115/82 2F	-	+few%	+10%	+	+
6	7275/82F	+20%	+50%	+	+/-	-
7	4122/82E	+100%	+	+	+	+
8	2034/82C	+	+70%	+60%	+	+
9	2594/82F	+	+	+(30%)	-	+
10	4430/82E	-	+	+	-	+

Chapter 4

Reaction of M8, 77.1, 8.30.3, 48.1 and 3.48.2. on normal colon.

Normal colon		Monoclonal antibody designation				
		M8	**77-1**	**8-30-3**	**48-1**	**3-48-2**
1	83/4223	-	-	-	+	+
2	83/506	+	+	-	+	+
3	84/171	+few%	-	+few%	+	+
4	84/418	-	-	-	+	+
5	84/912	+	+	-	+	+
6	84/914	+	-	-	+	+
7	84/1096	-	-	-	+	+
8	84/1336	+	-	-	+	+
9	84/1864	+	+	-	+	+
10	83/4109	-	-	-	+	+

Chapter 5

The tumour/organ to blood ratios of ^{125}I labelled 77-1 on XK1 at 24 hours

Tumour	1.38	1.5	1.3	1.3
Skin	0.19	0.46	0.22	ND
Muscle	0.11	0.07	0.10	0.30
Lung	0.17	0.38	0.15	0.30
Heart	0.30	0.30	0.30	0.25
S.I.	0.16	0.13	0.12	0.23
Colon	0.18	0.06	0.05	0.15
Bone	0.16	0.19	0.14	ND
Brain	0.02	0.02	0.02	0.04
Kidney	0.25	0.40	0.27	0.30
Liver	0.20	0.30	0.18	0.36
Spleen	0.14	0.48	0.24	0.51
Pancreas	0.08	0.52	0.09	0.21
Fat	ND	ND	0.22	ND

Chapter 5

The tumour/organ to blood ratios of ^{125}I labelled 77-1 on XK1 at 48 hours

Tumour	1.9	1.4	1.6	2.6
Skin	0.5	0.34	0.39	0.33
Muscle	0.12	0.11	0.16	0.09
Lung	0.12	0.11	0.16	0.09
Heart	0.4	0.28	0.26	0.23
S.I.	0.15	0.12	0.15	0.17
Colon	0.12	0.12	0.16	0.16
Bone	0.14	0.12	0.13	0.16
Brain	0.03	0.02	0.02	0.02
Kidney	0.28	0.31	0.31	0.31
Liver	0.26	0.25	0.28	0.31
Spleen	0.38	0.21	0.23	0,48
Pancreas	0.25	0.15	0.19	0.20
Fat	0.92	0.17	0.23	0.13

Chapter 5

The tumour/organ to blood ratios of ^{125}I labelled 77-1 on XK1 at 72 hours

Tumour	2.2	2.8	2.0	2.5
Skin	0.54	0.39	0.12	0.44
Muscle	0.18	0.13	0.50	0.10
Lung	0.36	0.33	0.14	0.35
Heart	0.27	0.20	0.25	0.29
S.I.	0.21	0.16	0.02	0.20
Colon	0.20	0.15	0.004	0.16
Bone	0.25	0.16	0.15	0.16
Brain	0.03	0.03	0.03	0.03
Kidney	0.38	0.28	0.26	0.36
Liver	0.87	0.35	0.34	0.30
Spleen	0.73	0.33	0.34	0.30
Pancreas	0.21	0.15	0.14	0.17
Fat	0.28	0.10	0.18	0.30

Chapter 5

The tumour/organ to blood ratios of ^{125}I labelled 48-1 on XK1 at 24 hours

Tumour	0.34	0.53	0.40	0.35
Skin	0.56	0.36	0.35	0.41
Muscle	0.19	0.11	0.15	0.11
Lung	0.30	0.36	0.43	0.27
Heart	0.25	0.22	0.21	0.20
S.I.	0.001	0.16	0.17	0.18
Colon	0.39	0.11	0.16	0.13
Bone	0.07	0.12	0.11	0.14
Brain	0.08	0.02	0.03	0.03
Kidney	0.03	0.32	0.32	0.30
Liver	0.11	0.03	0.02	0.02
Spleen	0.25	2.3	2.4	2.3
Pancreas	1.01	0.31	0.28	0.26
Fat	0.5	0.48	0.41	0.24

