INVESTIGATION OF TWO METHODS TO IMPROVE THE THERAPEUTIC POTENTIAL OF RADIOLABELLED ~~ANTITUMOUR~~ ANTIBODIES: USE OF AN ANTI-ANTIBODY AND SUPPRESSION OF THE HOST ANTI-ANTIBODY RESPONSE.

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To my parents

Abstract

The aim of targeting therapy with radiolabelled antitumour antibody is to increase the quantity of cytotoxic agent delivered to the tumour relative to normal tissues. But the administration of antibody conjugates with 131-iodine (^{131}I) is limited by myelosuppression. The purpose of this thesis was to investigate two methods of reducing myelotoxicity so that a larger amount of radiolabelled antibody could be given.

An anti-antibody ('second antibody') that binds to the primary antitumour antibody was given to patients with colorectal cancer 24 hours after 131-iodine (^{131}I) labelled antibody to carcinoembryonic antigen (CEA). This led to an acceleration in the removal of radioactivity from blood but not the body. The β dose to bone marrow, estimated from the radioactivity in blood was reduced after 'second antibody' was given ($p<0.05$). Although, the total bone marrow radiation dose was not significantly reduced by 'second antibody' up to 152 mCi ^{131}I anti-CEA was given without life-threatening toxicity.

A second approach examined whether ^{131}I anti-CEA could be given in divided doses allowing the bone marrow to recover between treatments. However, a single intravenous injection of polyclonal or monoclonal anti-CEA led to an increase in the concentration of serum human IgG anti-antibody in 13 out of 24 (54.2%) patients. Treatment in the presence of anti-antibodies detected before therapy did not prevent uptake of radioactivity in the tumour. But therapy

repeated following a rise in the concentration of anti-antibody led to allergic reactions and rapid elimination of the radiolabelled antibody. Cyclosporin A was found to suppress the anti-antibody response in rabbits and man. Up to four injections of approximately 50 mCi of anti-CEA were given before small amounts of human anti-antibody appeared. However, myelosuppression was seen in 4 out of 6 patients.

As almost half the bone marrow radiation was delivered before 24 hours a greater improvement in therapeutic ratio may result from giving 'second antibody' earlier and in combination with repeated courses immunconjugate and cyclosporin A.

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Declaration

All the work reported in this thesis was performed by the author except as stated above or as mentioned in the text.

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SECTION I INTRODUCTION

Chapter 1

INTRODUCTION AND BACKGROUND TO THE TREATMENT OF CANCER WITH ANTITUMOUR ANTIBODIES.

Progress in the treatment of advanced tumours over the last thirty years has largely been due to the development of new chemotherapeutic agents. In some patients survival has been prolonged but the prognosis of the vast majority with advanced cancer has changed little with most tumours exhibiting resistance, after responding only briefly to chemotherapy or radiotherapy. Many of the reasons for the failure of treatment are unclear but they may in part be due to an inability to deliver an adequate dose of radiation or drug to the tumour without compromising the normal tissues of the host. The therapeutic ratio could be improved by the selective delivery of cytotoxic conjugates; on this hypothesis rests the basis for developing targeted therapy with antitumour antibody conjugates.

Antibody Targeting

The notion that antibodies might be raised against tumours and used for therapy was first proposed and

investigated by Héricourt and Richet (1895 a,b). They reported patients who responded to treatment with serum from animals that had been immunised with an osteosarcoma. However, it is unclear how these antibodies were able to distinguish cancer cells from normal tissues as little was known about the biochemical differences that exist between malignant and normal cells.

Further interest in targeted therapy of cancer arose following Ehrlich's proposal, in 1913, that antibodies raised against receptors specific to parasites might be able to seek out and destroy the organisms without any deleterious effect on the host.

Nearly half a century elapsed before Pressman and Korngold (1953) demonstrated that an injection of an antitumour antibody, developed in rabbits that had been immunised with the Wagner osteosarcoma cell extracts, and conjugated with radioiodine could localise in the same tumour growing in mice. Their work and that of Bale et al. (1955) showed that there was a greater accumulation of radioactivity in the tumour than in other organs of the animal. The selectivity of antibody uptake was poor but it could be improved by prior adsorption of the antibody with cross-reacting tissues (Pressman and Korngold 1953). By using a paired-labelling technique Pressman et al. (1957) were able to demonstrate that accumulation of specific antibody in the tumour was small but greater than the uptake of nonspecific antibody. These experiments established the principle of targeting with antitumour antibodies. Day et

al. (1965) were the first to demonstrate specific uptake of antiglioma antibodies in patients. Antibodies to glioma tissue were raised in rabbits, radioiodinated and injected into the carotid artery of patients 3-5 days before a second craniotomy. A dual labelling technique was used to show that the uptake of radiolabelled control proteins was less than the antitumour antibody.

As a result of several key developments over the last thirty years it has become possible to use radiolabelled antibodies to detect tumours in patients. Therapy is a further application of radiolabelled antibodies and it is the subject of the work that will be described.

Tumour markers

The discovery of human tumour associated antigens such as the oncofoetal proteins, alpha foetoprotein (AFP) and carcinoembryonic antigen (CEA), and the ability to grow human tumours as xenografts in the hamster cheek pouch or immunodeprived mouse facilitated the study of antibody targeting of human tumours. It was initially thought that oncofoetal proteins were tumour specific; later they were shown to be present in the tissue and serum of some patients with nonmalignant disease so that they can be considered as physiological compounds expressed inappropriately. For instance, human chorionic gonadotrophin (hCG) is normally produced in pregnancy but it is also associated with tumours exhibiting trophoblastic differentiation. However, increased

expression of these substances in tumours relative to normal tissues makes them potential targets for antitumour antibodies.

True tumour markers have so far only been found in lymphoproliferative diseases and myeloma that express immunoglobulin or part of the immunoglobulin molecule in the serum or cell surface. Antibodies to these molecules (the anti-idiotype) identify a unique malignant clone (Stevenson and Stevenson 1975). The production of monoclonal antibodies (Köhler and Milstein 1975) has aided the exploration of the cell surface and has helped to identify several new tumour associated antigens. These include glycoproteins, oncoproteins and growth factor receptors (Virji et al. 1988).

Localisation of human tumours with radiolabelled antibodies

Quinones et al. (1971) showed that radiolabelled antibodies to hCG could localise in choriocarcinoma xenografts growing in the hamster cheek pouch. The ratio of radioactivity in tumour to liver by the third or fourth day after injection was better with anti-hCG than nonspecific antibody. However, the difference in uptake between radiolabelled anti-hCG and normal rabbit immunoglobulin G (IgG) was not sufficient to demonstrate specific localisation in the tumour by whole body scanning. Similar findings were reported by Primus et al. (1973) using radiolabelled antibodies to CEA, targeted to human colon

carcinoma xenografts in hamsters. By using dual-labelling they were able to show that 131-iodine (^{131}I) antitumour antibodies were retained longer in the tumour than 125-iodine (^{125}I) nonspecific radiolabelled antibody injected at the same time. The absolute amount of antibody taken up by tumours was small but retention of the antibody could be prolonged by using antibodies that had been immunopurified on a CEA immunoabsorbent column (Primus et al. 1977).

The ability to demonstrate human colon cancer xenografts by external photoscanning was a considerable step forward as it identified the possibility of applying these antibodies to the detection and treatment of tumours (Goldenberg et al. 1974, Mach et al. 1974).

A few years later external scintigraphy was used to image a variety of CEA-producing tumours in patients following injection of a polyclonal anti-CEA antibody conjugated with ^{131}I (Goldenberg et al. 1978, Mach et al. 1980). At first, the detection of tumours in man was less sensitive than in animals. This was largely because interference of background radioactivity was greater in man and xenograft tumours were larger in relation to the body mass and more superficial. A variety of techniques have been developed to reduce the background radioactivity due to nonspecific retention of radiolabelled antibody. These have helped to increase the ratio of radioactivity in the tumour relative to normal tissue (and blood), improve the clarity of the tumour images and increase the sensitivity of radioimmunolocalisation in man (for review see Begent 1985).

Tumours as small as 1 cm in lymph nodes of normal size have been identified by radioimmunolocalisation (Moldofsky et al. 1984). Studies are being performed in patients with colorectal cancer and gestational trophoblastic diseases to assess whether imaging with radiolabelled antibody improves the outcome of patients (Begent et al. 1986, Begent et al. 1987, Patt et al. 1988).

Antibody therapy

The ability to produce antibodies to tumour antigens renewed interest in the concept of antibody-targeted therapy. It was thought that serotherapy, which hitherto had been largely unsuccessful (Currie 1973), might become effective if antitumour antibodies could activate the immune system of the host. Hamblin et al. (1980) showed that polyclonal anti-idiotypic antibody led to a temporary fall in the circulating lymphocyte count of a patient with lymphocytic leukaemia. Further serotherapy studies with monoclonal antibodies were stimulated by the report that one patient with a cutaneous T cell lymphoma responded to therapy with an anti-T cell antibody (Miller and Levy 1981) and another patient had a complete clinical response following therapy of a B cell lymphoma with an anti-idiotypic antibody (Miller et al. 1982).

However, other trials with anti-idiotypic antibody (Meeker et al. 1985, Miller et al. 1987, Rankin et al. 1985), anti-T cell antibodies (Miller et al. 1983), or

antibodies to B cell lymphoma (Foon et al. 1984) have led only to a short-lived response in a few patients.

Serotherapy trials have also been conducted in patients with melanoma and gastrointestinal cancer (Oldham et al 1984, Sears et al. 1982, Sears et al. 1984, Douillard et al. 1986). Single injections of up to 1000 mg of antibody have been given but few durable tumour responses have been seen. However, these studies have provided important information on the distribution of antitumour antibodies in patients, the binding of antibodies to tumour cells and the host reaction to xenogeneic antibodies. These will be discussed more fully in relation to immunoconjugate therapy (*vide infra*). Broadly, the conclusions drawn from these trials indicate that under present circumstances mouse monoclonal antitumour antibodies given alone are not able to stimulate the host defence mechanisms adequately to eradicate tumours. However, the combination of antitumour antibodies with lymphokines, such as interleukin-2 may lead to an enhanced action of serotherapy (Berinstein et al. 1988).

Alternatively, antitumour antibodies can be used as a targeting vehicle to carry a drug, toxin or radionuclide to the tumour cell. For this treatment to be successful it is necessary to deliver a dose of immunoconjugate that is sufficiently large to eradicate the tumour without producing unacceptable toxicity in the host.

In the earliest study of immunoconjugate therapy intraperitoneal injections of chlorambucil conjugated to an antitumour antibody were shown to be more effective in

prolonging the survival of mice inoculated with lymphoma than were normal rabbit IgG conjugates, antitumour antibody, or chlorambucil alone (Ghose et al. 1972). Other drug-antibody immunoconjugates have also been shown to have superior cytotoxic activity than antibody or free drug in a xenograft tumour model systems (Arnon and Hurwitz 1985, Rowland et al. 1985, Baldwin et al. 1987). In most cases a delay in the growth of tumours occurred when immunoconjugates were given shortly after the inoculation of the tumour cells. The effectiveness of drug immunoconjugate therapy is also dependent on the tumour burden (Ghose et al. 1972). Improvements are likely to depend on delivering a larger quantity of drug to the tumour.

One way of achieving this is to use bridging molecules to link the drug and antibody (Garnett and Baldwin 1985). Whilst in cytotoxicity assays these complexes exhibit increased potency it is not clear whether their biodistribution will be altered by an increase in the molecular size and chemical modification of the antibody.

In an attempt to increase the potency of immunoconjugates plant toxins, such as ricin have been linked to antitumour antibodies. As these molecules act as enzymes entry of as little as one molecule into the cytosol can be lethal (Eiklid et al. 1980). Recognition of ricin by carbohydrate binding receptors on cells is reduced by removal of the B chain of the toxin. The toxicity resides in the A chain which can be conjugated to an antitumour antibody and targeted to the tumour cells. (Thorpe and Ross

1982, Vitetta et al. 1983). Prolonged remission of a BCL_1 lymphoma in mice has been seen following intravenous administration of immunotoxin (Krolick et al. 1982). Recently, ricin immunotoxins have been given to patients with melanoma (Spitler et al. 1987). Clinical improvement was seen in some patients with little toxicity but further controlled studies are needed to determine whether the response is directly due to the effects of the immunotoxin.

The effectiveness of drug or toxin conjugates is influenced by access to the tumour cell. Tumour vascularity is one major factor that influences antibody distribution but uptake by the cell is also affected by factors such as the heterogeneity of antigen expression. The binding of antibody to a cell surface may induce antigenic modulation, preventing further binding of antibody, and resistance to cytotoxicity (Gordon et al. 1981, Miller and Levy 1981, Foon et al. 1984, Meeker et al. 1985). In some tumours antigen is secreted into the surrounding tissue spaces and this can interfere with access of the antibody to the tumour cell. For example, one study showed that anti-CEA antibody was concentrated in the extracellular matrix of the tumour (Lewis et al. 1982) although in another study autoradiographs demonstrated anti-CEA bound to the cell surface of xenograft tumours (Buchegger et al. 1983).

These local factors are less important when radionuclides are used as the cytotoxic conjugate. The radiation 'field' from radioimmunoconjugates on or around tumour cells can damage cells which either fail to express

the tumour antigen or are inaccessible to the antibody. This is the principal benefit of radioimmunotherapy. A further advantage is that gamma radiation from a radioisotope allows the distribution of the radioimmunoconjugate to be monitored by external scintigraphy.

Radioimmunotherapy

The first investigation of this form of antibody therapy used an antifibrinogen antibody conjugated with ^{131}I (Spar et al. 1967). Nine patients with various tumours were given 55 to 168 mCi of ^{131}I and a remission of symptoms was reported in some patients. Most studies in man over the last ten years have used ^{131}I labelled antitumour antibodies as considerable experience exists from the use of this isotope for therapy of thyroid cancer. The methods of conjugating ^{131}I to antibodies are well known and radioiodinated antitumour antibodies have been used to image tumours in patients.

The largest clinical experience of radioimmunotherapy comes from the group at the Johns Hopkins Hospital, Baltimore. They have used radiolabelled anti-CEA and antiferritin antibodies to treat patients with hepatoma, cholangiocarcinoma and Hodgkins disease (Ettinger et al. 1979, Order et al. 1980, Ettinger et al. 1982, Order et al. 1985, Lenhard et al. 1985, Sitzmann et al. 1987). Tumour responses, determined from serial radiographs and changes in AFP have occurred in nearly half the patients (Order et al.

1985). However, radioimmunotherapy was part of a multimodal treatment programme which included cytotoxic chemotherapy and external beam radiotherapy. The rôle of radiolabelled antibody in these diseases can only be determined by a randomised study. This has now been completed (Order, personal communication) and the results are awaited.

Tumour responses to ^{131}I labelled antibody have also been reported in melanoma (Carrasquillo et al. 1984), cutaneous T cell lymphoma (Rosen et al. 1987) and B cell lymphoma (DeNardo et al. 1988). Some success has been observed following intracavity therapy with radiolabelled antibodies (Hammersmith Oncology Group 1984, Pectasides et al. 1986, Coakham et al. 1986, Lashford et al. 1988).

Although limited, these responses have provided some encouragement for further studies, particularly as more data accumulates on the therapeutic effect of radiolabelled antibodies in animals. Early studies in xenograft tumours demonstrated a greater delay in growth following radiolabelled antitumour antibodies than radiolabelled nonspecific antibody (Goldenberg et al. 1981, Zalcberg et al 1981). More recent studies have confirmed these observations (Badger et al. 1986, Cheung et al. 1986, Sharkey et al. 1987, Buchegger et al. 1988a, Ceriani and Blank 1988, Chiou et al. 1988, Lee et al. 1988, Yoneda et al. 1988).

In many studies it is not uncommon to find as much as 10% of the injected radioactivity per gram of tumour. This is considerably greater than the amount found in the tumours of patients. The antibody concentration depends on the time

of the biopsy; at best it has been shown to be only 0.03% of the injected activity per gram (Carrasquillo et al. 1986).

Both the absolute amount of antibody and the ratio of antibody in tumour compared with normal tissues are important considerations for successful radioimmunotherapy. The latter provides a measure of the therapeutic ratio and the former relates to the potency of the conjugate. Large doses of radioactivity need to be delivered to the tumour if radioimmunotherapy is to be effective in man. In animals an increase in the accumulation of antibody in the tumour is seen as the dose of antitumour antibody is escalated. With less than 100 μg of antibody the increase in uptake is proportional to the injected dose, but there is no increase in the proportion of the injected dose in the tumour. Saturation of the tumour by antibody (791T/36) was seen when 1-2 mg were given (Pimm et al. 1984). This may vary depending on the density of antigen receptors but it has been estimated that 200 mg of an immunoglobulin M (IgM) anti-idiotypic antibody would be needed to saturate 10^{12} lymphoma cells. The amount of antibody required may be greater for secreted antigens (Stevenson et al. 1984). Thus far, none of the studies of radioimmunotherapy in patients have examined the distribution and saturation kinetics.

Before these issues can be addressed it is necessary to examine the degree to which toxicity limits therapy with radiolabelled antibodies. Myelosuppression is the principal toxicity seen when ^{131}I is given for the treatment of thyroid cancer. It arises from radiation damage to myeloid

precursor cells, most of which are in the bone marrow. These cells are damaged as ^{131}I in the blood perfuses the bone marrow and by the effects of gamma radiation from ^{131}I in other areas of the body acting as a 'radiation source'. Damage to stem cells in the circulation and spleen may also contribute to myelosuppression. Bone marrow suppression, mainly thrombocytopenia, occurs quite commonly if more than 200 mCi are given. Severe and sometimes fatal myelotoxicity has been observed when the radiation dose to the blood exceeded 200 cGy (Benua et al. 1962). Similar side-effects are likely to be encountered following therapy with ^{131}I labelled antibody. For instance, the frequency and degree of bone marrow suppression was increased when more than 100 mCi of antiferritin were given to treat hepatocellular carcinoma (Ettinger et al. 1982). The implementation of measures to reduce myelotoxicity, such as accelerating the removal of circulating ^{131}I without reducing the concentration of radioactivity in the tumour might usefully improve the therapeutic ratio of radioimmunotherapy.

Acceleration of the removal of radiolabelled antibody

There are two main approaches to consider. One involves the use of Fab' immunoglobulin fragments. They lack the Fc region of immunoglobulin that binds to cells with Fc receptors and because of their low molecular size they are cleared rapidly through the kidney. A difference in clearance rate has been shown in mice with melanoma

xenograft tumours following injection of whole immunoglobulin and Fab' fragments to the p97 melanoma antigen (Beaumier et al. 1985). The clearance of an Fab'$_2$ fragment to CEA was also more rapid than whole immunoglobulin but slower than an Fab' fragment (Wahl et al. 1983). Comparative studies have not been performed in man but radiolabelled Fab' fragments have been used for tumour imaging (Larson et al. 1983a) and therapy (Carrasquillo et al. 1984).

Because of the rapid clearance of radiolabelled Fab' fragments uptake in tumours of animals or man, expressed as a percentage of the injected activity per gram, is less than that observed with intact immunoglobulin (Buchegger et al. 1983, Larson et al. 1983b). The uptake of Fab'$_2$ fragments in xenograft tumours is also lower than whole immunoglobulin (Buchegger et al. 1983, Harwood et al. 1985, Searle et al. 1986). It is likely that large quantities of radiolabelled Fab' fragments will be needed for therapy. Up to 342 mCi of ^{131}I Fab' antimelanoma antibody has been given and myelotoxicity was reported to be unusual unless a cumulative activity of more than 500 mCi was given (Carrasquillo et al. 1984). Renal toxicity may become important if large amounts of radiolabelled Fab' fragments are given as there is greater accumulation in the kidney than with intact immunoglobulin (Buchegger et al. 1983, Beaumier et al. 1985).

Using an alternative approach, one could give an anti-antibody directed against the antitumour antibody to form an

immune complex that might be cleared more rapidly than the first antibody alone. Anti-antibody ('second antibody'), entrapped in liposomes has been shown to accelerate the clearance of radiolabelled antitumour antibodies in animals (Barratt et al. 1981) and this approach has been successfully applied to enhance the imaging of tumours in man (Begent et al. 1982). Subsequent studies have shown that free 'second antibody' is equally effective (Sharkey et al. 1984, 1988, Bradwell et al. 1983, Goodwin et al. 1984, Goldenberg et al. 1987, Begent et al. 1989a). Maximum improvement in the clearance rate of antitumour antibody in animals was seen when the dose of 'second antibody' was five or more times that of the primary antibody. 'Second antibody' resulted in an 8 fold improvement in tumour to blood ratio (Begent et al. 1989a).

The radiation dose to the host might be reduced if 'second antibody' could improve the clearance rate of therapeutic injections of radiolabelled antibody. Evidence to support this hypothesis has recently been published by Pedley et al. (1989). They showed that the radiation dose to bone marrow in nude mice bearing human colon cancer xenografts could be reduced by giving 'second antibody' 6 or 24 hours after ^{131}I anti-CEA. The first part of this thesis will examine whether a reduction in the bone marrow dose occurs in patients with colorectal tumours who are treated with ^{131}I anti-CEA and 'second antibody'.

This common disease results in approximately 17,000 deaths annually in England and Wales and there has been

little improvement in survival over the last 30 years (Office of Population Censuses and Surveys 1983, 1985). A new approach to treat this tumour is needed and study of the effect of ^{131}I anti-CEA in this condition serves as a useful model for targeted-therapy of tumours. CEA can be found in nearly all colorectal cancers and the injection of small doses of radiolabelled anti-CEA antibodies have identified deposits of tumour in patients (Goldenberg et al. 1978, Dykes et al. 1980, Mach et al. 1980, Begent et al. 1986, Allum et al. 1986). The results of therapy with ^{131}I anti-CEA antibody with 'second antibody' will be presented in SECTION III. The distribution of radiolabelled antibody will be assessed by gamma camera imaging and as part of a separate project methods of dosimetry will be developed using the serial scintigraphic images.