Chapter 5

The tumour/organ to blood ratios of ^{125}I labelled 48-1 on XK1 at 48 hours

Tumour	0.48	0.47	0.43	0.58
Skin	0.32	0.24	0.30	0.35
Muscle	0.13	0.09	0.12	0.10
Lung	0.46	0.33	0.30	0.39
Heart	0.20	0.22	0.23	0.26
S.I.	0.16	0.16	0.17	0.28
Colon	0.16	0.14	0.11	0.15
Bone	0.16	0.15	0.14	0.06
Brain	0.03	0.04	0.03	0.06
Kidney	0.37	0.34	0.35	0.29
Liver	0.28	0.26	0.21	0.23
Spleen	0.22	0.53	0.21	0.21
Pancreas	0.22	0.23	0.19	0.15
Fat	0.20	0.24	0.15	0.47

Chapter 5

The tumour/organ to blood ratios of ^{125}I labelled 48-1 on XK1 at 72 hours

Tumour	0.47	0.54	0.61	0.50
Skin	0.31	0.34	0.32	0.31
Muscle	0.16	0.12	0.11	0.20
Lung	0.35	0.45	0.43	0.37
Heart	0.20	0.22	0.23	0.23
S.I.	0.18	0.12	0.15	0.18
Colon	0.12	0.10	0.16	0.14
Bone	0.14	0.14	0.14	0.14
Brain	0.35	0.03	0.03	0.03
Kidney	0.32	0.32	0.31	0.35
Liver	0.22	0.24	0.31	0.29
Spleen	0.15	0.24	0.24	0.28
Pancreas	0.12	0.14	0.20	0.17
Fat	0.09	0.14	0.14	0.17

Chapter 5

The tumour/organ to blood ratios of ^{111}In labelled 77-1 on XK1 at 24 hours

Tumour	2.47	3.8	2.8	4.6
Skin	0.79	0.38	0.55	0.69
Muscle	2.66	0.77	0.48	0.51
Lung	0.66	0.43	0.52	0.54
Heart	0.38	0.41	0.04	0.47
S.I.	0.96	0.49	0.49	0.68
Colon	1.25	0.52	0.56	0.66
Bone	0.67	0.28	0.34	0.57
Brain	0.06	0.04	0.04	0.04
Kidney	2.33	1.4	1.34	1.2
Liver	1.8	0.81	0.80	0.87
Spleen	2.4	0.82	0.07	1.18
Pancreas	5.3	0.98	0.98	0.79
Fat	2.9	1.06	1.3	0.65

Chapter 5

The tumour/organ to blood ratios of ^{111}In labelled 77-1 on XK1 at 48 hours

Tumour	10.89	2.67	9.11	5.89
Skin	1.11	0.98	0.68	1.08
Muscle	0.48	0.62	0.75	0.25
Lung	0.74	0.59	0.68	0.72
Heart	0.53	0.36	0.36	0.49
S.I.	0.89	0.66	0.54	0.97
Colon	0.10	0.73	0.75	1.43
Bone	0.63	0.35	0.54	0.51
Brain	0.06	0.06	0.06	0.06
Kidney	2.83	1.93	2.14	2.68
Liver	2.28	1.49	1.13	1.24
Spleen	3.52	1.21	1.04	1.40
Pancreas	1.72	0.89	0.90	0.96
Fat	0.93	2.92	0.80	0.75

Chapter 5

The tumour/organ to blood ratios of ^{111}In labelled 48-1 on XK1 at 24 hours

Tumour	0.74	0.97	0.77	1.01
Skin	0.67	0.46	0.38	0.67
Muscle	0.15	0.14	0.15	0.14
Lung	0.38	0.39	0.33	0.60
Heart	0.25	0.21	0.18	0.36
S.I.	0.25	0.35	0.24	0.46
Colon	0.21	0.27	0.22	0.38
Bone	0.24	0.21	0.18	0.32
Brain	0.04	0.04	0.02	0.04
Kidney	1.6	1.8	1.3	2.4
Liver	0.45	0.47	0.40	0.57
Spleen	0.44	0.62	0.42	0.72
Pancreas	0.41	0.53	0.46	0.66
Fat	0.46	0.44	0.37	0.86

Chapter 5

The tumour/organ to blood ratios of ^{111}In labelled 48-1 on XK1 at 48 hours

Tumour	1.8	1.18	0.99	1.05
Skin	0.92	0.61	0.62	0.78
Muscle	0.59	0.20	0.15	0.16
Lung	0.53	0.47	0.49	0.68
Heart	0.41	0.32	0.30	0.33
S.I.	0.61	0.36	0.38	0.39
Colon	0.48	0.38	0.39	0.34
Bone	0.44	0.27	0.21	0.26
Brain	0.09	0.04	0.02	0.05
Kidney	3.05	2.46	2.07	1.9
Liver	1.29	0.56	0.56	0.56
Spleen	1.62	0.64	0.67	0.75
Pancreas	1.04	0.53	0.40	0.49
Fat	1.18	0.65	0.68	0.88

APPENDIX 2

BUFFERS AND STOCK SOLUTIONS

Citrate buffer (pH 6.0)

15 mls of 0.15 M Tri Sodium Citrate and 1.5 mls of 0.15 M Citric Acid.

Citrate buffer (pH 3.0)

2.5 mls of Tri Sodium Citrate and 15 mls of 0.15 M Citric Acid.

Citrate buffer (pH 4.5)

15 mls of 0.15 M Tri Sodium Citrate and 11 mls of 0.15 M Citric Acid.

Carbonate-bicarbonate coating buffer for ELISA plates (pH 9.6)

Na_2CO_3	1.5g
$NaHCO_3$	2.93g
NaN_3	0.2g

Make up to 1 litre with distilled water.

DAB substrate

Tris-HCl 0.5 M (pH 7.6)	200 ml
DAB (diaminobenzidine)	100 mg
H_2O_2 (30% stock)	40.0 ul

Fast Red substrate

Solution A : Veronal acetate buffer pH 9.2

Na acetate (trihydrate)	0.9715g
Na barbitone	1.4715g
Double glass distilled water	247.5ml

N/10 hydrochloric acid - adjust pH to 9.2

Solution B : substrate solution

Napthol AS: B1 phosphoric acid salt	5mg
N,N dimethyl formamide	2 drops

Mix 10 ml solution A with 5 mg Brentamine Fast Red TR salt.

Mix this with solution B. Filter and use. Endogenous alkaline phosphatase activity blocked by addition of 1 mM levamisole.

Hepes buffer

Hepes (Sodium salt)	13.0g
NaCl	9.0g

Make up to 1 litre with distilled water.

Lowry et al. protein estimation - Reagents A and B

A 2g Na_2CO_3 in 100 ml 0.1N NaOH

B 100 mg sodium potassium tartarate; 50 mg $CuSO_4$ and 10 ml water

Phosphate buffer (pH 6.0)

0.15 M NaH_2PO_4 is added to 50 mls of 0.15 M Na_2HPO_4 until the pH is 6.0.

Phosphate buffered saline (pH7.4)

Na_2HPO_4. $12H_2O$	57.2g
$NaH_2PO_4.2H_2O$	5.2g
NaCl	84.4g
NaN_3	4.8g

Make up to 10 litres with distilled water.

Tris buffer

NaCl	80.0g
Tris (Trishydroxymethyl methylamine)	6.05g
N HCl	44ml

Make up to 10 litres with distilled water

Tris buffer for membrane preparation

Tris	6.05g
NaCl	5.84g
NaN_3	2g
$MgCl_2$	0.2g

Make up to 1litre with distilled water.

Trypan Blue solution

PBS (pH7.4)	250 ml
Trypan blue	1.0g

TISSUE CULTURE MEDIUM AND REAGENTS

Aminopterin stock solution

Aminopterin	2.5g
L15	20 ml

Ammonuim chloride solution

0.8% NH_4Cl in PBS

DMEM in agar

Bactoagar 2% (w/v)	9.0 ml
10 x DMEM	1.0 ml

Adjust to pH 7.4 by the dropwise addition of sterile M NaOH.