Polyclonal antisera were used in the early anti-CEA studies. The development of hybridoma technology has led to the production of a number of antibodies that react with different epitopes on the CEA molecule. These can be produced in large quantities without variation in quality. However, localisation of monoclonal anti-CEA antibodies in human colon tumour xenografts either individually or as a mixture has not been shown to be superior to polyclonal antisera (Sharkey et al. 1988a). The anti-CEA antibodies used in these studies were developed in the Cancer Research Campaign laboratories at Charing Cross Hospital. The patients in SECTION III will receive polyclonal anti-CEA antibody (PK2G,PK4S) raised in goat or sheep, as described

by Searle et al.(1980), and those in SECTION IV will be given a mouse monoclonal antibody (A5B7) (Harwood et al. 1986). Localisation of these polyclonal and monoclonal anti-CEA antibodies in MaWi human colon tumour xenografts have been shown to be similar in the animal model (Pedley et al. 1987).

The human immune response to antitumour antibodies

It is likely that repeated injections of immunoconjugates will be required for effective therapy and that the interval between therapy will be determined by the degree of toxicity in the host. While the frequency of injections can be reduced to allow recovery of the normal bone marrow the development of a host immune response to the injected antibody is likely to preclude further therapy.

Anaphylactic reactions to foreign compounds were formally described by Richet in 1902 although observations of the phenomenon had been made by Héricout and Richet in 1895 and by Magendie, more than 60 years earlier (see Richet 1913). During the early part of this century it was shown that the adverse reactions seen in children treated with antibodies against diphtheria toxin were related to the formation of anti-antibodies in the host.

The repeated administration of horse antithymocyte serum has produced the clinical features of serum sickness (Lawley et al. 1984). Although this has not been observed following therapy with antitumour antibodies, an

antiglobulin response is often detected after one or more courses of therapy. Further treatment can lead to an acute hypersensitivity reaction and ineffective tumour localisation in some patients (Sears et al. 1982, Miller et al. 1983, Carrasquillo et al. 1984, Meeker et al. 1985). Even small doses of radiolabelled antibody, given to localise tumour have been shown to be immunogenic (Pimm et al. 1985).

The degree to which anti-antibodies interfere with repeated courses of antitumour antibody therapy was unclear at the start of this work. For instance, Schroff et al. (1985) did not find a reduction in the binding of antitumour antibody to melanoma in the presence of raised antiglobulins. Koprowski et al. (1984) have suggested that the anti-tumour effect following therapy with 17-1A, an antibody to a gastrointestinal cancer antigen, is improved by the presence of anti-idiotypic antibodies.

Not all patients form anti-antibodies following therapy with antitumour antibodies. The absence of an immune response is unlikely to be due to a state of generalised immunosuppression as this is unusual in patients with cancer. However, immunosuppression associated with chronic lymphatic leukaemia may explain why in two studies an anti-antibody response was absent in seventeen patients treated with the mouse monoclonal antibody T101 (Schroff et al. 1985, Shawler et al. 1985). Anti-antibodies did develop in one half of the patients given T101 for cutaneous T cell lymphoma (Shawler et al. 1985).

It has been known for more than 40 years that patients with rheumatoid arthritis have antibodies which react with self and animal IgG. Antibodies reacting with animal immunoglobulins are also known to exist in healthy subjects (Hay et al. 1975, 1976). Immune complexes can be found in the serum following a single injection of anti-CEA. It has been suggested that these complexes consist of CEA and anti-CEA antibody or the anti-CEA antibody and human immunoglobulin. Furthermore, the presence of these complexes do not interfere with immunoscintigraphy of tumours (Primus et al. 1980).

It is important to study whether 'pre-existing' anti-antibodies in the serum have an adverse effect on treatment with radiolabelled antibodies and whether their presence increases the likelihood of a specific anti-antibody response.

In the second part of this thesis assays will be developed to measure the human antibody response to antitumour antibodies. Antiglobulins reacting with sheep, goat and mouse immunoglobulins will be measured in healthy subjects, and in patients before and after single and repeated injections of radiolabelled antitumour antibodies. The studies will examine whether 'pre-existing' anti-antibodies alter the distribution and localisation of radiolabelled antibodies and whether the pattern of clearance of antitumour antibody differs from that seen after repeated therapy following an anti-antibody response.

Prevention of the anti-antibody response

There are strong arguments in favour of investigating measures to prevent the host immune response to antitumour antibodies, particularly in the case of immunoconjugate therapy where the antibody acts as a targeting vehicle and not as a 'trigger' for the immune system of the host.

The induction of immunological tolerance to radiolabelled antibody conjugates by clonal deletion of antibody-forming lymphocytes is an attractive approach. It has been suggested that this was the mechanism responsible for the induction of tolerance to flagellin in lethally irradiated mice that had received spleen cells previously incubated with ^{125}I labelled flagellin (Ada and Byrt 1969). However, the development of anti-antibodies following the injection of large doses of ^{131}I Fab' antimelanoma antibody (Carrasquillo et al. 1984) suggests that this mechanism does not operate in man. But Fab' immunological fragments did appear to be less immunogenic; the median number of courses given before anti-antibodies developed was greater than in patients treated with whole immunoglobulin (Carrasquillo et al. 1984).

It is possible to induce immunological tolerance to immunoglobulins in animals but much harder to achieve this in man (Weigle 1973). However, it was suggested that tolerance was responsible for the absence of an immune response to equine antilymphocyte globulin in a patient pretreated with horse gamma-globulin (Taub et al. 1969).

An 'operational tolerance' or unresponsiveness has been seen in about one third of patients treated with antilymphocyte globulin following pretreatment with 2-18 daily intravenous injections of 50 μg/kg of aggregate-free equine gamma globulin and maximum doses of immunosuppressive agents (Rossen et al. 1971). It has been suggested that ultracentrifugation of immunoglobulin removes aggregates which act as an adjuvant for the immune response and that injection of 'deaggregated' immunoglobulin facilitates the induction of immunological tolerance (Dresser 1962). 'Deaggregated' antibody given without immunosuppression can induce tolerance in mice and rabbits (Dresser 1962, Dresser and Gowland 1964, Biro and Garcia 1965) but not in man (Rossen et al. 1971).

A combination of large a dose of cyclosphosphamide, and smaller amounts of azathioprine and prednisone has been given to patients receiving OKT3, a mouse monoclonal anti-T cell antibody used to treat renal allograft rejection. This regimen reduced the proportion of patients developing antimouse (Thistlethwaite et al. 1986). However, in some situations cyclophosphamide can enhance the immune response in animals (for review see Turk and Parker 1982).

It therefore seems reasonable to examine the effects other drugs; for example, cyclosporin A which is a powerful immunosuppressive agent, first described by Borel et al. (1976). It is was discovered whilst examining the antibiotic activity of fungal extracts from cylindrocapum lucidum or Trichoderma polysporum. Initial studies showed that

cyclosporin A, a highly lipophilic cyclic endecapeptide, suppressed antibody production in mice. The ability of the drug to suppress the cell-mediated immune response associated with allograft rejection and graft versus host disease has attracted much more attention than its effect on humoral immunity (Borel et al. 1976, Borel et al. 1977, Smith 1982). In contrast to cyclophosphamide or azathioprine cyclosporin A produces profound suppression of humoral immunity in animals without bone marrow suppression (Borel et al. 1977). Cyclosporin A has not previously been used to suppress humoral immunity in man.

The main site of action of cyclosporin A is on T lymphocytes, interfering with production and or response to interleukin-2 (for review see Hess 1986). Study of the mechanism of action has centred on its effect on helper and cytotoxic T cells but there is evidence to suggest it also has some action on B lymphocytes (Kunkl and Klaus 1980) and antigen-presenting cells (Varey et al. 1986). The precise subcellular mode of action remains unclear. Cyclosporin A has a high affinity for cyclophilin, a cytosolic protein found in many eucaryotic cells, including those of lower phylogenetic orders (Handschumacher et al. 1984, Harding and Handschumacher 1988). Recently cyclophilin been shown to be identical to an enzyme involved in protein folding that is essential for protein synthesis in the cell (Takahashi et al. 1989, Fischer et al. 1989).

In mice T helper cell priming occurs in the presence of cyclosporin A (Klaus and Kunkl 1983) and this is likely to

account for the failure of cyclosporin A to prevent the secondary immune response *in vivo* (Lindsey et al. 1982). It is unclear whether antibody production remains suppressed following repeated administration of an immunogen and cyclosporin A. The third part of this work (SECTION IV) will investigate whether cyclosporin A can prevent the anti-antibody response to antitumour antibodies given on repeated occasions. It will also examine whether ultracentrifugation of monoclonal antibodies reduces the immune response and facilitates the action of cyclosporin A. The drug will be given to rabbits and then patients given repeated injections of an antitumour antibody.

The aim of this thesis is to investigate two approaches that might improve the therapeutic effectiveness of radiolabelled antibodies. The first examines whether the use of an anti-antibody ('second antibody') reduces the toxicity in the host and the second considers whether the human anti-antibody response can be prevented so that repeated therapy with radiolabelled antibodies is possible.

SECTION II MATERIALS AND METHODS

Chapter 2

PREPARATION AND ADMINISTRATION OF RADIOLABELLED ANTIBODIES TO PATIENTS.

2.1 Introduction

This chapter will describe a novel method of administering therapeutic quantities of radiolabelled antibodies and will outline the methods used to monitor the distribution, toxicity and therapeutic efficacy of radiolabelled antibodies. The results of therapy will be presented in SECTIONS III and IV.

The anti-CEA antibodies were produced in the department of Medical Oncology at the Charing Cross Hospital. The patients in SECTION III received polyclonal anti-CEA antibodies that were raised in goat (PK2G) or sheep (PK4S) and affinity purified on CEA-Sepharose as previously described by Searle et al. (1980). These antibodies have been shown to bind to tumours expressing CEA (Lewis et al. 1982) and are used in a routine double-antibody radioimmunoassay to measure the concentration of CEA the serum of patients (Lewis and Keep 1981). Small quantities of

radiolabelled polyclonal anti-CEA antibody have been injected into patients and they have been shown to localise in CEA-producing tumours (Begent et al. 1982).

Antisheep or antigoat antibodies ('second antibody'), given to some of the patients, were raised in a donkey and purchased from CMD, Bournemouth. The antibodies were immunopurified on goat IgG-Sepharose by T. Adam and were shown to precipitate complexes of PK2G or PK4S and CEA (T. Adam unpublished observations).

The patients in SECTION IV received radiolabelled mouse monoclonal anti-CEA antibody (A5B7) that was developed during the clinical studies with polyclonal anti-CEA. A5B7 is an IgG_1 antibody raised against purified heat-treated CEA; its properties have been described by Harwood et al. (1986).

All the antitumour antibodies were prepared by P.Keep, P. Harwood or T. Adam for clinical use according to the guidelines set out in the Operation Manual for Control of Production, Preclinical Toxicology and Phase I Trials of Antitumour Antibodies and Drug Antibody Conjugates (1986).

The antibodies were radioiodinated by F. Searle, T. Adam or S. Riggs using the chloramine T method. A single protein peak was seen when the sample was passed through a G-200 Sepharose column. The radiolabelled antibody was passed through a 0.2 μm filter (Millipore) and stored overnight at 4 °C before it was injected into patients.

2.2 Administration of radiolabelled antibodies to patients

Patients

The clinical studies were approved by the ethical committee at Charing Cross Hospital. Written consent was given by patients who had histologically proven CEA-producing tumours that were unresectable or metastatic. Patients had to fulfil the criteria in table 2.1 in order to be eligible for the studies.

table 2.1

1. No antitumour therapy in the preceding 4 weeks
2. Measurable tumour or raised serum tumour markers
3. Performance status 0,1,2 (WHO,1979).
4. Haemoglobin ≥ 8 g/dl.
5. White blood cells > 3.5 x 10^9/l.
6. Platelets > 100 x 10^9/l.
7. Plasma creatinine < 120 µmol/l.
8. Plasma bilirubin < 30 µmol/l
9. Negative intradermal skin testing for hypersensitivity to antitumour antibody (and second antibody, if applicable).
10. No history of allergy to iodine or animal proteins
11. No history of asthma or active eczema.

Thyroid protection

Potassium iodide, 180 mg 8 hourly, orally, starting 48 hours before the iodinated antibody and continuing for 4 weeks after the last injection was given to compete with the uptake of ^{131}I by the thyroid gland. Patients were also given oral potassium perchlorate, 200 mg 6 hourly for five days, starting 24 hours before the radiolabelled antibody to reduce the active uptake of ^{131}I by the thyroid, salivary glands and stomach. These drugs have been used to reduce the uptake of iodide in these tissues following the injection of diagnostic doses of radiolabelled antibody. Quantitative data on the uptake of ^{131}I in the presence of these drugs are lacking and the long-term effects of radiolabelled antibodies on thyroid function are unknown. Regular thyroid function tests were performed following radioimmunotherapy.

Intradermal skin testing.

10 µg of antibody in 0.1 ml of 0.9 % saline were injected into the volar aspect of the forearm. A similar volume of 0.9 % saline was injected as a control. The presence of erythema greater than the control was deemed to be a positive reaction. Intradermal skin testing was performed before each course of therapy.

Injection of radiolabelled antibodies.

The patients were all treated in single rooms with an integral bathroom and toilet. The floor and furnishings were all covered with protective polythene to reduce contamination in the event of accidental spillage. Monitoring of the radioactivity in the patient and the room was carried out by the department of Medical Physics at Charing Cross Hospital.

Staff entering the room wore plastic gowns, overshoes and gloves to avoid contamination. A remote method of injection was developed to reduce the dose of radiation to the hands. A simplified diagram of the apparatus is seen in figure 2.1. An intravenous infusion of 0.9 % saline was started. An E-Z catheter (14 gauge; 24 inches) was inserted into the 50 ml pot containing the radioactive antibody and attached via a three-way tap to the intravenous cannula. A 16 gauge Butterfly needle attached to the giving set of a 250 ml bag of 0.9 % saline was inserted into the lead-lined pot. Air was removed from the line by retrograde flow of 0.9 % saline from the patient, placing the 250 ml bag of saline on the floor. Following this manoeuvre the 250 ml infusion bag of 0.9% saline forced the radiolabelled antibody out of the lead pot under hydrostatic pressure. The method was first tested by observing the time and volume of fluid required to wash out a solution of lissamine green from the pot.

The antitumour antibody was infused over 30 minutes, although the readings from a dose meter showed that more

than 90% of the activity had entered the patient after 15 minutes. Less than 1 mCi remained in the pot after the infusion. Patients were encouraged to take oral fluids and intravenous fluids were given for 24 to 60 hours to maintain a urine output of 2.5 to 3.0 litres.

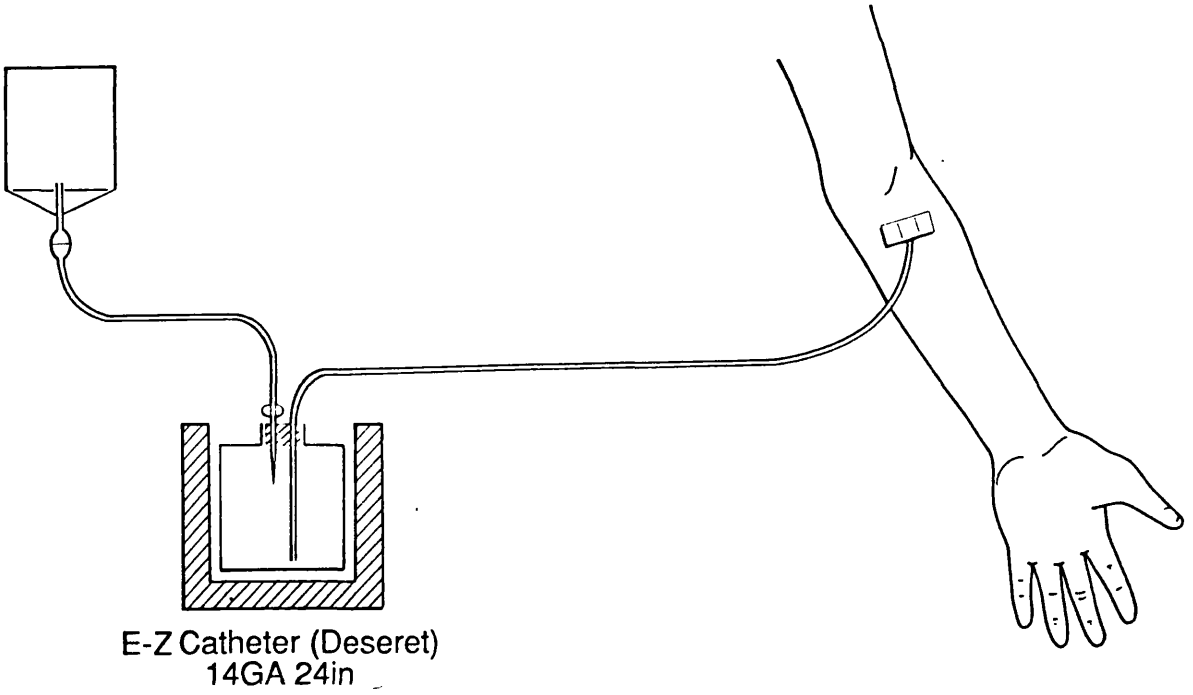


figure 2.1

The apparatus to infuse radiolabelled antibodies into patients. For details see text.

Medical staff were always in attendance during the infusion and stood in the room behind a protective lead shield. Following the infusion all contaminated equipment was removed and stored until it could be safely disposed.

Injection of 'second antibody'

'Second antibody' was given intravenously to some of the patients with colorectal cancer 24 hours after PK2G or PK4S. 600 mg of aspirin and 8 mg of chlorpheniramine were given one hour before the infusion of 'second antibody' and as a precaution 10 % of the dose in approximately 30 ml of 0.9 % saline was given over 10 minutes. If there was no adverse reaction the remainder of the antibody was infused in 100 ml 0.9 % saline over 20 minutes.

2.3 Blood and urine samples of 131-I anti-CEA.

Whole blood samples from patients were collected in preweighed tubes containing lithium heparin and the radioactivity was counted on a LKB 1282 Compugamma. Blood was sampled immediately following the infusion of antitumour antibody and at regular intervals over the next 3 days as stated in chapters 6 and 9. The gamma counter was calibrated with a known activity of ^{131}I so that the counts per minute could be expressed as μCi per gram of blood or as μCi per ml of urine by multiplying the counts per minute by 1.8×10^{-6}. Values were corrected for physical decay of the isotope

using the formula:

$$A = A_o \times e^{-\lambda t}$$

(where A_o is the activity at time zero; λ = transformation constant; t = time in days.).

All urine passed was collected for 72 hours after administration of the radiolabelled antibody. Samples were collected 12 hourly for 48 hours and then 24 hourly. The amount of ^{131}I excreted was calculated by measuring the radioactivity in aliquots of urine.

Measurement of serum protein bound iodine.

This was performed on serial samples of serum from patients by adding 100 µl of a 20 % solution of trichloroacetic acid (BDH) to 100 µl of blood or urine. The mixture was placed on ice for 30 minutes and then centrifuged at 11500 revolutions per minute for 15 minutes (Eppendorf microcentrifuge). The supernatant was removed and the precipitate was washed twice with 95 % ethanol. The radio-activity in the precipitate was counted and the results were expressed as a percentage of the counts per minute measured in the first sample taken immediately after the infusion of the antitumour antibody.

The distribution of the protein-bound radioactivity in the serum was determined by autoradiography of serum separated by 8.5 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (see appendix A). Samples of serum were diluted in 0.05 M PBS and sample buffer (appendix A) so that

each sample contained between 16,500 to 33,000 counts per minute per 100 µl.

60 µl were loaded onto a 5 % sodium dodecyl sulphate polyacrylamide stacking gel and run at 20 mA for 1 hour and then 40 mA through the gel. The details of the electrolyte buffer are given in appendix A. The gel was then stained with Coomassie blue (appendix A), washed with 'destain' and dried (Biorad dryer) on filter paper after 1 % glycerol had been added for at least 1 hour. The gels were exposed for 18 to 24 hours on Kodak X-OMAT X-ray film at - 20 °C and developed by the X ray developer in the department of Radiology at Charing Cross Hospital.

2.4 Gamma camera imaging

The images obtained from scans were used to determine the distribution of radiolabelled antibody and organ dosimetry. Patients no: 1 to 9 were scanned on a Nuclear Enterprises LFOV gamma camera linked to a Nodecrest computer. A Gemini 700 (Technicare/IGE) camera linked to a Saturn computer was used for all the other patients. This camera has a 400 KeV high resolution collimator and can acquire planar and tomographic data. The gamma counts obtained from an elliptical orbit of the collimator were used to reconstruct the SPET image (single photon emission tomography). The camera was operated by S.Riggs and A. Green. The first scans were obtained five to eight hours after the injection of radiolabelled antibody. In most cases

further scans were obtained daily for the first three days and then two to three times per week for as long as sufficient radioactivity remained in the patient. For SPET this was up to 120 hours after therapy.

2.5 Bone marrow dosimetry

The β dose to bone marrow was derived from the cumulative radioactivity in blood. A computer program, written by S.Riggs calculated blood activity in μCihr by fitting an exponential decay curve between each pair of points and integrating the activity with respect to time. An exponential curve was fitted through the last three points to calculate the activity to infinity. The calculation of the β dose to bone marrow was made using the method proposed by Bigler et al. (1986), which will be discussed more fully in chapter 5. The main assumption of this method is that radioactivity in blood and bone marrow are equivalent. The mass of the red bone marrow, taken as a proportion by weight of the calculated mass of red marrow in standard man (70 kg) was used to calculate the β dose to the bone marrow.

2.6 Tumour response

Serial estimations of the concentration of serum tumour markers and X-ray computerised tomography (CT) of tumour deposits were used to assess the response to radioimmunotherapy. The latter were performed in the

Department of Radiology at Charing Cross Hospital.

Most patients had tumours that were refractory to treatment with chemotherapy or radiotherapy. In this situation one might anticipate the tumour response rate following radioimmunotherapy, measured by criteria such as those proposed by the World Health Organisation (WHO) (1979), to be low. The demonstration of a change in the concentration of serum tumour markers would help to support evidence for therapeutic activity of radioimmunotherapy and refute the concept that treatment is ineffective because a reduction in the size of the tumour is not seen. Serial measurement of serum tumour markers has provided considerable information on the optimal management of gestational trophoblastic and germ cell tumours (Bagshawe and Begent 1981,1983) and the introduction of new drugs (Newlands and Bagshawe 1977).

Monitoring of the concentration of serum CEA, alone, following anti-CEA therapy may be unreliable as ^{131}I anti-CEA might interfere with the radioimmunoassay for CEA and the presence of immune complexes of CEA and anti-CEA in the serum could affect the assay for free CEA in the serum. Therefore measurement of CEA and an unrelated serum tumour marker such as CA19-9, a carbohydrate-rich glycoprotein found in the serum of almost one third of patients with advanced colorectal cancer (Ritts et al. 1984), was performed to support evidence of a therapeutic effect of radiolabelled antibodies.

Serum tumour markers

Carcinoembryonic antigen (CEA)

The concentration of CEA in the serum was measured by the assay laboratory in the Department of Medical Oncology using the double-antibody radioimmunoassay described by Lewis and Keep (1981). The upper limit of normal for the serum CEA concentration is 10 μg/ml. Patients' sera was stored at - 20 °C until the radioactivity had decayed as falsely low serum CEA values were seen when samples containing ^{131}I were assayed. An example is shown in figure 2.2. This was probably due to binding of radiolabelled anti-CEA antibody to the CEA-antibody and polyethylene glycol complex. The increase in the radioactive counts precipitated on the filters gives a falsely low CEA value.

CA 19-9

The carbohydrate antigen CA 19-9 was measured by radioimmunoassay using a kit purchased from Abbott Laboratories. After allowing sufficient time for radioactive decay of ^{131}I, 100 μl samples of serum were added to wells in the reaction tray followed by a further 100 μl of buffer. Beads coated with a mouse monoclonal antibody to CA 19-9 were dispensed into each reaction tray. Serum, containing high concentrations of CA 19-9 were first diluted tenfold or more in a serum diluent (30 μl in 270 μl).Extra supplies of diluent were obtained from Abbott laboratories as the kit did not contain sufficient diluent to measure serial samples with a high CA 19-9 concentration. The reaction trays were

incubated for 3 hours at 37 °C. The beads were then washed with distilled water using a Pentawash II automatic well washer. 200 µl of mouse monoclonal antibody to CA 19-9 conjugated to ^{125}I were then added and incubated for 3 hours at room temperature. Following further washing the beads were counted on the LKB compugamma. A standard curve was constructed from 5 standard samples and a known internal standard. The assay was able to measure 5 U/ml or more of CA19-9.

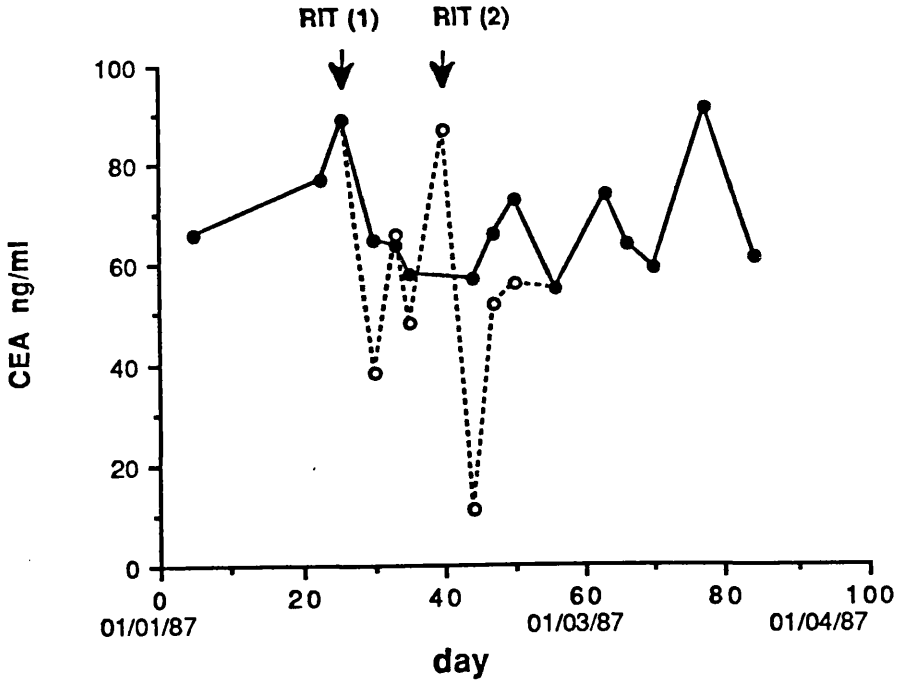


figure 2.2

Effect of ^{131}I on the determination of CEA concentration in serum by radioimmunoassay. CEA concentrations were measured before o and after • radioactive decay of ^{131}I in the serum.

2.7 Toxicity.

The toxicity of radioimmunotherapy was recorded according to the criteria established by the World Health Organisation (WHO 1979). Samples of blood were taken at regular intervals for full blood count, plasma urea, creatinine, electrolytes and liver function tests. Plasma thyroxine and thyroid stimulating hormone were measured before and at monthly intervals after radioimmunotherapy. The concentration of serum complement (C3 and C4) was measured twice weekly during the first 4 weeks after therapy. The concentration of immunoglobulins and rheumatoid factor in serum were measured before and four to six weeks after antibody therapy. Samples were analysed by technical staff in the Departments of Haematology and Chemical Pathology at Charing Cross Hospital. Rheumatoid factor was measured by the technical staff in the Department of Microbiology at Charing Cross Hospital, using latex agglutination (Behring kit) and red blood cell agglutination by rabbit IgG (RAHA) (Fujirebo Inc.).

Chapter 3

SUPPRESSION OF THE IMMUNE RESPONSE: ULTRACENTRIFUGATION OF ANTITUMOUR ANTIBODIES AND ADMINISTRATION OF CYCLOSPORIN A.

3.1 Introduction

This chapter will describe the preparation of ultracentrifuged anti-CEA antibodies and the preparation of cyclosporin A that was used to suppress the immune response to repeated injections of antitumour antibody in animals and patients. Details of the drug administration in animals and in patients will be given in chapters 8 and 9 respectively.

3.2 Preparation of antitumour antibodies

Antihuman chorionic gonadotrophin antibody (W14)

W14 is an IgG_1 antibody raised against intact hCG. It was used for the animal experiments as a monoclonal antibody to CEA was unavailable. Following injection, radiolabelled W14 has been shown to localise in hCG producing tumours growing in animals or patients (Searle et al. 1984, Begent et al. 1985). The antibody was grown either in mouse ascites or cell free supernatant.

Immunopurification was performed with some assistance from C. Bier, according to the method of described by Searle et al. (1984). The antibody was heated to 56 °C for 30 minutes, centrifuged at 3600 rpm for 15 minutes and passed through a 0.5 µm filter before being added to an hCG-cyanogen bromide activated Sepharose 4B column (9 x 2.6 cm)(Pharmacia). Column chromatography was performed at 4°C. The immunoaffinity column was packed above a desalting column of G-25 Sephadex (26 x 70 mm)(Pharmacia). The column was washed with 0.05 M phosphate buffered saline (PBS)(see appendix A). Bound material was eluted with 15 ml of 3 M ammonium thiocyanate. The absorbance of this peak at 280 nm was separated from the peak containing ammonium thiocyanate. The latter was identified by a change in colour of strips of filter paper which had been soaked in saturated copper sulphate. The first peak was concentrated by pressure ultracentrifugation (Amicon) and dialysed against 0.05 M phosphate buffer and stored at -70 °C. An aliquot of material was added to a Sephacryl S-200 column (1.6 x 100 cm)(Pharmacia) and washed with PBS. A single peak was eluted in the same void volume as normal IgG (62 ml). The protein content was determined by absorbance of the solution at 280 nm.

Radioiodination of W14

A small amount of anti-hCG antibody given to animals was radiolabelled with ^{125}I to monitor the clearance of W14 in the blood. The sample was taken from antibody that had been ultracentrifuged. Radiolabelling was performed in a hood by the iodogen method. A 1 mg/ml solution of iodogen (1, 3, 4, 6-tetrachloro-3a, 6a diphenylglycoluril) (Pierce) was diluted to 20 µg/ml with dichloromethane. 150 µl was added to a glass tube and air-dried. The tubes were stored in a desiccator until use. Radioiodination was performed at room temperature. 125 µg of ultracentifuged W14 in 100 µl of 0.2 M phosphate buffer, pH 7.5 (appendix A) was added to the tube followed by 1 mCi of ^{125}I (Sodium iodide, Amersham). The tubes were shaken regularly for 30 minutes. The sample was added to a Sephadex G-25 column (Pharmacia, 30 x 0.9 cm) to separate the protein from free iodine and the column was washed with sterile 0.9% saline (figure 3.1). Aliquots of approximately 1 ml were collected and the radioactivity counted in the gamma counter. Virtually all of the radioactivity was contained in the protein peak which appeared with the void volume. It was pooled and diluted in 0.9% saline before injection into rabbits.

Ultracentrifugation of antibodies

Ultracentrifugation was performed after the method of Rossen et al. (1971). Samples of antibody in 0.2 M phosphate buffer were centrifuged at 48,000 g (Sorvall 55B) in two

stages, each for one hour, using a fixed angle rotor at 4 °C. After the first stage the top two-thirds of the original sample were carefully removed with a pipette and re-centrifuged under the same conditions. A small amount of white precipitate was often seen at the bottom of the centrifuge tube. After the second centrifugation the top-third was removed and injected. The protein content of each fraction following centrifugation was calculated from the absorbance of the solutions at 280 nm and related to the known absorbance of IgG at this wavelength. Table 3.1 shows a typical distribution of the ultracentrifuged fractions of W14.

The composition of the various centrifuged fractions of W14 were studied by high pressure liquid chromatography (Gilson) within 4 hours of ultracentrifugation. They were passed through a 0.2 μm filter (Acrodisc, Gelman UK) and 20 μl was added to a SW300 (Waters) gel filtration column. The absorbance was measured at 280 nm and the peaks are shown in figure 3.2. No difference in the elution profile was seen.

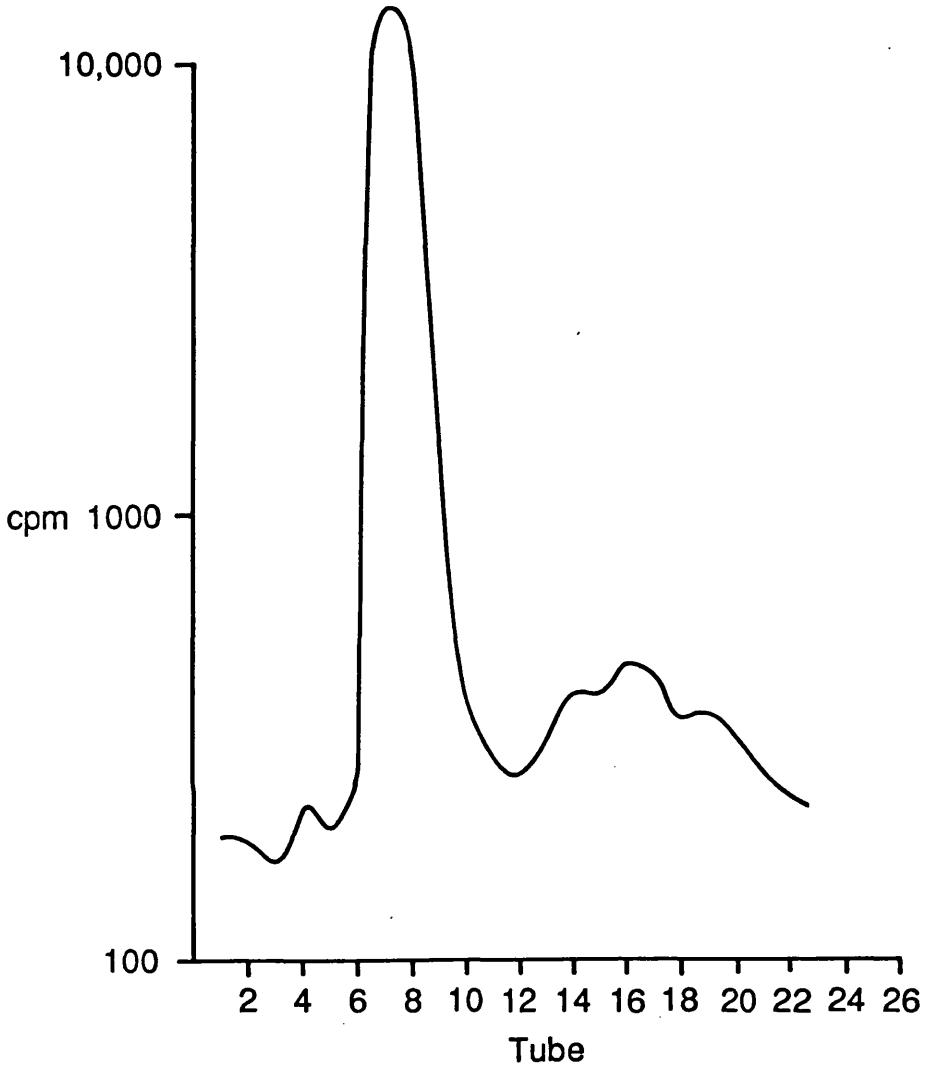


figure 3.1

Separation of radioiodinated anti-hCG (W14) by G-25 Sephadex chromatography. Samples were collected in aliquots of 40 drops, approximately 1 ml. The labelled protein appeared in the void volume, usually tubes 6 to 9. The small broad peak of free iodide (tube 15 to 18) was not always seen.

Ultracentrifugation of A5B7 was performed by a similar method except that the antibody was dissolved in 0.05 M phosphate buffer. Because the supply of A5B7 was limited it was necessary to radiolabel 40 to 60 % of the solution from the second centrifugation in some of the patients who were given more than 5 mg of ultracentrifuged antibody for conjugation with 50 to 80 mCi ^{131}I.

table 3.1 **PROTEIN CONCENTRATION OF FRACTIONS OF W14 SEPARATED BY ULTRACENTRIFUGATION**

Sample	vol(ml)	conc. (mg/ml)	total (mg)
<u>first centrifugation</u>			
TOP 2/3	4.66		
LOW 1/3	2.33	1.29	1.02
<u>second centrifugation</u>			
TOP 1/3	1.55	0.79	1.22
MID 1/3	1.55	1.00	1.55
LOW 1/3	1.55	1.38	2.14
			7.93

Starting concentration of W14 = 1.23 mg/ml in 7 ml.(8.61 mg)

92.1 % of the antibody was recovered following ultracentrifugation.

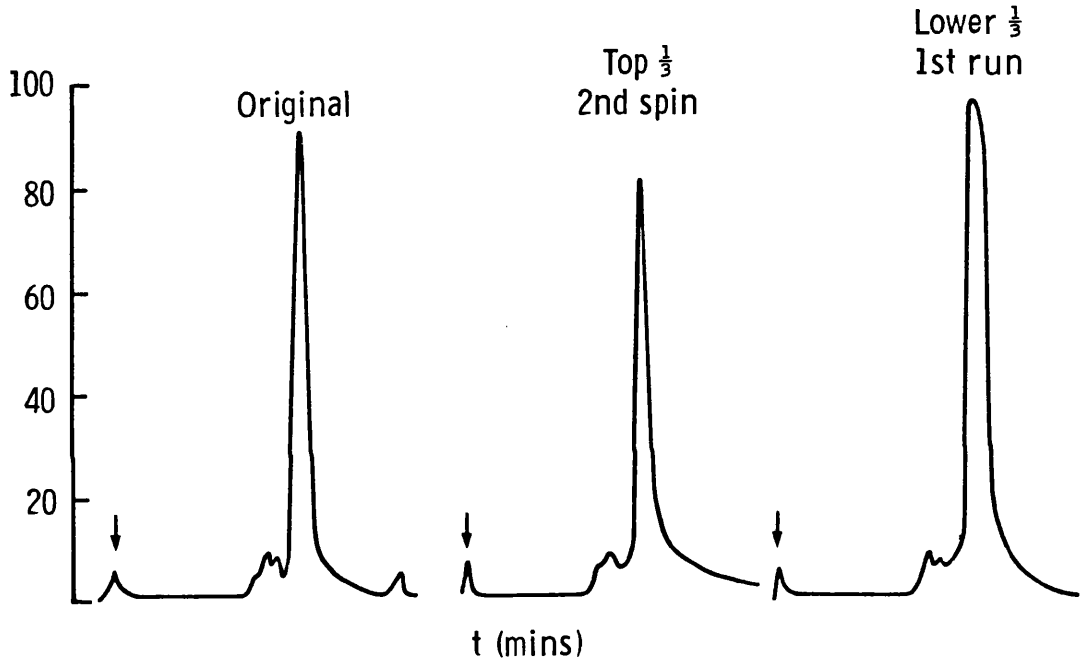


figure 3.2
Composition of fractions of W14 after ultracentrifugation. The absorbance of the fractions were measured at 280 nm following separation by high pressure liquid chromatography. The sample was applied a time 0 minutes (arrow). Most of the antibody appeared in a single peak, co-eluting with IgG. A small higher molecular weight peak was seen in all samples.

Administration of W14 to rabbits

32 New Zealand white rabbits weighing from 1.5 to 3.3 kg (mean 2.3 kg) were used. They were immature and all but five were female. They were kept under standard conditions in the animal house and weighed weekly.

The animals received an intravenous injection of 200 µg W14 into a lateral ear vein 24 hours after the first injection of cyclosporin A. The antibody was diluted in 0.9% saline to 250 to 400 µl; the volume injected varied for each batch of experiments. Each animal also received approximately 400 µl of 0.9% saline containing 8 µg W14 conjugated with 80 µCi ^{125}I.

Blood clearance of ^{125}I W14 in rabbits

Samples of blood were taken from the lateral ear vein 30 minutes after injection and then at regular intervals over the next 28 days. Radioactivity was measured on the gamma counter (LKB 1280 Compugamma) at the same time and therefore not corrected for radioactive decay. The radioactivity was expressed either as counts per minute per gram blood or as a percent of the initial counts per minute per gram of blood, where experiments performed at different times were compared.

3.3 Immunosuppression of animals and patients with cyclosporin A.

For the animal experiments cyclosporin A (Sandoz AG, Basel) was dissolved in Miglyol 812 (Dynamit Nobel, UK) and ethanol to 100 mg/ml. Typically, 100 mg of cyclosporin A was dissolved in 0.6 ml Miglyol and 0.04 ml ethanol which had been heated to 50 °C. The solution was made up to 1 ml with Miglyol 812 and stored at 4°C. The drug was given by intramuscular injection into the hind quarter of the rabbits. A fresh solution was made for each set of experiments. Miglyol 812 has previously been shown not to affect the immune response (Smith 1982).

An oral preparation of cyclosporin A (Sandoz Ltd, UK) was given to patients. The whole blood concentration of the drug was measured on the third, fourth and fifth day by a polyclonal radioimmunoassay (Sandoz. AG) by Dr. D. Holt New Cross Hospital.

Chapter 4

MEASUREMENT OF ANTI-ANTIBODIES IN HUMAN AND ANIMAL SERUM

4.1 Introduction

This section will describe the methods that were developed to detect antibodies to animal immunoglobulins following the administration of antitumour antibodies to patients and animals. For many years the Rose-Waaler test has been used to detect human antibodies reacting with IgG coated onto sheep erythrocytes (rheumatoid factor). Using a radioimmunoassay, which is more sensitive, it has been shown that antibodies to animal immunoglobulins exist in the serum of healthy subjects as well as patients with rheumatoid arthritis (Hay et al. 1975, 1976).

This technique and that of enzyme immunoassay can also be applied to measure anti-antibodies before and after administration of antitumour antibodies. Other methods have also been used, such as column chromatography which demonstrated the presence of human IgG and antitumour antibody immune complexes in serum (Primus et al. 1980, Pimm et al. 1985, Davies et al. 1986) and autoradiographic Ouchterlony gel precipitation (Klein et al. 1986). These are

sensitive but time-consuming and are unsuitable for the analysis of a large number of samples. In this study an enzyme immunoassay was developed, largely to avoid any possible interference that might have occurred from radiolabelled antitumour antibody in the assay system. The methods were based on an enzyme immunoassay first described by Voller et al. (1975). The assay was adapted to measure antibodies to sheep, goat or mouse IgG in animals or man.

At the start of this work only semiquantitative results of anti-antibody assays could be found in the literature. Many groups have continued to publish results as a dilution titre, optical absorbance of an enzyme substrate, optical absorbance in relation to binding of the enzyme conjugate to known quantities of human IgG bound to plates, or the amount of antihuman antibody bound per ml of serum (Hay et al. 1975, Sears et al. 1984, Foon et al. 1984, Schroff et al. 1985, Courtenay-Luck et al. 1986, Spitler et al. 1987). Meeker et al. (1985) and Shawler et al. (1985) were the first to quantify the results of a human anti-antibody assay in relation to an immunopurified standard.

In this chapter the preparation of an immunopurified reference human antisheep/goat and antimouse antibody will be described. A reference standard enables the results of different assays to be compared. The results of the anti-antibody assays will be presented in SECTION IV.

4.2 The human IgG immune response to goat, sheep and mouse antibodies

Flat-bottom 96-well immunoassay plates (Alpha) were coated with a 100 µl of a 10 µg/ml solution of polyclonal sheep anti-CEA (PK4S) or mouse monoclonal anti-CEA (A5B7) in 0.01 M carbonate-bicarbonate pH 9.6 buffer (coating buffer; see appendix A) per well. Absorption of the antibody to the plastic in an alkaline solution was found to be better than in PBS. Coating solutions could be used for up to two weeks and maximum absorption of the antibody to the wells occurred by incubation for 90 minutes at room temperature. The plates were then washed 3 times with distilled water by flooding and eversion and used immediately or stored for up to 48 hours at 4 °C. No difference in nonspecific binding of serum to the plate was seen if 5% bovine albumin in PBS was added to block unbound sites on the plates.