Freezing medium

L15 containing 10% (v/v) fetal calf serum	9.0 ml
DMSO	1.0ml

HAT medium

Dulbecco's modified Eagle's medium containing:	
foetal calf serum	10% (v/v)
hypoxanthine (H)	13.6ug/ml
aminopterin (A)	0.18ug/ml
thymidine (T)	3.9ug/ml

L-15 HT medium

Leibovitz's medium L15 containing:	
foetal calf serum	10% (v/v)
hypoxanthine	13.6ug/ml
thymidine	3.9 ug/ml
kanamycin	100.0 ug/ml

Stock agar (2%)

Bactoagar	0.4g
Distilled water	18.0 ml
Autoclave.	

Trypsin/Versene

Versene(basic salt solution containing 0.8% EDTA)	100.0 ml
trypsin (0.05% w/v)	0.1g

APPENDIX 3

GENERAL REAGENTS AND SUPPLIERS

Animals

CBA/Ca Ola mice, BALB/c mice and nude mice were all obtained from Olac Ltd., Bicester, Oxon

ELISA

General Cell Cultures and Hybridoma Experiments

Aminopterin	Sigma Chemical Co., Poole, Dorset
Bactoagar	Difco Laboratories, East Molesey, Surrey
Bovine Serum Albumin (BSA)	Sigma Chemical Co.,Poole, Dorset
Dimethylsulphoxide (DMSO)	BDH, Poole, Dorset
Dulbecco's Modified Eagles Medium (DMEM)	Institute of Cancer Research, London
DMEM 10x	Gibco- Biocult, Paisley, Scotland
Foetal Calf Serum (FCS)	Flow Laboratories,Irvine, Scotland
Hypoxanthine	Sigma Chemical Co., Poole, Dorset
Kanamycin	E.R Squibb, Wirral, Cheshire
L15	Gibco-Biocult, Paisley, Scotland
Nunc tissue culture flasks	Gibco-Biocult, Paisley, Scotland
Nunc 6, 24 and 96 well plates	Gibco-Biocult, Paisley, Scotland
Penicillin	Glaxo, Greenford, Essex
Polyethylene ampoules	Sterilin, Teddington, Middlesex
Polyethylene Glycol (PEG 1500)	BDH, Poole, Dorset
Pristane	Aldrich Chemical Co., Gillingham, Kent
RT 30 polystyrene tubes	Sarstedt, Leicester

Streptomycin	Glaxo, Greenford, Essex
Thymidine	Sigma Chemical Co., Poole, Dorset
Trypan Blue	BDH, Poole, Dorset
Trypsin	Sigma Chemical Co., Poole, Dorset
Universal Tubes (30ml)	Sterilin, Teddington, Middlesex
Versene	Institute of Cancer Research, London

The myeloma cell line NSO was courtesy of Dr.C.Milstein.

Immunocytochemistry and immunofluorescence

CNP30	Penetone, Northumberland
Diaminobenzidine (DAB)	Sigma Chemical Co., Poole, Dorset
D.P.X.	Raymond Lamb, London
Glycerin-albumin	Raymond Lamb, London
Hydrogen Peroxide	Hopkin and Williams, Essex
Hydromount	Raymond Lamb, London
Lithium Carbonate	BDH, Poole, Dorset
Mayer's Haemalum	BDH, Poole, Dorset
OCT-compound	Raymond Lamb, London
Poly-L-lysine	Sigma Chemical Co., Poole, Dorset
Rabbit anti-mouse IgG peroxidase-conjugated	Dako, High Wycombe, Bucks
Rabbit anti-mouse IgG conjugated to fluorescein	Nordic Immunology, UK

Radiolabelling

Iodogen	Sigma Chemical Co., Poole, Dorset
Chloramine T	Sigma Chemical Co., Poole, Dorset
Sephadex G25	Pharmacia
Sodium metabisulphite	Sigma Chemical Co., Poole, Dorset

Radioisotopes

Iodine-125	Amersham International, Amersham, UK
^{111}Indium chloride	Amersham International, Amersham, UK