Samples of serum were diluted in PBS with Tween 20 and 0.1% bovine serum albumin (dilution buffer; see appendix A). Duplicate samples of 100 µl of a tenfold or greater dilution of serum were added to each well. Incubation overnight at 4 °C or for 2 hours at room temperature produced equivalent results. The latter method was used and the plates were agitated continuously on a rocker. The wells were then washed 3 times with PBS-Tween 20 (washing buffer), using a wash bottle and allowed to dry.

100 µl of a 1 in 1000 dilution of goat antihuman IgG-alkaline phosphatase (Miles-Yeda) in diluting buffer was

added to each well and incubated for 2 hours at room temperature on a rocker. The wells were then washed five times with washing buffer. The background binding of the enzyme conjugated antibody to the wells containing PK4S, measured by absorbance of the substrate (see below) was typically < 0.05. This was no greater than the binding to wells not coated with antigen.

The substrate for the enzyme was p-nitrophenyl phosphate (Sigma). 5 mg tablets were dissolved to make a 1 mg/ml solution in 10% diethanolamine-HCl buffer, pH 9.8 (see appendix A). Positive samples developed a yellow colour. The addition of 2 mM magnesium chloride increased the rate of the colourimetric reaction. The reaction was stopped after 15 to 20 minutes by adding 50 µl 3 M sodium hydroxide and the absorbance at 405 nm was measured in a Titertek Multiskan 340/2 (Flow Labs.).

Goat antihuman IgG antibody used for the human antisheep or goat assays produced an unacceptably high background in the human antimouse assay. Rabbit antihuman IgG-alkaline phosphatase (Dako) also gave a high background absorbance when added to wells containing mouse IgG but not to uncoated wells. The degree of background binding depended on the type of mouse IgG coated on to the wells. For example, binding to A5B7 was greater than to W14, although both were IgG_1 antibodies. The background reactivity could be reduced by incubating the antihuman IgG conjugate with a 1 in 10 dilution of normal mouse serum but this also reduced the specific binding of the enzyme-conjugated antibody.

Finally, a goat antihuman IgG conjugated to alkaline phosphatase (Sigma A0287) was selected as cross reacting antibodies to mouse IgG had been removed during preparation. Background absorbance due to binding of this antibody to A5B7 coated onto plates was < 0.05, when 100 µl of a 1 in 2000 dilution (in diluting buffer) was added for 2 hours at room temperature. The background absorbance was < 0.01 for W14 or PK4S bound to the plate. This antihuman antibody conjugate was used for all the quantitative determinations of human IgG antisheep, goat or mouse antibody.

Quantitation of human IgG anti-antibodies.

Immunopurification of human antisheep/goat IgG

Dilutions of a sample of pooled serum from a patient who had been treated with sheep anti-hCG serum for drug-resistant choriocarcinoma in 1978 was found to bind to plates coated with PK4S or normal goat IgG (Sigma). The titre of antiglobulin was high and binding to polyclonal goat or sheep anti-CEA (PK2G or PK4S) coated onto the wells of microtitre plates was equivalent. An immunopurified preparation of this serum was used as a standard for the human antisheep or antigoat assays.

7.5 ml of serum, stored at -20 °C was thawed, heated to 56 °C in a water bath and then centrifuged for 15 minutes at 3,000 rpm to precipitate large aggregates. The supernatant was removed, passed through a 5 µm filter (Acrodisc, Gelman UK) and added to a column containing of 7 ml cyanogen

bromide activated Sepharose 4B (Pharmacia) to which 20 mg goat IgG had been coupled. Goat IgG was dissolved in coupling buffer (0.1 M sodium bicarbonate containing 0.5 M sodium chloride, pH 8.3) and mixed with the gel which had first been washed with 1 M hydrochloric acid. After incubation for 2 hours at room temperature the remaining sites on the activated sepharose were blocked by 1 M ethanolamine buffer, pH 9.0. The mixture was left overnight at 4 °C and then washed three times with 3 M ammonium thiocyanate followed by PBS.

The affinity gel was placed in a 'basket' on top of a G-25 Sephadex column (26 x 700 mm; Pharmacia). The serum sample was washed through the column with 0.05 M PBS in a cold cabinet at 4 °C. Three absorbance peaks at 280 nm were detected using an LKB Uvicord (figure 4.1a).

Bound material was eluted by adding 20 ml of 3 M ammonium thiocyanate (figure 4.1b) and concentrated by pressure ultrafiltration (Amicon). The sample was then passed through 1.2 µm and 0.45 µm filters (Acrodisc, Gelman UK) and the protein content determined by the absorbance of the solution at 280 nm in a spectrophotometer (Pye Unicam SP8-100). The concentration of protein, determined from the extinction coefficient of IgG was 460 µg/ml. From the total yield of antiglobulin the original 7.5 ml of serum contained about 610 µg/ml of human anti-antibody.

The constituents of the immunopurified protein were examined by immunoelectrophoresis using a 1.5 % solution of Agar (Oxoid) in barbitone buffer, pH 8.6 (appendix A) on a

microscope slide. A current of between 8 to 10 mA was passed across the gel which contained the sample and 1% bovine serum albumin and 3 drops of 0.05 % bromophenol blue as a marker. Rabbit antihuman serum (Miles) was added to the trough and the gel was left in a humid box over night (see figure 4.2a).

Further analysis was performed by sodium dodecyl sulphate polyacrylamide gel electrophoresis using an 8.5% polyacrylamide gel and a 5% stacking gel (Biorad Apparatus)(see appendix A). Approximately 10.5 µg of putative IgG antisheep/goat antibody was diluted in an equal volume of sample buffer (see appendix A) containing 2-mercaptoethanol. The sample was boiled for 10 minutes and then loaded onto the gel apparatus. Electrolyte running buffer (see appendix A) was added and a current of 20 mA increasing to 40 mA after one hour was passed through the gel. Protein in the gel was stained with Coomassie blue (appendix A) and the constituents are shown in figure 4.2b.

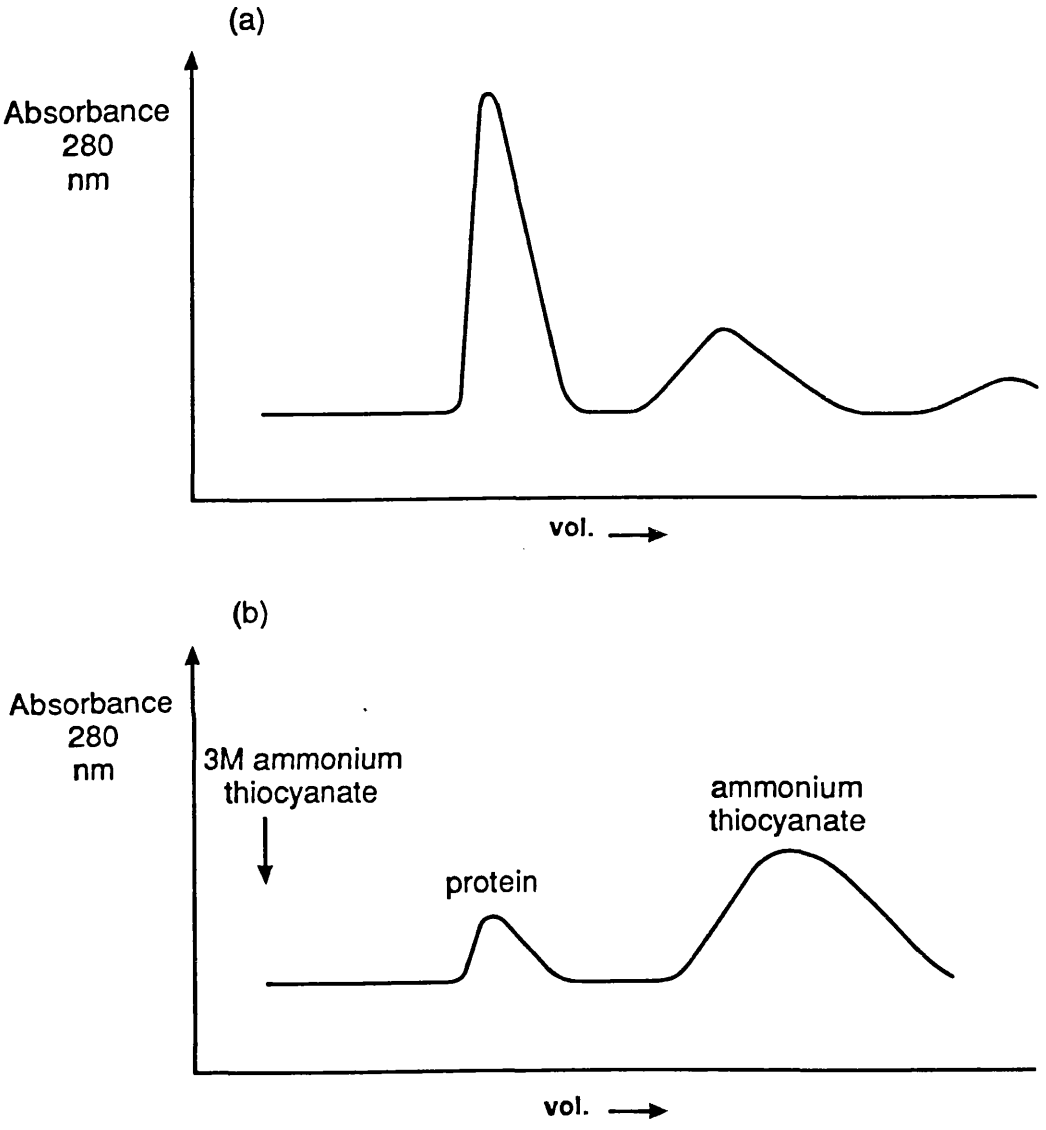


figure 4.1
Immunopurification of human IgG antisheep antibody by affinity column chromatography. (a) The absorption spectrum of human serum passing through human Sepharose 4B IgG affinity column placed on a Sephadex G-25 column. The first peak contains the majority of serum proteins. (b) Bound material, removed by adding 3 M ammonium thiocyanate, appeared in the same elution volume as serum proteins and separate from the ammonium thiocyanate peak.

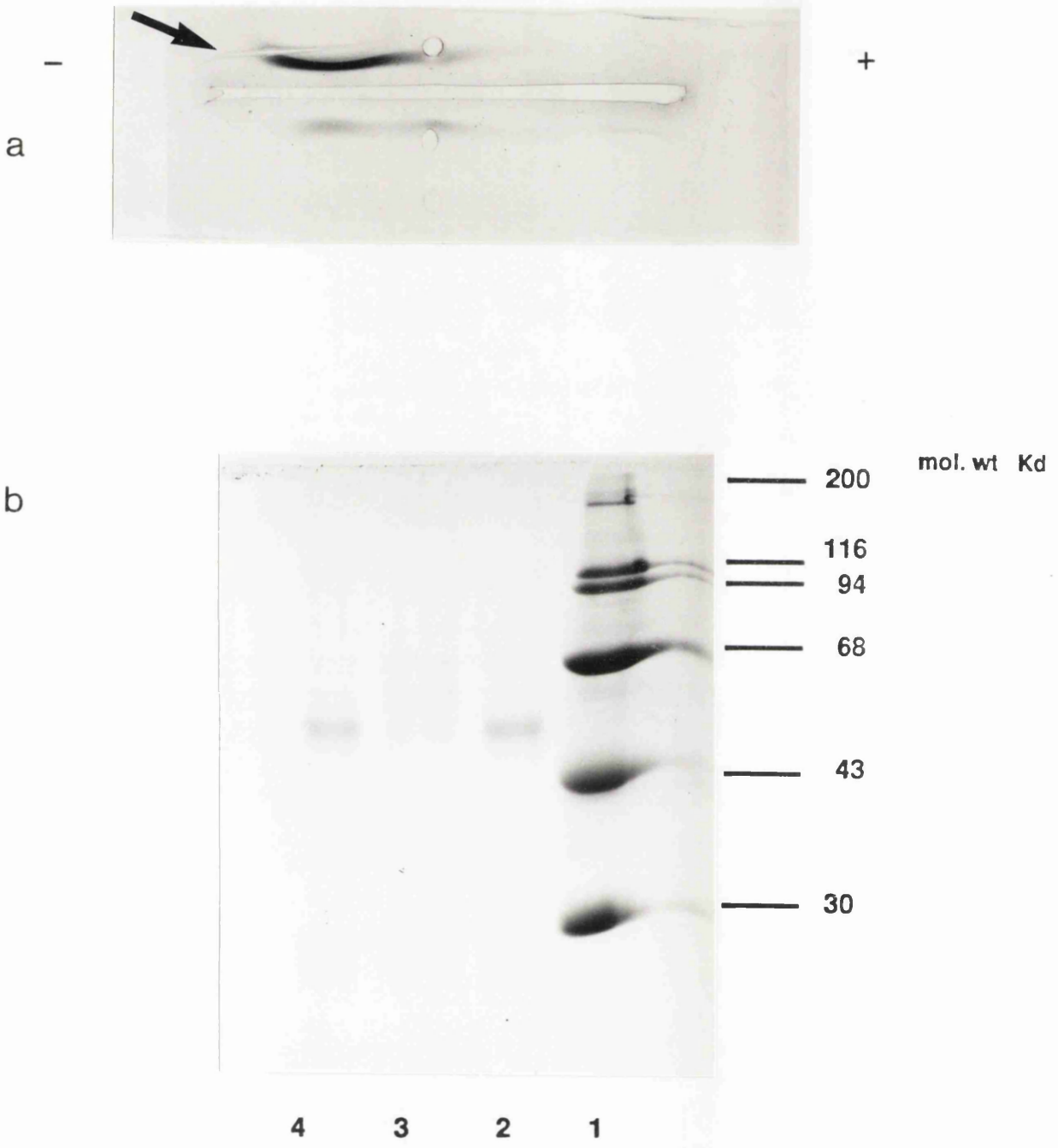


figure 4.2

a. Immunoelectrophoresis of human IgG antisheep.
A single precipitation band is seen following incubation with rabbit antihuman antiserum (arrow). The sample in the other well had been passed through a protein A column to remove IgG in an attempt to isolate human IgM/IgA antisheep.

b. sodium dodecyl sulphate polyacrylamide gel electrophoresis of a reduced sample of immunopurified serum demonstrates two bands consistent with heavy and light chain bands in lanes 2 & 4 (marked); lane 3 immunopurified sample that did not bind to protein A. There is a faint band of 70,000 daltons, consistent with μ chain.

Quantitation human antisheep/goat by binding inhibition to human IgG

A second independent method was used to quantify immunopurified human antisheep/goat antibody. This measured the degree to which dilutions of human antisheep antibody were able to inhibit the binding of antihuman IgG-alkaline phosphatase to human IgG bound to microtitre plates. The concentration of human IgG antisheep/goat was calculated by comparing the inhibition of binding with that of known amounts of normal human IgG added to antihuman-alkaline phosphatase.

150 ng of human IgG (Sigma) in 50 µl of 0.05 M phosphate buffer were added to each well of a microtitre plate and dried overnight at 37 °C. The protein was then fixed by exposing the wells to 100 µl of methanol for 2 minutes. The wells were then washed 3 times with washing buffer.

Dilutions of the immunopurified human anti-antibody and dilutions of a 1 mg/ml solution of human IgG (Sigma)(64 to 0.25 µg/ml) in 0.05 M phosphate buffer were made in diluting buffer. Samples were incubated with goat antihuman IgG conjugated to alkaline phosphatase (Miles-Yeda), diluted 1 in 500 in diluting buffer. The samples were mixed and left over night at room temperature.

100 µl of each sample in triplicate was added to each well and incubated for 3 hours at room temperature. The wells were washed three times with washing buffer before 100

µl of the alkaline phosphatase substrate was added.

The mean absorbance values were plotted and the results are shown in figure 4.3. The human anti-antibody concentration was derived by relating the absorbance of several dilutions of the immunopurified human antigoat antibody to that of the human IgG-antihuman conjugate reaction. The mean concentration of human IgG antisheep/goat antibody was 418 µg/ml (s.d. 71), which was similar to the value calculated by absorbance of the immunopurified sample at 280 nm.

For the enzyme immunoassay of human IgG antisheep/goat the top working dilution was 1 in 80. Small aliquots were diluted 80 fold in diluting buffer and stored at -70 °C until use.

Immunopurification of human antimouse antibody

A reference human IgG antimouse antibody was prepared from a patient no: 17 (see chapter 9) who developed a high antimouse antibody titre following three injections of ^{131}I radiolabelled A5B7. The first dose was given to localise tumour by radioimmunoscintigraphy and two subsequent therapeutic doses were given six months later. The samples were first immunopurified on a cyanogen bromide activated Sepharose 4B column conjugated to 50 mg of mouse IgG (Sigma). The methods were as described for the preparation of human IgG antisheep antibody.

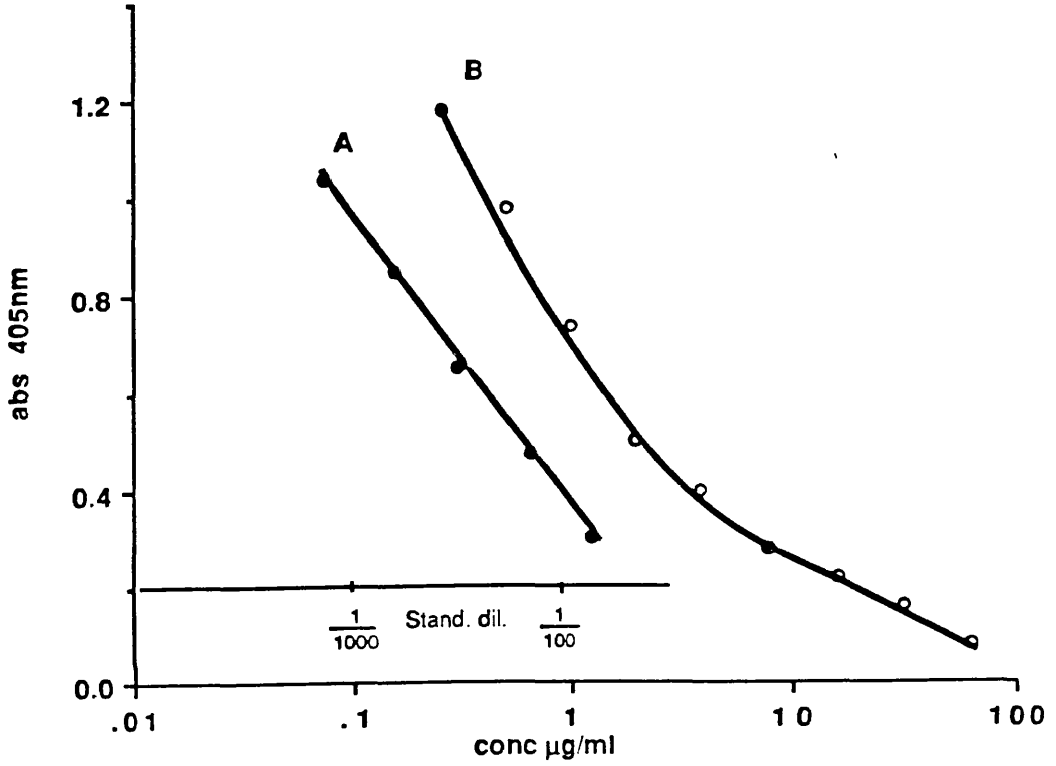


figure 4.3

Human IgG antigoat antibody was measured by a competitive binding inhibition assay. The reduction in binding of antihuman IgG-alkaline phosphatase to human IgG, in the presence of dilutions of the 'standard' human antigoat antibody (A), measured by optical absorbance of the enzyme substrate was parallel to the change in absorbance when antihuman IgG-alkaline phosphatase was incubated with dilutions of normal human IgG (B).

4 mls of serum were added to the immunoabsorbent column. The unbound material was collected and reduced back to its original volume by pressure ultrafiltration (Amicon). 3 M ammonium thiocyanate was added to elute the bound fraction and the elution profile was similar to that seen in figure 4.1. The protein peak was concentrated by pressure

ultrafiltration and the concentration determined by the absorbance at 280 nm.

Antimouse antibody in the eluted sample and the unbound fraction were determined by enzyme immunoassay. The volume of the eluted fraction was 60 % of the original volume of serum added to the column. However, more human antimouse antibody was found in the unbound fraction compared with the "immunopurified" material (figure 4.4a).

A second sample of serum was immunopurified on a column of cyanogen bromide activated Sepharose 4B coupled to 30 mg of A5B7. The amount of human antimouse antibody in the unbound fraction, restored to its original volume, was now much less (figure 4.4b). This suggests that much of the human IgG antimouse antibody in this patient was anti-idiotypic, or directed against variable portion(s) of the A5B7 molecule.

The sample immunopurified on the A5B7 immunoabsorbent column was used as the standard for the human IgG antimouse antibody assays and was diluted to 6 µg/ml in diluting buffer and stored at 4 °C until use. The composition of the sample was assessed by immunoelectrophoresis with rabbit antihuman serum (figure 4.5a) and by sodium dodecyl sulphate polyacrylamide gel electrophoresis (figure 4.5b).

Patterns of the human anti-antibody response

Three different patterns of human antimouse antibody response were observed. A prozone phenomenon was seen in patients with a high titre of antimouse antibody. Initially, dilutions of serum led to an increase in the optical absorbance. As further dilutions were made the dilution curve (figure 4.6) became parallel to that of the immunopurified standard human IgG antimouse antibody. This type A response differed from that of patients with a moderately high titre (type B), who showed no prozone effect. The dilution of serum from patients with a low titre of antimouse antibody (type C), typically the pretreatment sample, was different. The absorbance of dilutions, starting at 1 in 10 was not parallel to the standard. In this group the concentration of anti-antibody was determined from a 1 in 10 dilution of serum which may have resulted in an overestimation of the amount of IgG antimouse.

The pattern anti-antibody dilution curves from patients with human antisheep/goat antibody was similar to that of the antimouse antibody dilution curves but the differences were less marked. The IgG antisheep/goat antibody assay was one tenth as sensitive as the antimouse antibody assay. The range of dilution for the immunopurified human antisheep antibody standard was about 5 μg/ml to 0.3 μg/ml; for the antimouse antibody assay the range was 0.6 μg/ml to 0.025 μg/ml.