PUBLICATIONS

Presentations to learned societies

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Monoclonal Antibodies Reacting with Colorectal Tumours*

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Abstract. *25 mouse monoclonal antibodies were screened for cell surface reactivity with colorectal tumours using the indirect immunoperoxidase method. Five antibodies of the IgG subclasses were selected. LICR-LON-M8 was raised against human milk fat globule membrane (HMFGM). The remaining four antibodies 48-1, 3-48-2, 77-1, and 8-30-3 were raised against human bladder metastases. M8, 77-1, and 8-30-3 which showed epithelial membrane antigen (EMA) – like reactivity exhibited greater selectivity for colorectal cancer compared with normal colon. M8 and 77-1 are being evaluated for immunoscintigraphy of colorectal carcinoma.*

Immunoscintigraphy is potentially a valuable tool in cancer diagnosis. The sensitivity of this technique depends on a selective affinity of antibodies for cancer, their access to tumours, and biological stability of radiolabelled conjugates (1). Successful imaging of colorectal carcinoma has been reported (2, 3, 4). Some antibodies used were raised against colon cancer (2, 3) and others against osteogenic sarcoma (4). It is commonly found that antibodies raised against one tumour will also react with other tumour types (5). Thus, one approach to obtain monoclonal antibodies for immunoscintigraphy of colorectal cancer is by screening existing antibodies for colonic reactivity. Here we report the identification of five such antibodies by this means.

Materials and Methods

25 mouse monoclonal antibodies raised against human milk fat globule membrane (HMFGM) (6,7) and bladder cancer metastases (8) were screened for reactivity with colorectal cancer using the indirect immunoperoxidase method. Briefly, 5μ sections of formalin or methacarn (8) fixed, paraffin-embedded tissues were de-waxed in xylene. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 10 minutes. Sections were washed with Tris buffered saline (TBS pH 7.6) and incubated with undiluted hybridoma supernatants at room temperature for 1 hour. They were washed with TBS and incubated for $^1/_2$ hour with rabbit anti-mouse immunoglobulin conjugated to peroxidase (Sigma P260 1:100 TBS). The reaction product was developed using 1:2000 3'3' diaminobenzidine tetrahydrochloride in TBS (Sigma) for 10 minutes. The sections were counterstained with Mayers haemalum and mounted in D.P.X. (Raymond Lamb, UK).

Sections from 3 adenocarcinomas of the colon were used for initial screening. Antibodies with cell surface reaction with any of these tumours were selected for further evaluation. The second screen involved 17 colorectal carcinomas (CRC) of varying degrees of differentiation, 12 adenomatous polyps, 10 morphologically normal blocks of colon taken 10-15 cms from resection edges of resection specimens for either cancer or diverticular disease, 10 lymph node secondaries (LN mets) and 6 liver metastases (liver mets). The staining reaction was classified as positive, weak, and negative.

Results

Five antibodies with cell surface reactivity were selected following initial screening. LICR-LON-M8 (IgG1) which was raised against HMFGM (6, 7). The remaining four antibodies 48-1 (IgG1), 3-48-2 (IgG1), 77-1 (IgG2a), and 8-30-3 (IgG2b) were raised against bladder cancer metastases (8). All antibodies reacted heterogenously with primary tumours varying from focal to a near homogenous staining pattern. All five antibodies exhibited selective staining in the luminal membranes and there was thus a tendency for stronger staining in the better differentiated tumours. The same pattern was observed within the lymph nodes and liver metastases. Heterogenous staining was also observed in the polyps. Monoclonal antibodies 48-1 and 3-48-2 reacted with a greater proportion of the tumour cells compared with the other antibodies. 48-1 and 3-48-2 uniformly stained the surface of all normal samples of colonic epithelium while the other three antibodies tended to react preferentially with the bases of the crypts and only in some samples (Table I).

The comparative reactivity of the antibodies in the tissues examined is shown in Table I, from which it can be concluded

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Table I. *Positive staining of monoclonal antibodies with colorectal tumours and normal colons.*

	Monoclonal antibody designation				
Tissue	M8	48-1	3-48-2	77-1	8-30-3
CRC	13	16	17	15	15
Polyps	2	11	12	2	6
Normal colon	6	10	10	3	1
LN mets.	6	6	7	8	8
Liver mets.	6	6	6	6	6

The table indicates the number of specimens positively stained for each antibody studied in 17 carcinomas, 12 adenomatous polyps, 10 normal specimens, 10 lymph node metastases and 6 liver metastases.