Each sample of serum was assayed in duplicate, unless otherwise stated. The results were expressed as the mean of two assays. A standard sample of serum was included as a positive control. The coefficient of variation of the assay was less than 22%.

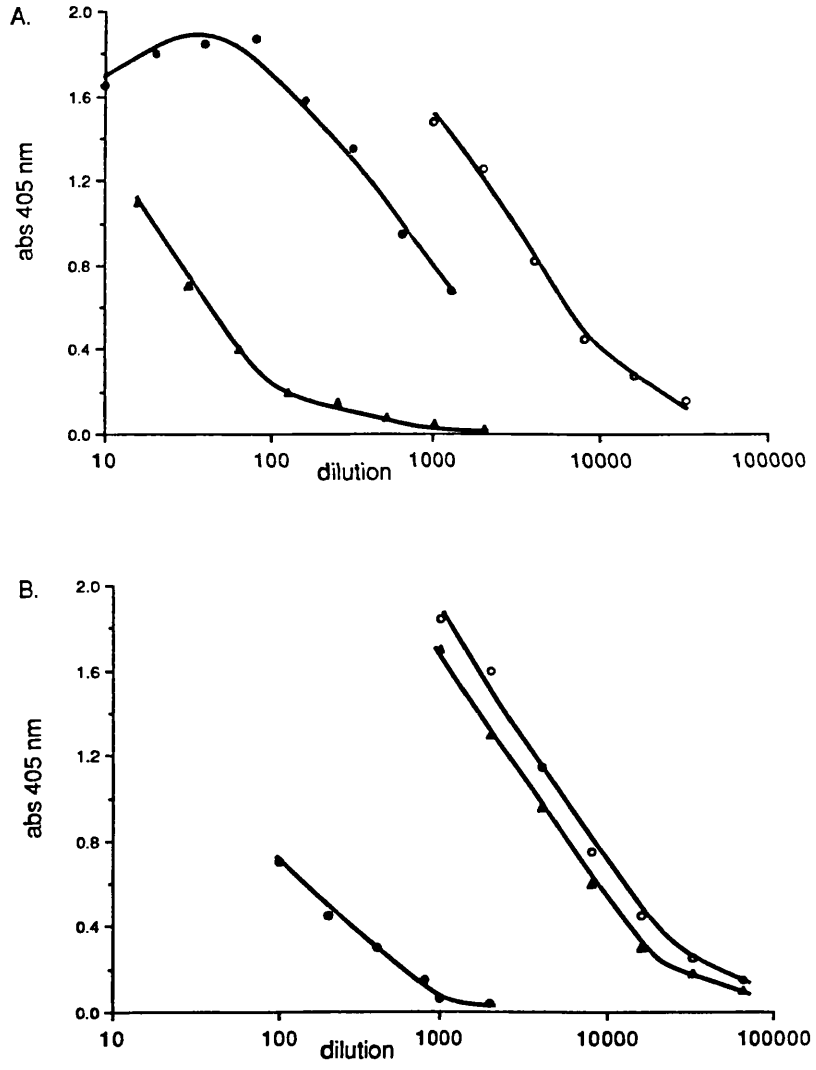


figure 4.4

Antimouse antibody following affinity chromatography of serum on A.) mouse IgG-Sepharose 4B and B.) A5B7-Sepharose 4B. Binding of whole serum o , material that had bound to the immunoabsorbent column ▲ , and unbound material ● , to mouse IgG (A5B7). In A.) most of the activity is in the unbound fraction and differs little from that of whole serum. In B.) most of the antimouse antibody is found in material eluted from the immunoabsorbent column.

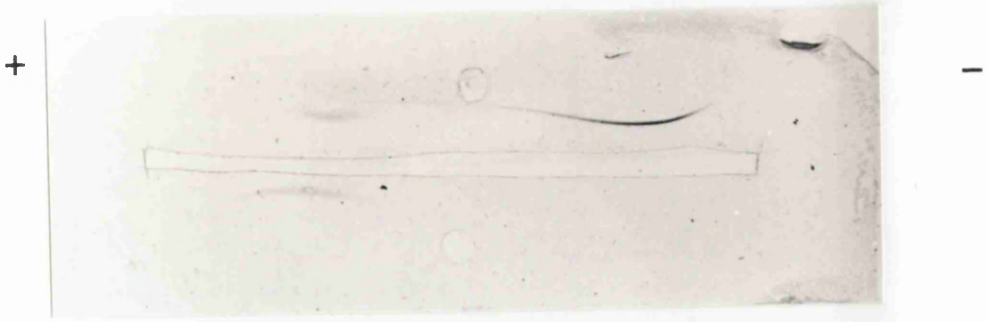


figure 4.5a Immunoelectrophoresis of immunopurified antimouse antibody demonstrates the presence of a single band consistent with IgG.

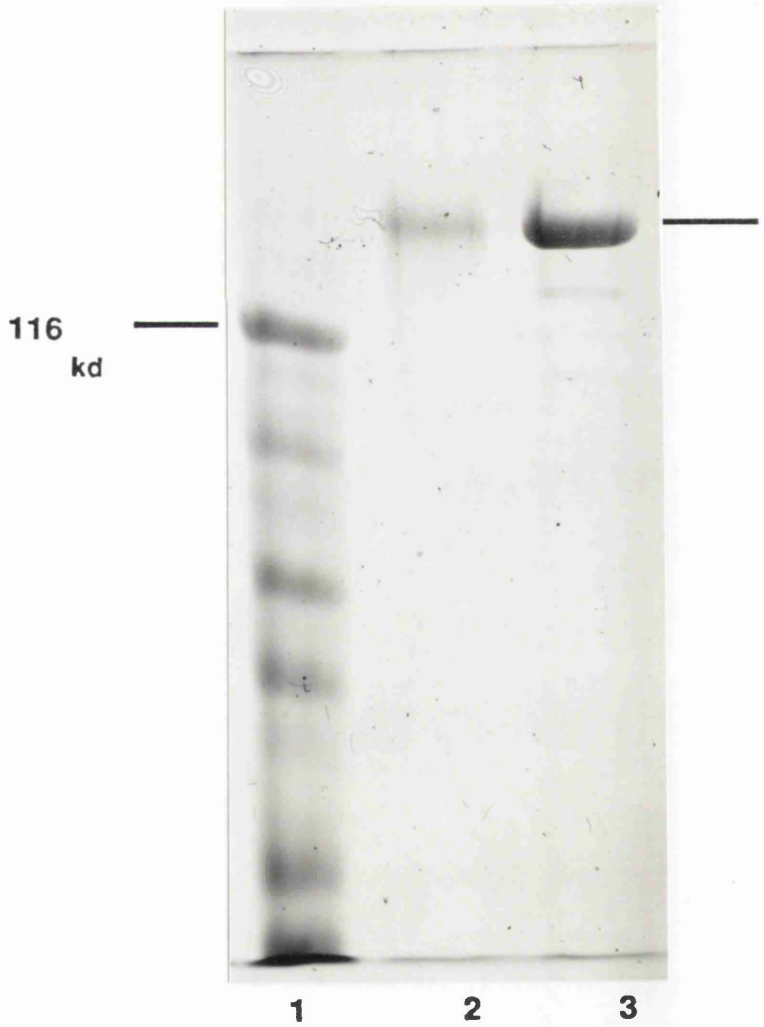


figure 4.5b.
Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the sample eluted from the A5B7-affinity column. PHAST system (Pharmacia) 7.5 % gel stained with Coomassie blue. Lane 1 molecular weight markers (Sigma); lane 2 immunopurified human antimouse antibody; lane 3 mouse IgG (A5B7).

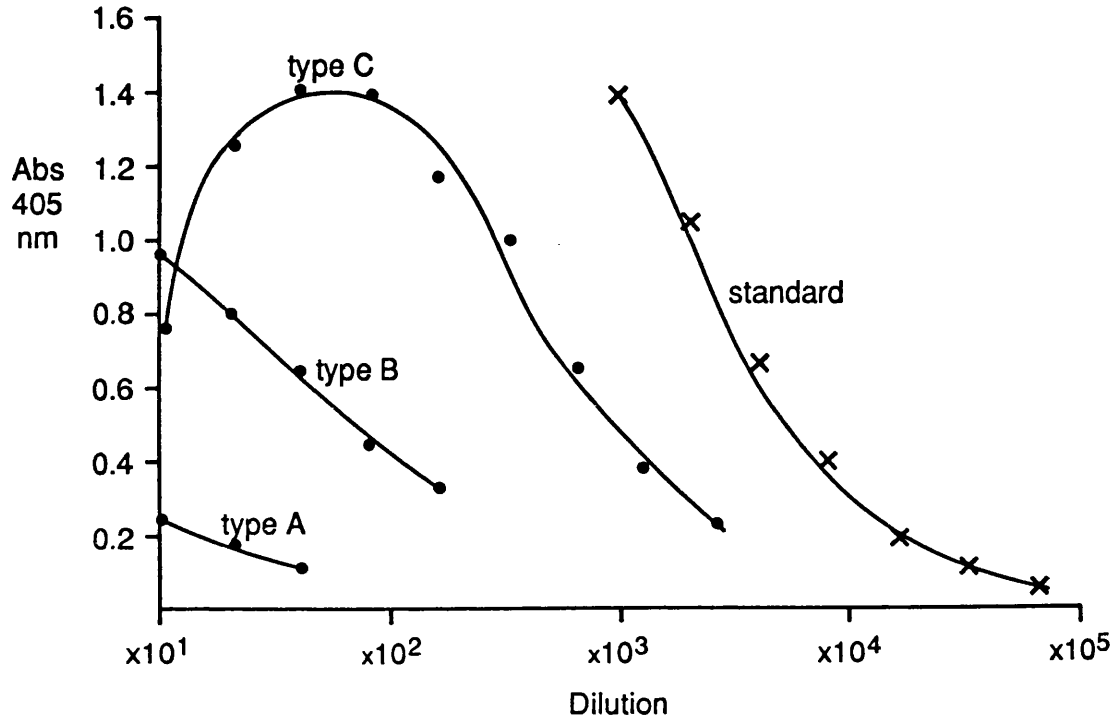


figure 4.6

Enzyme immunoassay of the human IgG antimouse antibody. Three different patterns are seen in dilution curves of samples from patients with a low (A), moderate (B) and high (C) response to mouse immunoglobulin. The concentration of human IgG anti-antibody was determined from dilutions that were parallel to the immunopurified standard.

Human IgG antidonkey antibody ('second antibody')

200 ng of horse IgG (Sigma) in PBS was dried onto the wells of microtitre plates. Dilutions of patients sera, in diluting buffer, were added to each well in duplicate. Following incubation with antihuman IgG conjugate the absorbance of several dilutions of the pretreatment samples was compared with dilutions of samples taken from 13 to 31 days after the injection of the antitumour antibody. It was

at this time that the highest titre of antisheep or goat antibody were seen. A result was considered to be positive if for a given absorbance value of a pretreatment sample the dilution of the post-treatment sample was $\geq 2 \times \log_2$ dilution of the pretreatment sample.

4.3 Anti-idiotypic antibodies

The difference in the titre of human IgG antimouse antibodies obtained from the A5B7 or mouse IgG immunoabsorbent columns suggested that much of the anti-antibody response was against the variable or idiotypic portion of the anti-CEA antibody. This was further supported by an inhibition assay which measured the binding of the human immunopurified antimouse antibody to A5B7 on the surface of microtitre wells following incubation with an excess of mouse IgG, A5B7 or SB10. SB10 is a mouse monoclonal antibody of the same isotype as A5B7, directed against human chorionic gonadotrophin and was produced by S. Sharma in the department of Medical Oncology at Charing Cross Hospital. Antibodies to shared regions and variable regions of the mouse immunoglobulin molecule were identified by comparing the differences in the degree of inhibition in the presence of the blocking antibodies.

Approximately 150 ng/ml of the sample of antimouse antibody, in dilution buffer, was incubated with the blocking antibody in a concentration ranging from 0.01 to 1000 µg/ml. The samples were allowed to stand overnight at

room temperature and then assayed for binding to A5B7, using the enzyme immunoassay methods described to measure human antimouse antibody. In the example shown in figure 4.7 the inhibition of binding of whole serum to A5B7 is greater in the presence of 1000 µg/ml A5B7 than in the same concentration of mouse IgG (Sigma). These antibodies inhibited the binding of human IgG antimouse, affinity purified on mouse IgG-Sepharose, to the same extent. Much of the anti-A5B7 antibody (anti-idiotype) remained in the fraction that did not bind to the mouse IgG affinity column. To test for anti-idiotypic antibodies in patients, samples of serum were incubated with 1000 µg/ml of SB10 or A5B7 before measuring the binding to A5B7. The results of these assays will be presented in chapter 7.

4.4 Human IgM anti-antibody assay

The method was similar to the IgG assay except that rabbit antihuman µ chain specific antibody conjugated to horseradish peroxidase (Dako), diluted 1 in 500 was used to identify human IgM. The substrate was O-phenyldiamine in 0.01 M citrate buffer, pH 4.8 containing 0.006 % hydrogen peroxide. 100 µl of substrate was added and left for 30 minutes in the dark. The colour was developed by adding 100 µl of 4 M hydrochloric acid to each well and the absorbance was read at 492 nm. The background absorbance with anti µ was less than 0.02. The results of IgM antiglobulin assays were expressed as a change in absorbance of serial samples.

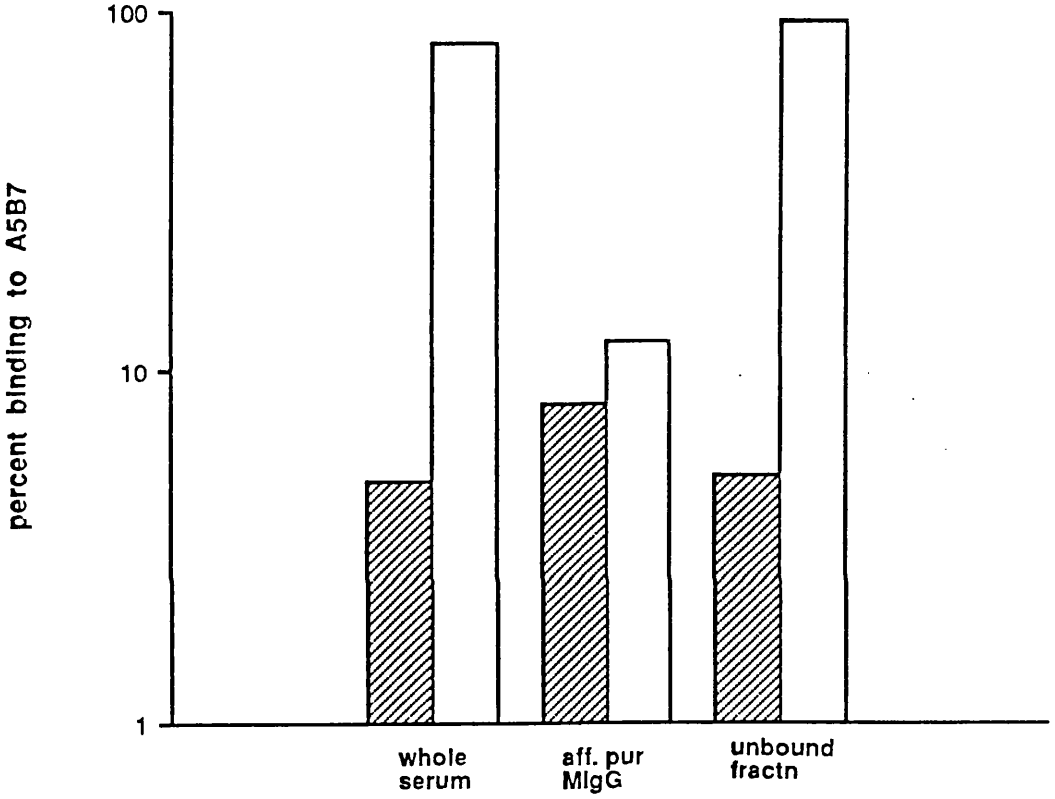


figure 4.7
The anti-idiotypic response to ant-CEA. The presence of anti-idiotypic antibodies to A5B7 was detected by comparing differences in the inhibition of binding of immunopurified fractions of human IgG antimouse antibody to A5B7 in the presence of an excess of mouse IgG □ or A5B7 ▨ .

4.5 Rabbit IgG antimouse antibody

The principle of this assay was similar to the one described in section 4.2. 50 µl of the mouse monoclonal antibody W14, 200ng per well, in 0.05 M phosphate buffer was dried at 37 °C onto polyvinyl microtitre plates and then fixed with methanol. Drying of antibody on to plates was more economical than the absorption method as it used less antibody and it produced equivalent results. 100 µl of a 1 in 1000 dilution of goat antirabbit IgG conjugated with alkaline phosphatase (Sigma) was used to identify rabbit IgG bound to W14.

Immunopurification of rabbit IgG antimouse antibody

The assay was standardised using a rabbit antimouse serum, raised against W14 immunopurified on a mouse IgG-Sepharose 4B column. The protein content was calculated from the absorbance at 280 nm. The composition of the standard was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (figure 4.8).

Rabbit antibovine serum albumin

IgG antibodies to bovine serum albumin (Sigma) were detected by measuring the binding of antirabbit IgG-alkaline phosphatase, diluted 1 in 1000, to rabbit IgG bound to a solution of 10 µg/ml of bovine serum albumin (Sigma) in coating buffer absorbed on wells of microtitre plates. The binding of tenfold dilutions of serum in buffer, free of

albumin were compared before and after injection of bovine serum albumin.

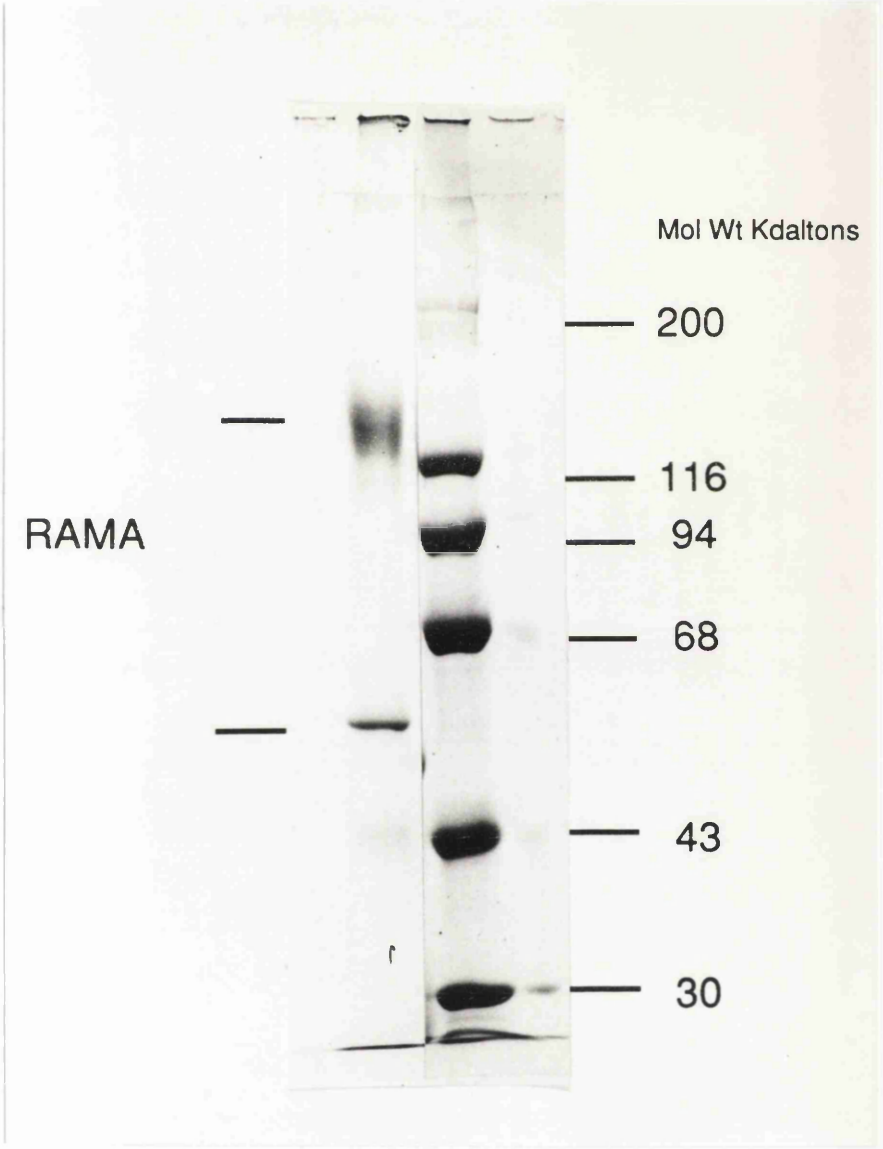


figure 4.8
Rabbit antimouse antibody. 7.5 % sodium dodecyl sulphate polyacrylamide gel electrophoresis of material eluted from mouse IgG-Sepharose 4B with ammonium thiocyanate. The gel was stained with Coomassie blue. There is a broad band of about 140 kd and a smaller lower molecular weight band.

4.6 Discussion

The enzyme immunoassays that have been described provide a means of measuring anti-antibodies in serial samples of serum. They provide a means of screening for human anti-antibodies, like the haemagglutination tests reported by Shawler et al. (1985) and Davies et al. (1986).

Results from serial samples can be compared using an immunopurified standard anti-antibody. The immunopurified samples were shown to comprise mainly of IgG and the two methods used to quantify the human IgG antigoat antibody assay produced comparable results. The enzyme immunoassay techniques and results of antiglobulin measurements in patients following treatment with monoclonal antibodies, reported by Shawler et al. (1985) and Meeker et al. (1985) were similar to the methods and results in this study (see SECTION IV). Schroff et al. (1985) found the concentration of human IgG antimouse antibody following therapy to be lower. They used a different technique in which the optical absorbance of the substrate following binding of antihuman IgG conjugate to the antitumour antibody was compared with the absorbance measured when the same concentration of enzyme-conjugated antibody was incubated with varying amounts of normal human IgG fixed to a plate. The results were expressed as μg IgG bound per ml of serum.