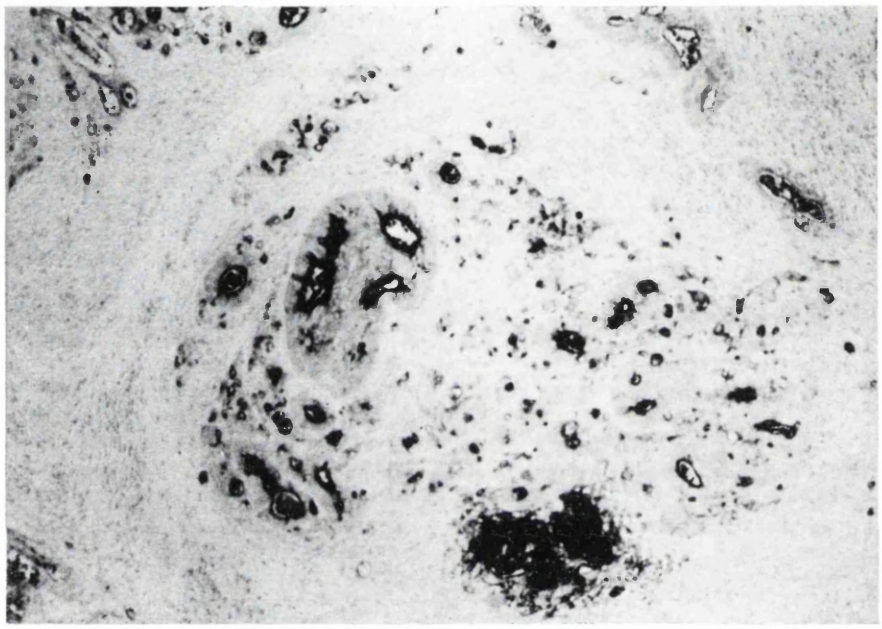

Figure 1. *Photomicrograph of colonic cancer stained with 77-1 showing strong positivity of tumour cells at their luminal surface. Magnification × 80.*

that M8, 77-1, and 8-30-3 are apparently more selective for the carcinomas, while 48-1 and 3-48-2 have a broad strong reactivity with the majority of the material examined.

Discussion

In the early stages of monoclonal antibody development, it was suggested that tissue specific tumour associated antibodies could be readily obtained. This has not proven to be the case (9). Thus antibodies raised against other tissues, for example, HMFGM (10), breast cancer (5), and osteogenic sarcoma (4), react with colorectal cancer. Our observations that M8, 48-1, 3-48-2, 77-1, and 8-10-3 raised against HMFGM and bladder cancer metastases react with colorectal cancer is consistent with these findings.

M8 recognises epithelial membrane antigen (EMA) (11, 12, 13), while 77-1 and 8-30-3 have EMA-like reactivity (8). EMA is present on most normal epithelia and is widely expressed in epithelial and mesothelial neoplasms (11, 12, 13). Our staining results with these antibodies suggest that the expression of epitopes recognised by these antibodies are "present" in higher concentrations on tumour cells compared with normal cells, but immunocytochemistry is a rather insensitive technique for antigenic detection and negative staining cannot be taken as absolute evidence of lack of antigenic expression. Despite reactivity with normal epithelia, M8 has been used successfully in immunoscintigraphy of breast cancer (14) and colorectal cancer (unpublished observation). The tissue distribution of 77-1 and 8-30-3 (8) is similar to M8 (6) so that imaging of colorectal cancer should be possible with these antibodies. Clinical studies are now underway with M8 and 77-1.