Schroff et al. (1985) have used this approach to measure the human IgM response, using pure IgM, dried onto wells as a standard but they have not published results of

the assay. Some experiments were performed using their method. Either mouse IgM or monoclonal IgM from a patient with Waldenströms macroglobulinaemia, obtained following separation of high molecular weight protein on a Sephacryl S-300 column, were fixed in varying concentrations to the wells of a microtitre plate. Immunopurification of IgM anti-antibody was not attempted as the titre of IgM antiglobulin was low. The absorption of enzyme substrate, following incubation with ant-μ antibody was proportional to the amount of IgM bound to the plate over a range of 30 to 250 ng of IgM but there was considerable interassay variation. For this reason a change in optical absorbance was used to measure IgM anti-antibodies.

Interpretation of the concentration of the IgG anti-antibody levels requires some caution. While it gives an indication of the amount of IgG anti-antibody the values could also be affected by the affinity of the antiglobulin. For instance, a sample containing high-affinity antiglobulin may appear to contain a higher concentration of anti-antibody than one with low-affinity antibody. The concentration of anti-antibody is determined from a dilution curve over a range where the absorbance is proportional to the dilution. In this part of the 'dilution curve' the antibody is no longer in excess, so that the amount of high-affinity antibody bound to a plate for any given dilution would be greater than that for low-affinity antibody.

Thus, both affinity and concentration are likely to account for the three different patterns of response to

antitumour antibodies. The type A response indicates that it is important to examine several dilutions of serum to make sure that the 'dilution curve' is parallel to that of the standard. Courtenay-Luck et al. (1986) have shown that the prozone effect (type A response) was due to IgM anti-antibody that interfered with the binding of IgG anti-antibodies. The effect could be abolished by removal of IgM antibodies using column chromatography.

Evidence from two sources suggested that most of the immune response in the patient whose serum was used as the standard for the IgG antimouse antibody assay was anti-idiotypic. Firstly, there was a considerable difference in the yield of anti-antibody following separation on mouse IgG or A5B7 affinity columns; secondly, there was a difference in the inhibition of binding of the antibody to A5B7 in the presence of an isotype-matched antibody. Study of the contribution of the anti-idiotypic response following therapy with antitumour antibodies is important if therapy with immunological fragments or human antibodies is considered. The results of the anti-idiotypic antibody response will be presented in SECTION IV.

SECTION III THERAPY WITH POLYCLONAL RADIOLABELLED ANTIBODIES.

Chapter 5

EFFECT OF 'SECOND ANTIBODY' ON THE DISTRIBUTION AND EXCRETION OF ^{131}I ANTI-CEA AND THE RADIATION OF BONE MARROW

5.1 Introduction

The two chapters in SECTION III describe the effects of radiolabelled polyclonal anti-CEA in patients with colorectal cancer. This chapter examines whether 'second antibody' reduces the radioactivity in blood by accelerating the clearance of ^{131}I anti-CEA and lowers the radiation dose to the bone marrow.

'Second antibody' was given 24 hours after the primary antibody. The optimal time of administration of 'second antibody' in patients is unknown but studies in animals have shown an improvement in the ratio of radioactivity in the tumour relative to blood following the injection of 'second antibody' at 6, 24 or 48 hours after the primary antibody (Sharkey et al. 1988a, Pedley et al. 1989, Begent et al. 1989a). Liposomally entrapped 'second antibody', given 24 hours after the antitumour antibody has been shown to accelerate the removal of polyclonal anti-CEA antibody from the circulation of patients (Begent et al. 1982).

Calculation of the bone marrow radiation dose was based on measurement of blood radioactivity. This approach has been used by Bigler et al. (1986) who have suggested that radioactivity in the blood closely approximates that of the bone marrow as blood circulates freely through the sinusoidal vasculature of bone marrow which has large fenestrae and a discontinuous basal lamina. Examination of bone marrow aspirates demonstrated rapid equilibration of radioactivity in the blood and bone marrow, with activity in the marrow typically 90 % that of the blood (Bigler et al. 1986). While measurement of blood radioactivity may give a good approximation of the short-range β radiation dose to bone marrow it is much harder to calculate the contribution of gamma dose. Measurement of the gamma effect on bone marrow requires knowledge of the distribution of radioactivity in all the surrounding organs.

Only the β dose to bone marrow was estimated in patients treated with radiolabelled antibody, alone or with 'second antibody'. The side-effects of ^{131}I anti-CEA and the results of therapy will be presented in chapter 6.

5.2 Treatment outline

Ten patients with advanced colorectal cancer were given 40-60 mCi ^{131}I (median 54) conjugated to 2.5 mg sheep anti-CEA (PK4S). Five patients were given donkey antisheep ('second antibody') 24 hours after the antitumour antibody. The distribution of radiolabelled antibody was studied by counting radioactivity in serial blood samples, continuous collections of urine, and serial gamma camera images. A description of the patients is found in table 5.1. The preparation and administration of antibodies has been described in SECTION II.

Table 5.1

Radioimmunotherapy with polyclonal sheep anti-CEA with or without 'second antibody' (group 2)

Pt. no:	age	sex	tumour date diag.	previous therapy	tumour sites	CEA µg/l	anti-CEA mg.	date	^{131}I mCi	sec. Aby. mg	antibody localisation
					'SECOND ANTIBODY'						
8 (EM)	56	M	colon 11/81	surgery	liver lung	27	2.5	1/86	52	10.0	-
10(CC)	75	M	colon 2/81	surgery radiotherapy	lung	27	2.5	2/86	55	10.0	lung
11(DK)	46	M	rectum 8/83	surgery	pelvis	240	2.5	2/86	60	10.0	pelvis
12(PB)	50	M	rectum 8/84	surgery radiotherapy 5FU	mediast. liver	60	2.5	3/86	42	10.0	pelvis liver
13(AD)	32	F	colon 4/85	surgery 5FU;EMA/CO radiotherapy	lung liver pelvis	171	2.5	3/86	40	10.0	liver pelvis
					NO 'SECOND ANTIBODY'						
6 (JB)	60	M	colon 4/85	surgery radiotherapy	neck Rt.iliac fossa	4574	2.5	10/85	48	-	pelvis
7 (GP)	65	M	colon 5/84	surgery	liver	12390	2.5	11/85	51	-	-
9 (MC)	67	M	colon 10/82	surgery radiotherapy	liver	135	2.5	1/86	60	-	liver
14(RS)	56	M	anal canal 12/84	surgery radiotherapy POMB	pelvis	51	2.5	5/86	55	-	pelvis
15(HA)	56	F	colon 10/81 (Ovary)	surgery radiotherapy DDP,cyclo,MTX	pelvis peritoneum	9	2.5	5/86	38	-	-

POMB = cisplatin, vincristine, methotrexate, bleomycin
MTX = methotrexate; 5FU = 5 fluorouracil; DDP = cisplatin; cyclo = cyclophosphamide
EMA/CO = etoposide, methotrexate, actinomycin D/ cyclophosphamide, vincristine

Pt no: = accession number (initials)
sec. Aby = second antibody

5.3 Removal of ^{131}I anti-CEA from blood after administration of 'second antibody'

The rate of removal of ^{131}I from the circulation was similar in the two groups up to 24 hours. The radioactivity in the blood in figure 5.1 is expressed as a percentage of the amount measured immediately after injection, usually 30 minutes after the start of the infusion of antitumour antibody. Calculation of the actual amount of radioactivity at the time of the first blood sampling indicated that there was a rapid early equilibration phase of the radiolabel in the blood which cannot be seen in figure 5.1. Assuming that all of the injected activity is in the circulation 10 minutes after the start of the infusion a mean of 51.7 (sd 9.6) % of the injected activity per kg blood had disappeared from the circulation by a mean of 42.6 (sd 12.0) minutes after the start of the infusion. After 6 hours there was a fairly uniform rate of clearance of the radiolabel with a mean half-life of 17.25 hours (sd 3.9).

Following the injection of 'second antibody' there was a rapid acceleration in the removal of radioactivity from the circulation. A mean of 11.6 % (sd 3.6) of the initial activity remained 3 hours after 'second antibody' which was significantly less than the mean of 29.2 % (sd 8.0) seen in the five patients who did not receive 'second antibody' ($t = 4.48$; $p < 0.01$). Beyond 27 hours, 3 hours following 'second antibody', the rate of clearance of ^{131}I was similar in the two groups.

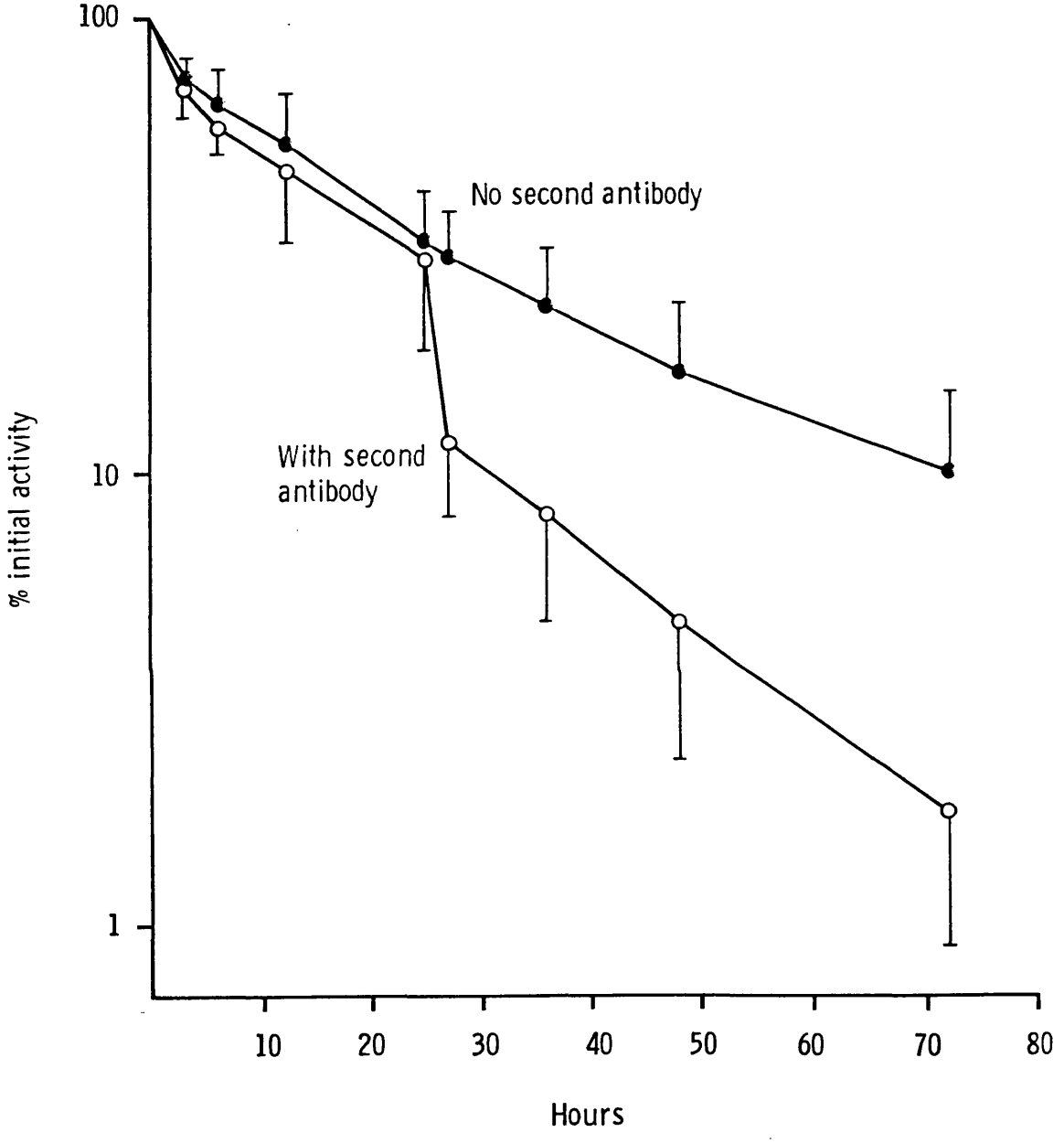


figure 5.1
The effect of 'second antibody' (donkey antisheep) on the removal of ^{131}I sheep anti-CEA (PK4S) from the circulation. Results are the mean and standard deviation of 5 patients per group. Initial activity was measured immediately after the infusion of radiolabelled antibody.

The amount of radioactivity bound to protein in the serum was measured in six patients by precipitation with 10 % (final concentration) trichloroacetic acid (TCA) (table 5.2).

table 5.2

PROTEIN BOUND IODINE IN SERUM AFTER RADIOLABELLED ANTIBODY

	second antibody (n = 5)	no second antibody (n = 1)
hours after PK4S	% ^{131}I ppt. by TCA (sd)	% ^{131}I ppt. by TCA
1	92.4 (1.8)	93
23-25	84.8 (9.8)	95
26-29	67.6 (11.1)	-
46-48	56.6 (12.4)	97
66-72	72.6 (19.4)	95

An autoradiograph of serial samples of serum, separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis shows the distribution of radiolabelled protein (figure 5.2). During the first 24 hours most of the radioactivity migrated with a protein band between 116 to 160 kd, compatible with IgG. A higher molecular weight band at the origin of the gel was also present. This could represent complexes of anti-CEA. Some radioactivity was seen in the region of lower molecular weight proteins, possibly immunological fragments.

The gamma camera images in figure 5.3 show the distribution of radioactivity before and after 'second antibody'. By 8 hours after 'second antibody' the radioactivity in the heart and great vessels was greatly reduced and much of the radioactivity remaining was seen in the liver and spleen.

A comparison of the relative rates of clearance of ^{131}I through the liver and spleen is found in figure 5.4. These data were obtained from the planar gamma camera images by counting the radioactivity over sections of tissue. In each of the five patients who were not given 'second antibody' the rate of fall of radioactivity in the liver was more or less the same as that seen in the spleen. Measurements in four of the patients who were given 'second antibody' show that in three the clearance through the spleen lagged behind that of the liver. Radioactivity in the spleen increased in one patient after 'second antibody'. There was no evidence to suggest that the rate of clearance of ^{131}I through either the liver or spleen was faster in the patients who received 'second antibody'.

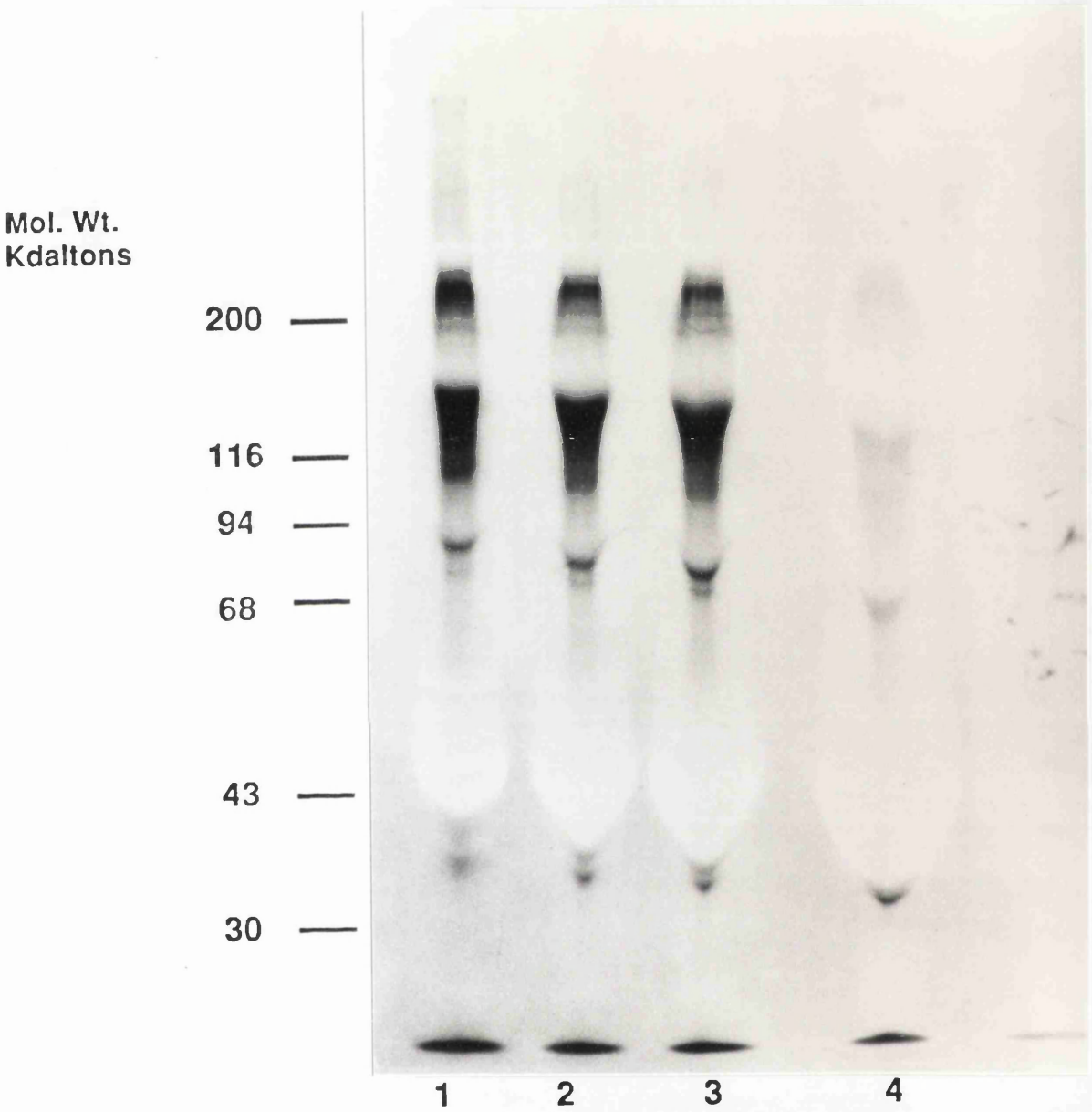


figure 5.2
The distribution of protein bound ^{131}I within the first 24 hours after injection of ^{131}I PK4S. Separation of the proteins was performed by 8.5 % sodium dodecyl sulphate polyacrylamide gel electrophoresis. Proteins were stained with Coomassie blue. An autoradiograph shows the distribution of radioactivity in serum proteins: 30 minutes (lane 1); 12 hours (lane 2); 24 hours (lane 3) after PK4S; and 28 hours, 4 hours after second antibody (lane 4).

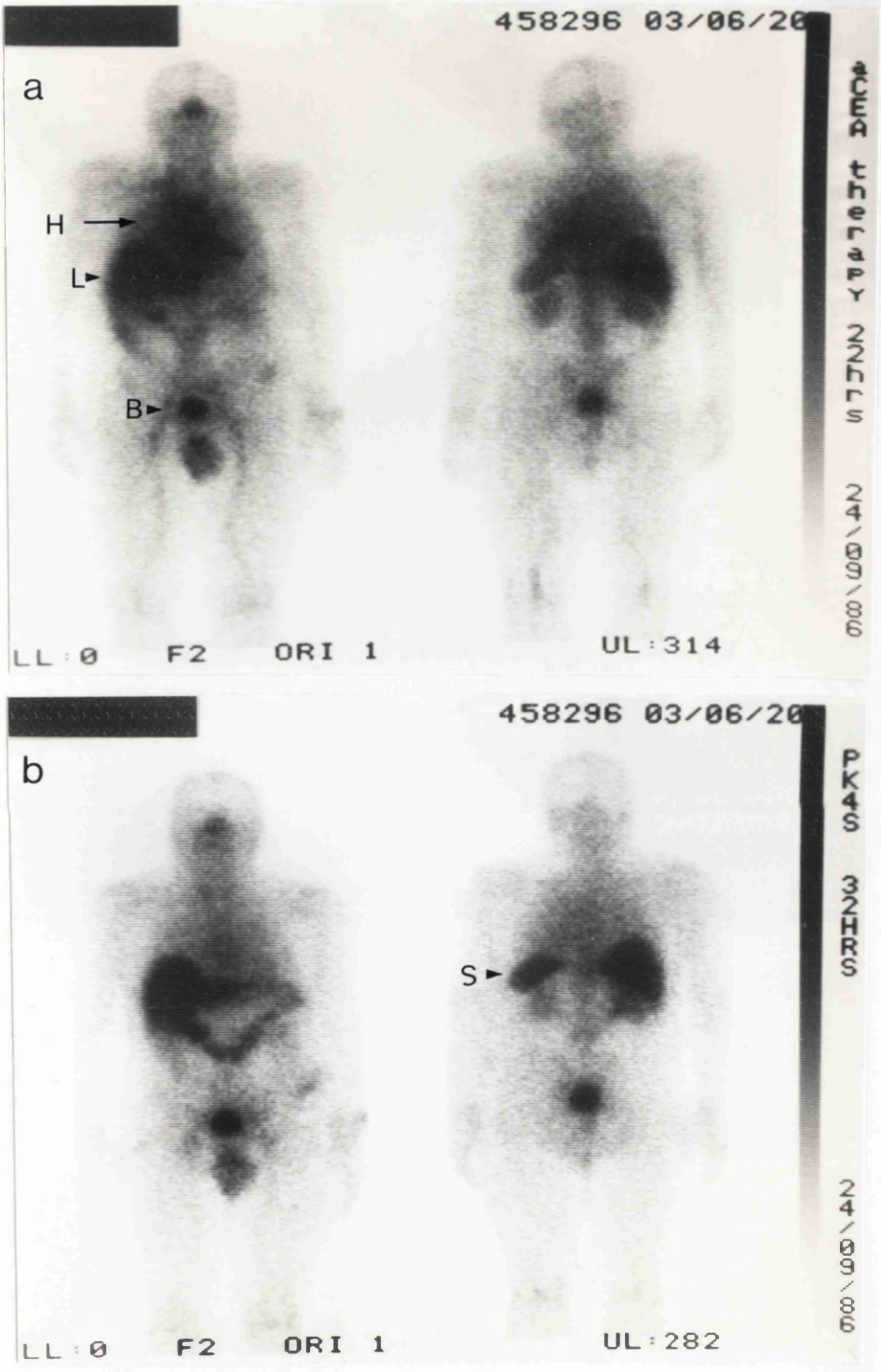


figure 5.3

Planar gamma camera image showing the distribution of radiolabelled antibody. a.) At 22 hours most of the radioactivity is in the heart (H), liver (L) and great vessels. Iodine is seen in the bladder (B). b.) 8 hours after 'second antibody' (at 32 hours) most of the radioactivity is now in the liver and spleen (S).

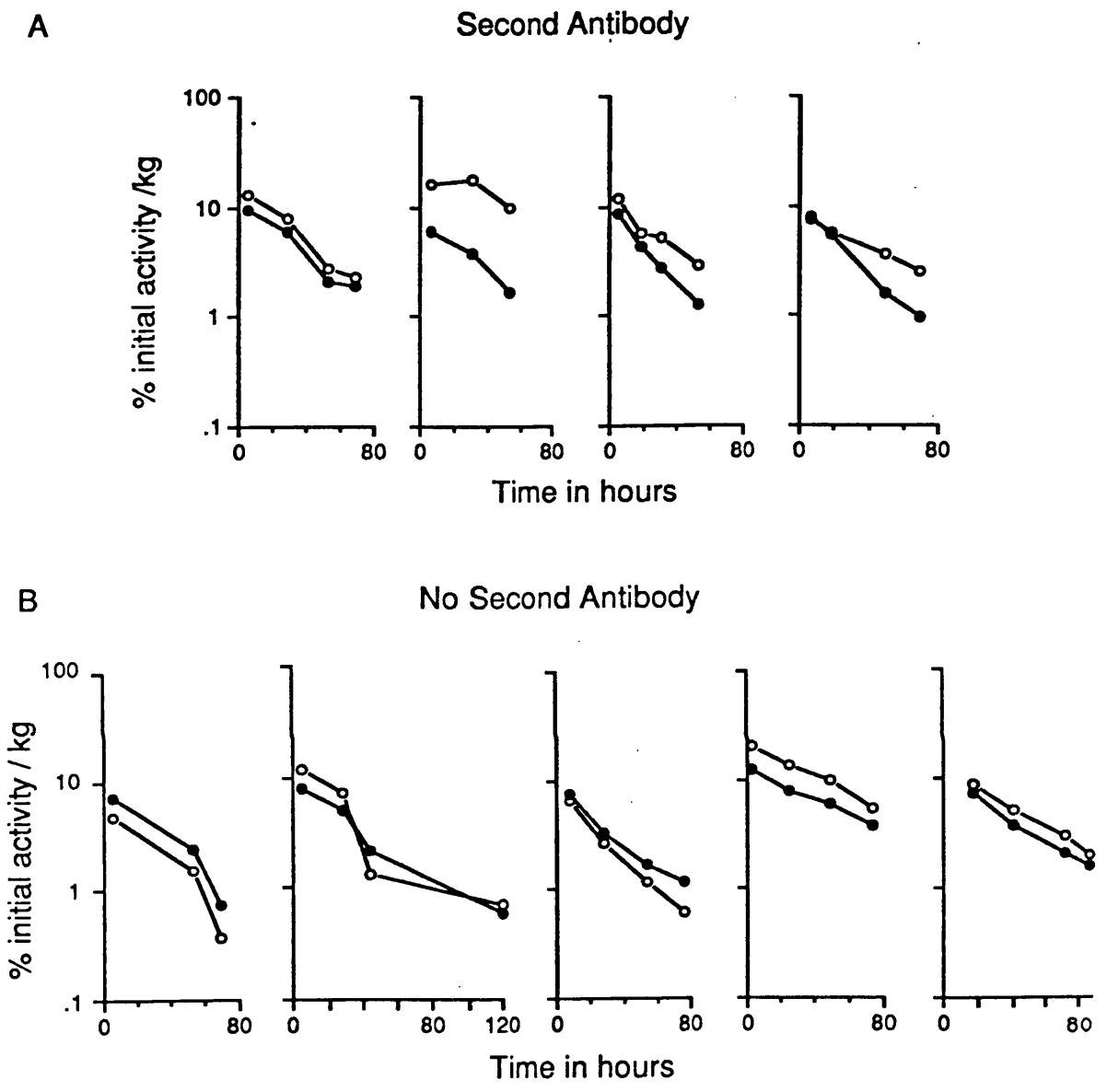


figure 5.4
Clearance of radiolabelled antibody through the liver and spleen. The data for radioactivity in sequential images were available in 4 patients who were given PK4S followed by 'second antibody' (A) and in 5 patients given PK4S alone (B). The percent of the injected activity per gram of tissue was calculated from serial planar gamma camera images. The change in the activity in the spleen o and liver • is plotted over the first 80 hours. In A. 'second antibody' was given at 24 hours.

5.4 Urinary excretion of ^{131}I

Urine was collected continuously for the first 72 hours. During this time 71 % (sd 0.12) of the injected activity of ^{131}I PK4S was recovered in the urine of patients who received 'second antibody' compared with 70 % (sd 0.13) in the patients who were not given 'second antibody'. The radioactivity in urine could not be precipitated by 10 % trichloroacetic acid, indicating that iodine in the urine was not bound to protein.

All patients had normal renal function but the volume of urine produced over the measured time-periods varied considerably. The rate of excretion of ^{131}I shown in figure 5.5 was adjusted to take account of this and the results are expressed as the percentage of the residual radioactivity excreted per litre of urine per hour. It was assumed the only route of excretion of ^{131}I was through the renal tract. The amount excreted in faeces, measured in two patients with colostomies (by Mr. Battacharya, in the Department of Medical Physics) accounted for less than 1 % of the injected activity of ^{131}I. The rate of excretion of ^{131}I was greatest during the first 24 hours after the administration of radiolabelled antibody. In the 12 hour period after 'second antibody' the rate of excretion of ^{131}I in the urine was not significantly greater than in the patients given PK4S alone.

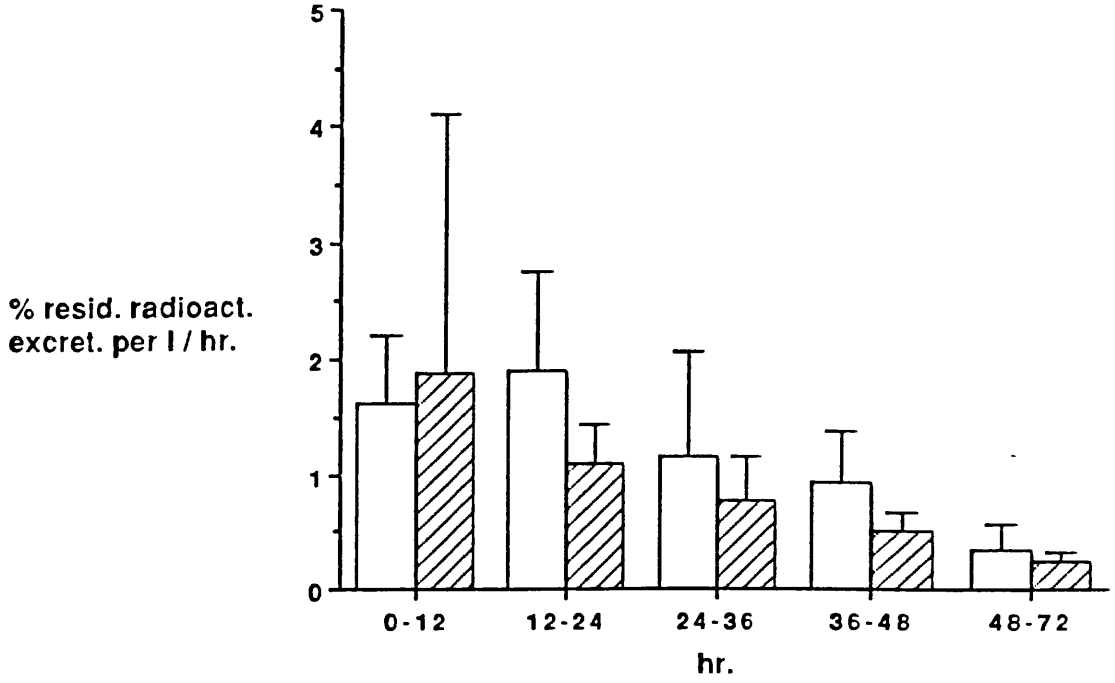


figure 5.5

Urinary excretion of ^{131}I during the first 72 hours following therapy with PK4S alone ☐ or with 'second antibody' ▨ .
The results are the mean of 5 patients per group. There was no difference in the plasma creatinine concentration in the two groups (91.4 vs 93.2 µmol/l).

5.5 Radiation dose to bone marrow

The β dose to the bone marrow was determined by integration of the area under the graph in figure 5.1. There was no significant difference in the amount of radioactivity in the blood of the two groups of patients at 24 hours, expressed as the percent of the injected activity per kg

blood (2.9 % (sd 1.3) versus 3.5 % (sd 1.2). A difference between the area under the two curves in figure 5.1 was seen after the injection of 'second antibody'. The area was smaller in the group given 'second antibody'. The mean radiation dose to the bone marrow following ^{131}I PK4S alone or with 'second antibody' is given in table 5.3.

table 5.3

RADIATION DOSE TO BONE MARROW (β)

PK4S ALONE		PK4S + 'SECOND ANTIBODY'	
pt no:	cGy/mCi	pt no:	cGy/mCi
6	1.43	8	0.45
7	0.95	10	0.55
9	1.01	11	0.75
14	1.00	12	0.84
15	0.46	13	0.39
mean	0.97 sd 0.34		0.60 sd 0.19

The β dose to bone marrow was lower in the patients who were given 'second antibody' but the difference, by the Wilcoxon rank sum test was not statistically significant ($p > 0.05$). The numbers in each group are small and the large standard deviation, mainly due to patient no:15, in the group that did not receive 'second antibody' had a marked affect on the whole group. However, the β dose to bone marrow after 24 hours was significantly less in the patients who were given 'second antibody' (0.131 versus 0.457 cGy/mCi $p < 0.05$ Wilcoxon sum of ranks). In the absence of 'second antibody' patients received about 50 % of the total β dose to bone marrow by 24 hours.

5.6 Discussion

The clearance of therapeutic doses of radiolabelled antibody from the circulation was accelerated by 'second antibody' in a manner similar to that seen in animals and patients following diagnostic injections of radiolabelled antibody for tumour localisation (Bradwell et al. 1983, Sharkey et al. 1984, Goodwin et al. 1984, Goldenberg et al 1987, Sharkey et al. 1988, Begent et al. 1989a).

In animals the dose-saving effect of 'second antibody' was 3.5, much higher than in the patients in this study (Pedley et al. 1989). This can be explained by the slower blood clearance of anti-CEA in mice; the reduction in area under the antibody clearance curve after 'second antibody' is greater than in patients. Following 'second antibody' there was a significant reduction in the bone marrow radiation dose. However, no overall dose-saving effect was seen. The delivery of nearly half the bone marrow dose before 24 hours suggests that the benefit of 'second antibody' might be improved if it were given earlier.

Gamma camera images showed that the reticuloendothelial system accumulated radioactivity following 'second antibody'. The cumulative radioactivity in the spleen, estimated from serial planar gamma camera images, was increased by 62 % in the patients who were given 'second antibody'(Begent, personal communication). This finding is in keeping with the results of Pedley et al. (1989) who

showed that after 'second antibody' there was a 43 % reduction in radiation dose to the spleen of mice bearing colon cancer xenografts.

Relative differences in radioactivity in the spleen were given as measurement of the absolute amount of radioactivity in this organ by planar gamma camera imaging has been shown to give falsely high values. The radioactivity determined by SPET is lower. However, these two imaging techniques give comparable estimates of activity in the liver (Green et al. 1989).

Any increase in spleen activity following 'second antibody' is likely to be due to a prolonged residence of ^{131}I labelled immunoglobulin complexes. The clearance of radioactivity through the liver did not appear to be altered by the administration of 'second antibody' but the clearance of radioactivity through the spleen was slower than the liver in 3 out of 4 patients given 'second antibody' (figure 5.4). Images of the spleen were seen more clearly in patients who were given 'second antibody' and they often persisted up to the third week after therapy. In animals there was an accumulation of radioactivity in the liver at some time between 3 and 24 hours after 'second antibody' (Pedley et al. 1989). The infrequent imaging of patients may have accounted for the failure to detect a transient increase in liver radioactivity.

The rapid removal of ^{131}I PK4S from the circulation after 'second antibody' did not result in any increase in the urinary excretion rate, indicating that there was no

acceleration in the clearance of ^{131}I from the body.

It is interesting to consider why this might be the case. It is unlikely to be due to retention of radioactivity in the spleen as the amount of radioactivity in this organ represents only a small fraction of the total administered activity. As there was no evidence to suggest that clearance through the liver was altered by 'second antibody' it is possible that excretion of ^{131}I by the kidney may be the rate-limiting step. The transient increase in free circulating ^{131}I after 'second antibody' (table 5.2) supports this argument. Further studies are needed to examine whether diuretics which inhibit chloride reabsorption in the kidney improve the urinary clearance of ^{131}I.

In summary there is evidence that a reduction in radiation to the bone marrow occurs following therapy with 'second antibody'. The failure to observe a significant effect in overall radiation may be due to the small number of patients and choice of the time to administer 'second antibody'. A further study was undertaken in which patients were given 100 or 150 mCi ^{131}I PK4S followed by 'second antibody'. The results of this study as well as the therapeutic and toxic effects of 50 mCi of PK4S alone or with 'second antibody' will be presented in the next chapter.

Chapter 6

RADIOIMMUNOTHERAPY OF COLORECTAL CANCER: THERAPEUTIC EFFECT AND TOXICITY OF SINGLE INJECTIONS OF ^{131}I ANTI-CEA AND 'SECOND ANTIBODY'.

6.1 Introduction

While the toxic effects of free ^{131}I on the bone marrow and lung are known (Benua et al. 1962) the distribution of ^{131}I, conjugated to an antibody, may be different and so alter the pattern of toxicity. For example, diagnostic imaging studies have shown that much of the radiolabelled antibody passes through the liver. Additional hazards could result from the use of 'second antibody' if deposition of radioactive immune complexes occurs in tissues such as the kidneys.

The distribution, toxicity and therapeutic activity of increasing amounts of radiolabelled anti-CEA and 'second antibody' will be described in this chapter. Some indication of the likely maximum tolerated amount of ^{131}I labelled antibody that can be given comes from studies where single injections of radiolabelled antibody were given. Significant bone marrow suppression was seen when greater than 200 mCi

of free ^{131}I or 100 mCi of ^{131}I labelled antibody were given (Benua et al. 1962, Ettinger et al. 1982). For this study up to 150 mCi of ^{131}I labelled antibody was used. The analysis will include the patients described in the previous chapter.

6.2 Treatment outline

Single injections of radiolabelled polyclonal anti-CEA antibody were given to 21 patients. 5 patients were given goat anti-CEA as part of a pilot study to examine the distribution and toxicity radiolabelled antibody and 'second antibody'. They received a mean of 51 mCi (range 38-60) of ^{131}I goat anti-CEA (PK2G), followed by donkey antisheep or horse antigoat antibody (group 1)(table 6.1a). One patient received a second injection of ^{131}I PK2G. The details of the second group of 10 patients (group 2) was described in chapter 5 (see table 5.1). They were given a mean of 50mCi (range 40-60) of ^{131}I labelled sheep anti-CEA (PK4S) with or without 'second antibody'. The other two groups, each containing three patients (table 6.1b) received PK4S conjugated with a mean of 89 mCi (range 77-100) (group 3) and 147 mCi (range 143-152) (group 4) followed by 'second antibody'.

The distribution of polyclonal goat anti-CEA in tumour and normal organs was assessed by serial gamma camera imaging. In the patients in group 1 visual inspection of the image was used to identify tumours in sites previously shown to be abnormal by X-ray CT. For groups 2 to 4 quantitative

scintigraphic data were acquired (by S. Riggs and A. Green) and these were used to support the identification of tumour sites. A region was considered positive if the radioactivity per gram of tissue in that area was either greater than that found in an equivalent region of normal tissue, or the activity per gram of the mean whole body activity.

table 6.1a

Radioimmunotherapy with polyclonal goat anti-CEA antibody and 'second antibody' (group 1)

Pt. no:	age	sex	tumour date diag.	previous treatment	tumour sites	CEA µg/l	anti-CEA mg	anti-CEA date	^{131}I mCi	sec.Aby. mg	tumour sites localised
1 (EW)	57	M	rectum 9/83	surgery radiotherapy	pelvis	23	2.5 2.5	2/85 5/85	55 40	12.5 -	pelvis -
2 (BE)	43	M	colon 9/84	surgery	lung	227	2.5	3/85	49	7.0	-
3 (JP)	38	F	colon 10/84	surgery CAP	pelvis	15	2.5	4/85	52	7.0	pelvis
4 (MW)	59	F	colon 1/83	surgery radiotherapy 5FU	pelvis liver	87	2.5	6/85	48	5.0	liver, pelvis
5 (MH)	71	F	colon 3/84	surgery	liver pelvis	26	2.5	6/85	40	5.0	liver, pelvis

table 6.1b

Radioimmunotherapy with polyclonal sheep anti-CEA and 'second antibody' (group 3 & 4)

Pt. no:	age	sex	tumour date diag.	previous treatment	tumour sites	CEA µg/l	anti-CEA mg	anti-CEA date	^{131}I mCi	sec.Aby. mg	tumour sites localised
16(HD)	73	F	rectum 10/82	surgery radiotherapy	pelvis	38	5.0	7/86	91	26.0	pelvis
17(AG)	66	M	rectum 4/85	surgery radiotherapy	pelvis	32	5.0	9/86	76	24.0	pelvis
18(SH)	65	M	colon 11/83	surgery;FUDR MitC.;5FU	liver	92	5.0	9/86	100	24.0	liver
19(VG)	55	F	colon 8/82	surgery;5FU, CCNU;5FU,MitC	liver lung	475	7.5	12/86	143	40.0	liver
20(CS)	63	M	colon 5/86	surgery 5FU	lung	344	7.5	12/86	145	40.0	-
21(FD)	43	M	rectum 7/83	surgery radiotherapy	pelvis lung	264	7.5	2/87	152	40.0	pelvis

pt no: = treatment number (initials); sec. Aby. = 'second antibody'.

CAP = cyclophosphamide, adriamycin, cisplatin.
MitC = mitomycin C, CCNU = lomustine; FUDR = 5 fluorodeoxyuridine. 5FU = 5 fluorouracil

6.3 Localisation of radiolabelled antibody

By the above criteria tumour sites were visible in 4 of the 5 patients who were treated with PK2G. Tumour was not identified in patient no: 2 who had numerous small lung metastases on an X-ray CT scan of the thorax. Tumour sites were identified in 13 of the 16 patients who were treated with PK4S (see table 6.1 a,b.). Tumour was not identified in one patient with lung metastases and in two with liver metastases. In the latter two it was difficult to distinguish the edge of the tumour from the apparently normal hepatic tissues. In patient no: 12 an increase in the uptake of radiolabel behind the bladder suggested the presence of a pelvic deposit. It was not confirmed as X-ray CT scanning of the pelvis had not been performed.

Two examples in which an increase in the uptake of radiolabelled antibody in known tumour sites is seen on gamma camera images are shown figure 6.1 and 6.2. In patient no: 17 the planar image shows radioactivity in the region of the bladder. Images of the bladder were obtained following the injection of ^{99m}Tc DTPA (diethylene triamine penta-acetic acid) and were subtracted from the ^{131}I image to demonstrate the abnormal area lying behind the bladder. Tumour lying behind the bladder is seen more clearly on the SPET image; the increase in radioactivity in this region corresponds to the mass seen behind the bladder on the X-ray CT image. The second example shows increase in uptake of radioactivity in parts of the liver known to contain tumour.

One deposit is seen on the X ray CT scan of the liver.

Tumour biopsies were performed on 3 out of 5 patients treated with PK2G. In one, no tumour was obtained from a transrectal biopsy of a pelvic deposit. In the other two histology of the specimens confirmed the presence of tumour. The radioactivity in a biopsy of a perineal deposit from patient no: 1 obtained 21 days after the injection of radiolabelled antibody was 3.8 times the amount of an equivalent weight of blood taken at the same time. In a liver biopsy from patient no: 5 the ratio of radioactivity in the tumour to blood was 1.5:1, 10 days after injection of radiolabelled PK2G. The radioactivity in the tumour at this time was 0.064 μCi/g.