48-1 and 3-48-2 reacted with glycolipid epitopes with molecular weight of 300,000 (8). This antigen is expressed by

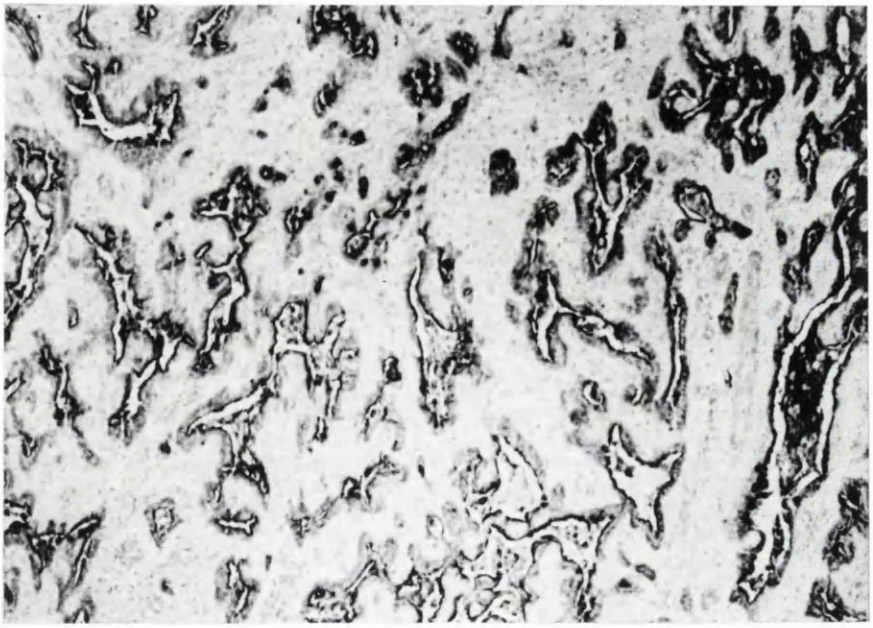

Figure 2. *Photomicrograph of colonic liver metastasis stained with 77-1 showing reactivity in most areas of the section. Magnification* × *80.*

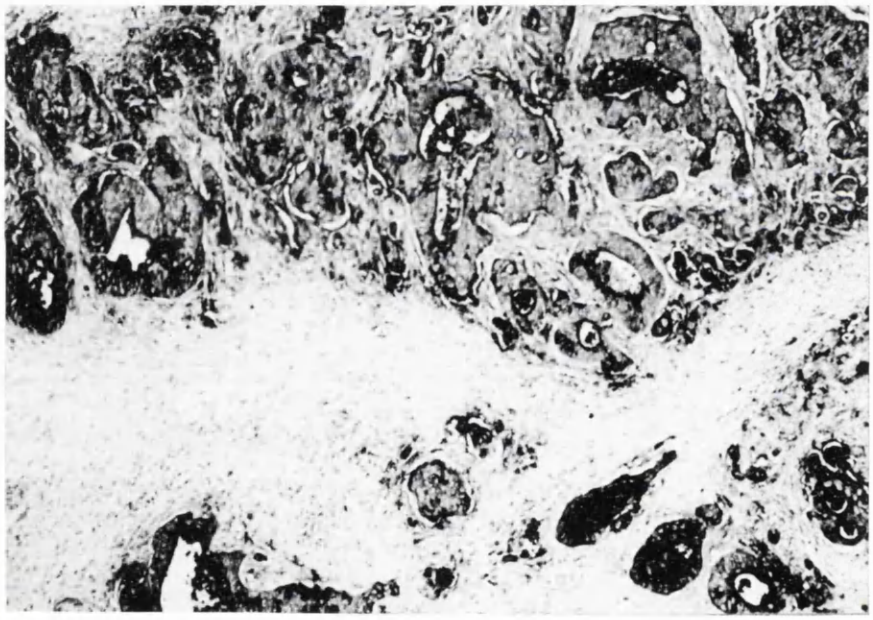

Figure 3. *Photomicrograph of colonic cancer stained with 48-1 showing heterogenous reactivity with tumour cells. Magnification* × *80.*

normal colon, polyps and colorectal cancer. Neither antibody showed preferential staining of colorectal cancer, and this would make them unsuitable for immunoscintigraphy of colorectal carcinoma.

This study illustrated that due to shared expression of epitopes by different tumours it is possible to select antibodies reacting with colorectal cancer or other tumours by screening existing antibodies.

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