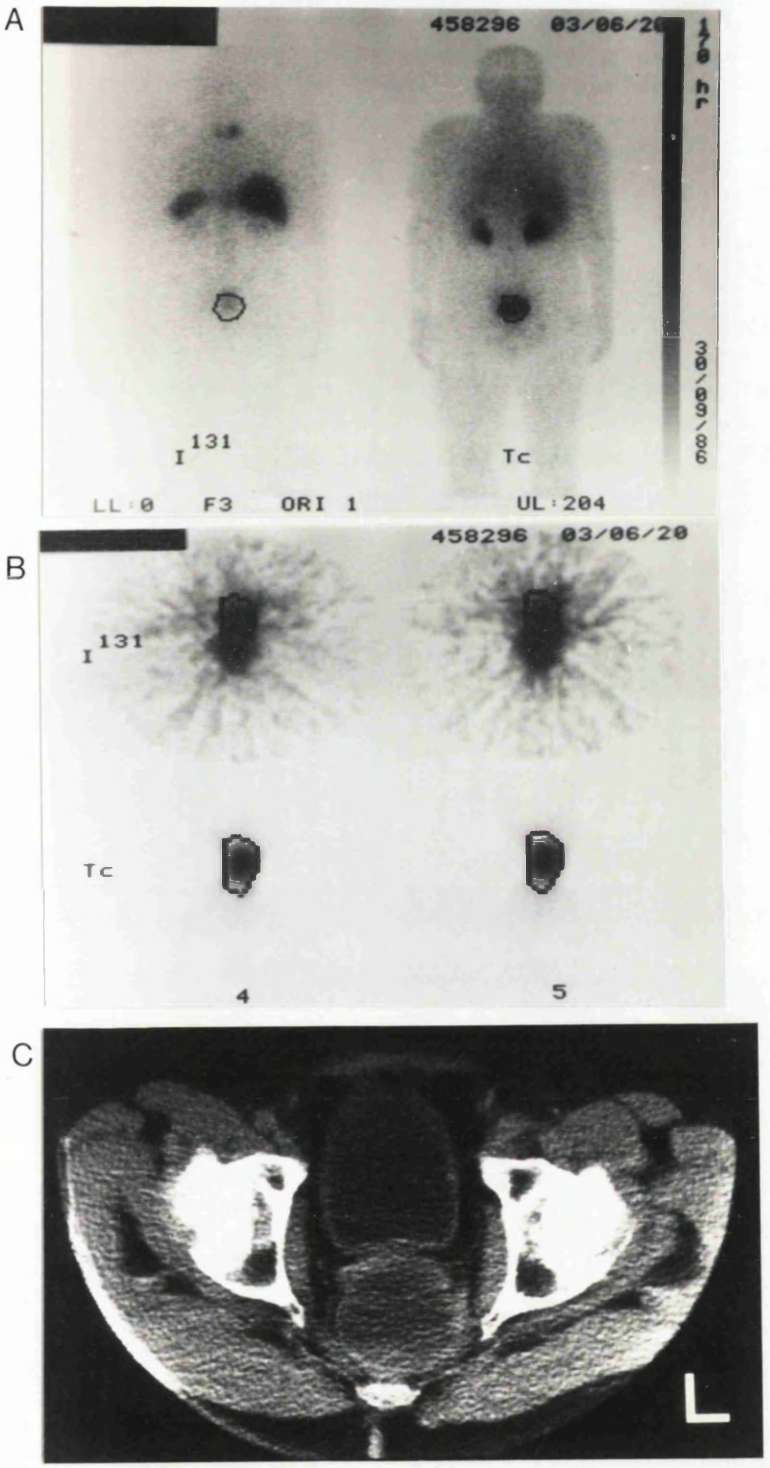
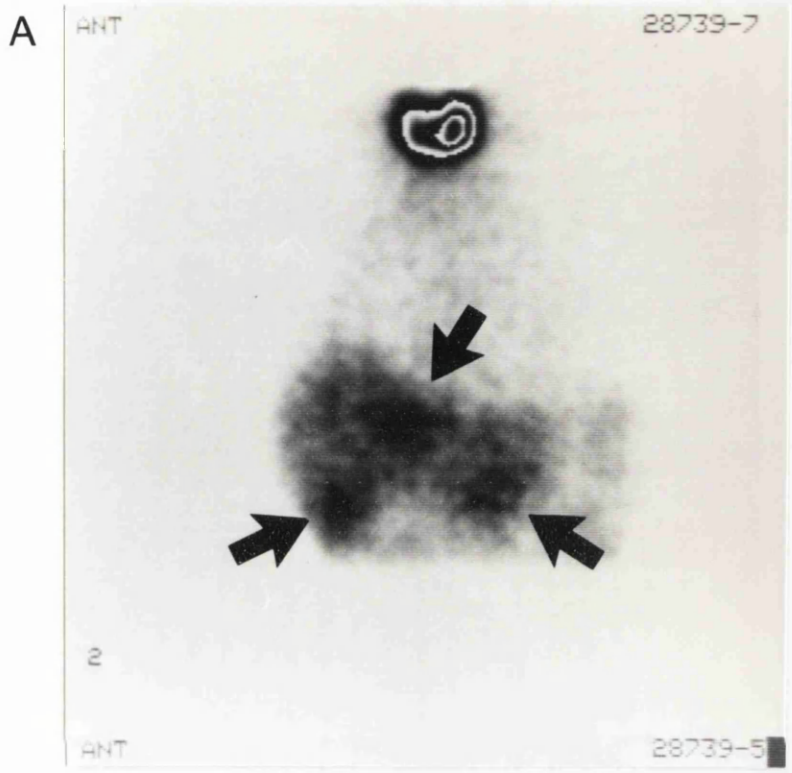


figure 6.1
A planar gamma camera image shows ^{131}I in the region of the bladder. Two methods were used to show tumour behind the bladder. An intravenous injection of ^{99m}Tc-DTPA was given to outline the bladder and the radioactive counts on the planar scan were subtracted from those in the ^{131}I image to show a mass separate from the bladder (A). SPET imaging shows there is an accumulation of radioactivity behind the bladder (B) which corresponds to the pelvic tumour seen on the CT scan of the pelvis (C).



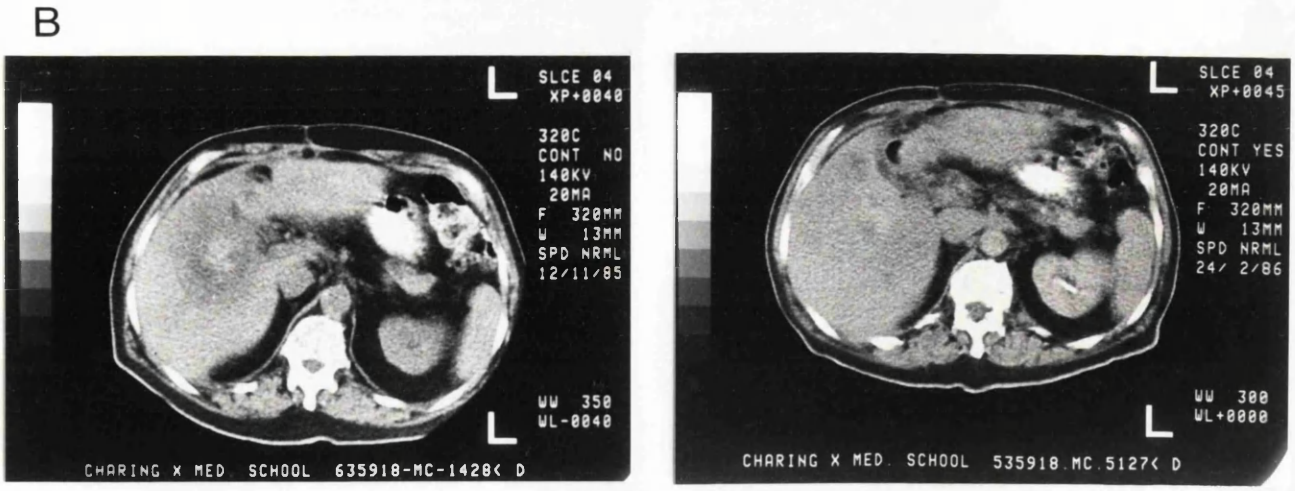


figure 6.2
A planar image of the liver (A) shows accumulation of radioactivity (arrows) in an areas known to contain tumour on CT (B). The tumour is less easily visible on a CT scan taken one month after radioimmunotherapy.

Quantitative external scintigraphic data rather than tumour biopsies were used to calculate the uptake of radioactivity in tumour and normal organs of patients following therapy with PK4S. These have been published separately (Begent et al. 1989b).

6.4 Tumour response to radioimmunotherapy

The tumour response was assessed by measuring changes in tumour volume on X-ray CT images taken before and one month after therapy and by serial measurements of serum CEA and CA19-9 concentration. By these criteria an objective tumour response was seen in 2 of the 21 patients.

The X-ray CT scan of the liver in figure 6.2 shows that there is a reduction in the size of deposit of tumour in the right lobe by 34 days after the injection of radiolabelled antibody. This change coincided with the disappearance of constant pain in the region of the liver. There was, however, no change in the abnormal liver function tests during this period, or a fall in the concentration of serum CEA. One month later an X-ray CT scan showed new lesions in both lobes of the liver. The health of the patient declined and he died 6 months after radioimmunotherapy.

The other patient, no: 1, from whom the perineal tumour biopsy was obtained, suffered constant pain in the perineum and difficulty with urination. These symptoms improved during the second to fourth week following radioimmunotherapy at a time when the concentration of CEA

and CA19-9 in the serum were both falling (figure 6.3). The addition of PK4S to samples of sera did not appear to affect measurement of the concentration of CA19-9. No change in the size of the tumour was seen on the X-ray CT image and his symptoms returned after one month. A second injection of radiolabelled antibody was given 3 months later. He developed an acute hypersensitivity reaction during the infusion and no uptake of radioactivity was seen in the region of the tumour.

The concentration of CEA and CA19-9 in serum was measured before and after radioimmunotherapy in most of the 21 patients. No significant change in the concentration of either tumour marker was found in any other patients (table 6.2)

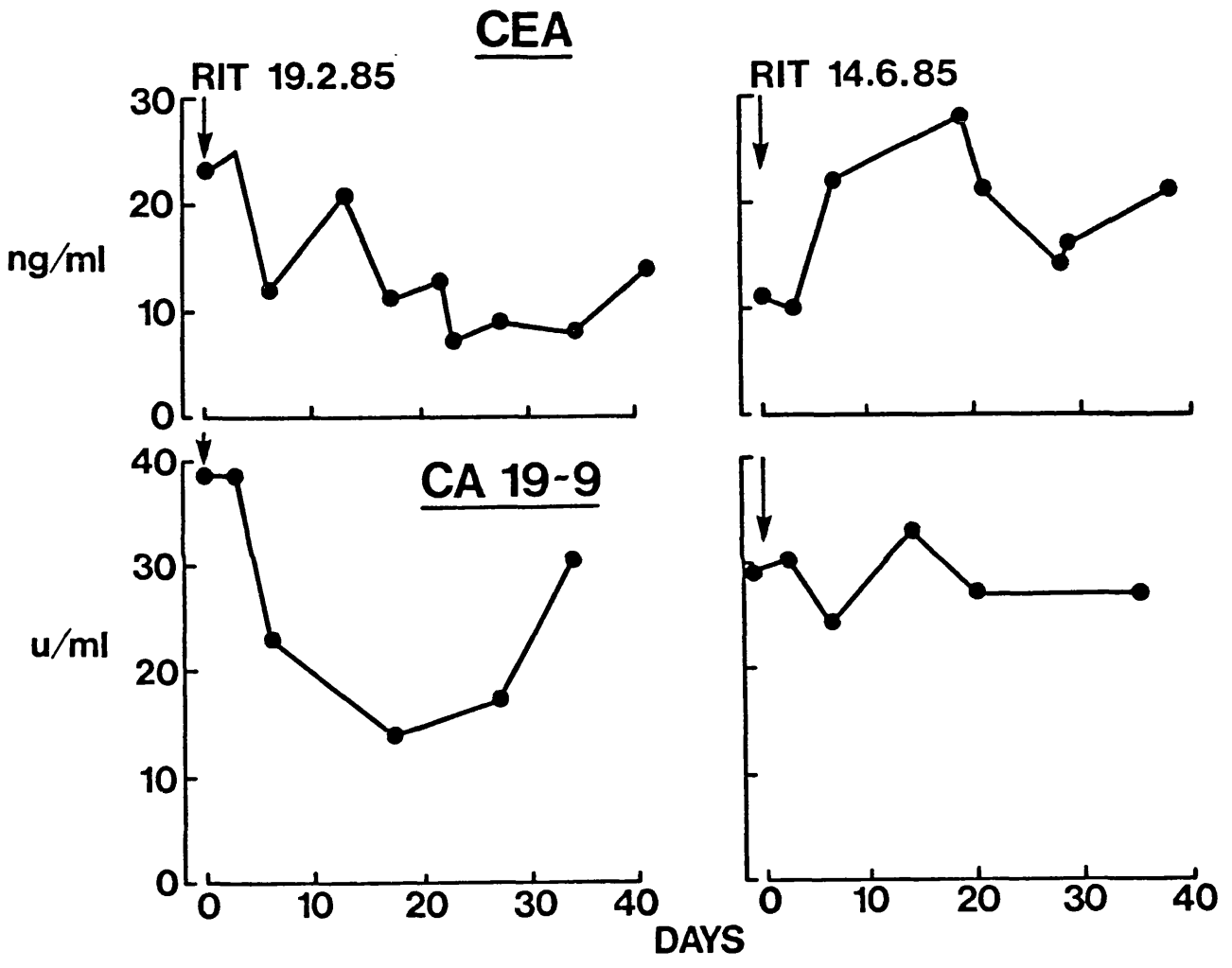


figure 6.3
Serum concentration of CEA and CA 19-9 in patient no:1 after the first injection of radiolabelled antibody. The concentration of the tumour markers fell during the period when his symptoms improved. No change in either tumour marker was seen after a second injection of ^{131}I PK2G 3 months later.

Table 6.2

SERUM CEA AND CA 19-9 CONCENTRATION IN PATIENTS RECEIVING RADIOIMMUNOTHERAPY

pt no:	CEA ng/ml		CA19-9 U/ml	
	pre	post	pre	post
1	23	9	40	17
2	227	347	23	23
3	15	31	<8	nd
4	87	121	475	765
5	22	35	1700	2350
6	4574	10540	3200	3900
7	12390	7937	13220	39390
8	27	30	4400	4100
9	135	163	<5	nd
10	27	26	58	82
11	240	262	535	565
12	60	120	26	nd
13	171	613	155	nd
14	51	84	<5	nd
15	9	12	145	nd
16	41	48	<5	nd
17	32	34	<5	nd
18	182	nd	<5	nd
19	475	347	295	485
20	344	876	355	810
21	222	688	<5	nd

post treatment values were taken at a median of 31 days after radioimmunotherapy; range 19-42 days for CEA and 23-35 days for CA19-9. nd = not done. Normal CEA < 10 ng/ml; for CA19-9 less than 1% normal individuals have more than 37 U/ml (Abbott Laboratories). For CA19-9 the lowest value reported was determined by the lowest concentration of CA19-9 supplied with the assay kit. The CEA assay is able to detect a concentration of CEA > 2 ng/ml.

6.5 Radiation dose to bone marrow following ^{131}I anti-CEA

The β dose to the bone marrow for the eleven patients, including the five from table 5.4, who were given PK4S and 'second antibody' is given in table 6.3. Between 40 to 152 mCi of ^{131}I the mean β dose to bone marrow was 0.65 cGy per mCi (sd 0.146).

table 6.3

ESTIMATED RADIATION DOSE TO BONE MARROW IN PATIENTS TREATED WITH RADIOLABELLED PK4S AND 'SECOND ANTIBODY'

Pt no:	mCi	cGy/mCi	β dose cGy
8	52	0.45	23.4
10	55	0.55	30.3
11	60	0.75	45.0
12	42	0.84	35.3
13	40	0.39	15.6
16	91	0.81	73.7
17	77	0.66	50.8
18	100	0.72	72.0
19	143	0.67	95.8
20	145	0.62	89.9
21	152	0.79	120.0

6.6 Toxicity of ^{131}I anti-CEA

The infusion of radiolabelled antibodies and 'second antibody' were generally well tolerated and did not result in any serious adverse effects. Rigors and fever were the only subjective side-effects experienced by 15 out of the 21 patients following the infusion of the primary radiolabelled

antibody. The symptoms appeared about 40 minutes after the end of the infusion and lasted 1 to 3 hours. This pyrogenic reaction ceased when a new tubing leading from the G-25 Sephadex resolving column was used for each iodination of antibody.

All but two patients were followed up for six or more weeks after therapy. One patient died after four weeks from progressive tumour growth in the liver and one patient was lost to follow up after four weeks. No changes in renal function, measured by serial samples of blood urea and creatinine concentration were detected during the six weeks after the infusion of antibody. Liver function tests were abnormal before radioimmunotherapy in 8 out of 21 patients and were attributed to the presence of liver metastases. The liver function tests (aspartate transaminase, alkaline phosphatase and bilirubin) continued to deteriorate in these patients, all of whom had advanced disease. Liver function tests became abnormal in a further 2 patients in the six weeks following antibody therapy. In both patients liver metastases were known to be present at the start of treatment.

Haematological toxicity was assessed by measurement of serial haemoglobin concentration, and white cell and platelet counts at not less than weekly intervals. The toxicity, using WHO grading (WHO 1979) (see appendix B) is presented in table 6.4.

table 6.4

HAEMATOLOGICAL TOXICITY OF RADIOLABELLED ANTIBODY THERAPY WITH AND WITHOUT 'SECOND ANTIBODY'.

^{131}I anti CEA (mg)	mCi	'sec. aby'	WHO Toxicity pts	Grade (pts) Hb	WBC	Plat
2.5	38- 60	No	5	1(1)	1(1)	0
2.5	40- 60	Yes	10	0	1(1)	0
5.0	77-100	Yes	3	0	0	0
7.5	143-152	Yes	3	0	0	3(1)

Grade 3 thrombocytopenia was the only major toxicity occurring in one patient (no:19), who had previously received a prolonged course of chemotherapy. The platelets fell to 44 x 10^9/l 31 days after antibody therapy. Four weeks later they were still only 55 x 10^9/l. No further blood samples were obtained. No difference in the platelet count was observed between the patients who were given 38 to 60 mCi of radiolabelled antibody with or without 'second antibody'.

A second injection of radiolabelled antibody given to patient no:1 resulted in an acute hypersensitivity reaction. He had developed human anti-antibodies after the first injection of radiolabelled antibody (see chapter 7). Retreatment in the presence of anti-antibodies identified a potential source of toxicity of radioimmunotherapy which will be considered more fully in SECTION IV of this work.

No long-term effects on thyroid function were observed. Two patients are alive more than two years after treatment.

6.7 Discussion

In general single injections of antitumour antibody were well tolerated. The infrequency of myelosuppression meant that it was not possible to establish whether bone marrow toxicity was reduced by 'second antibody'. Increasing the administered activity to 150 mCi ^{131}I did not lead to any alteration in the clearance of radioactivity from blood or result in any life-threatening bone marrow suppression.

Bone marrow suppression was reported in 21 % of patients in whom the radiation dose to blood, following therapy with free ^{131}I for thyroid cancer, was more than 200 cGy (Benua et al. 1962). They estimated the total blood dose to be 1.15 cGy/mCi and that on average 56 % of the dose was due to the effects of gamma radiation. Thus, the β dose to blood following free iodine therapy was about 0.5 cGy/mCi which approximates to the value of the bone marrow dose calculated in the patients who received 'second antibody'. Thus one would anticipate that myelosuppression would be frequent following 200 mCi or more of ^{131}I labelled antibody and 'second antibody'.

The presence of thrombocytopenia in only one of three patients in group 4 who received mean of 147 mCi contrasts with the results of dose escalation studies of radioimmunotherapy performed by other groups. Ettinger et al. (1982) reported thrombocytopenia ($< 100 \times 10^9/l$) in all of the eight patients who received more than 100 mCi of ^{131}I

antiferritin for primary liver cancer and in three out of five patients who received less than 100 mCi of ^{131}I labelled antibody. Treatment with 50 mCi of antiferritin for Hodgkin disease resulted in a fall in the platelet count to 50 x 10^9/l or less in three out of thirty seven patients (Lenhard et al. 1985). Rosen et al. (1987) described thrombocytopenia (< 120 x 10^9/l) in all of the three patients who received more than 145 mCi of ^{131}I T101 antibody.

Factors other than the administered activity are likely to contribute to the toxic effects of radiolabelled antibody. For example, bone marrow radiation will be increased by an antibody with a long circulatory half-life as the cumulative blood radioactivity will be greater. The half-life of polyclonal antiferritin was estimated to be 3 days (Order et al. 1980), approximately three times that of PK4S. The total body irradiation following polyclonal antiferritin was estimated to be approximately four times that of an affinity-purified antiferritin which has a much shorter half-life in the body (Leichner et al. 1983). This may explain the high incidence of myelosuppression seen with less than 100 mCi of ^{131}I antiferritin and it supports the argument for using 'second antibody' to accelerate the clearance of circulating radiolabelled antibody from the blood.

From biodistribution data obtained following localisation studies it has been estimated that the radiation dose to bone marrow following ^{131}I Fab' fragments,

that are cleared more rapidly from the blood than intact antibody is 0.3 cGy per mCi. (Larson et al. 1983a). The methods used to derive this figure were not stated but the estimate is less than that calculated following free ^{131}I. As there is no agreed methodology of measuring the total radiation dose to bone marrow a comparison of results between groups needs to be made with caution. For instance, Leichner et al. (1983) and Rosen et al. (1987) have related myelosuppression to total body irradiation rather than blood radioactivity. Nevertheless, Carrasquillo et al. (1984) have given a single injection of up to 342 mCi Fab' antibody and a cumulative activity of up to 861 mCi to patients with melanoma. In general, haematological toxicity was associated with the administration of greater than 500 mCi of ^{131}I Fab'.

Reduction of the 'bone marrow reserve' by previous treatment with cytotoxic drugs or radiation is another factor that can influence myelosuppression. This may have contributed to the toxicity of 50 mCi ^{131}I antiferritin in patients with persistent Hodgkin's disease following intensive treatment (Lenhard et al. 1985). Similarly, treatment of hepatocellular cancer with external beam radiation, doxorubicin and 5 fluorouracil before radiolabelled antiferritin may have increased the degree of myelosuppression (Order et al. 1985). These factors need to be considered before one can predict the adverse effect of different radiolabelled antibodies on the bone marrow.

Objective evidence of a tumour response was seen in two of the twenty-one patients. One of these received PK4S without 'second antibody'. A potential disadvantage of 'second antibody' therapy is that it reduces radioactivity in the tumour, either by removing antibody or clearing it from the circulation before maximum tumour uptake has occurred. However, the radiation dose to the tumour of the five patients who received 'second antibody' was not significantly less than in those who were given PK4S alone (Begent et al. 1989b). This observation contrasts the finding in mice with human colon cancer xenografts where a 58 % reduction in radioactivity in the tumour was seen after 'second antibody' (Pedley et al. 1989). This difference may be due to the longer time for radioactivity to reach a maximum in the xenograft tumours.

Most patients were treated at a late stage of their disease when the tumour bulk was large. The fall in the concentration of CA19-9 and CEA in one patient at a time when his symptoms were improving suggests that serum tumour markers may be a useful measure of therapeutic effect when clinical or radiological assessment fail to demonstrate a change in size of the tumour. However, the amount of radiolabelled antibody reaching the tumour was insufficient to result in a tumour marker or radiological response in the majority of patients.

A better therapeutic effect is likely to be achieved by increasing the amount of radiolabelled antibody delivered to the tumour. If the dose-saving effect of 'second antibody'

could be improved by earlier administration, it might be possible to give larger quantities of radioactivity without increasing the degree of myelosuppression. From serial measurement of the radioactivity in the tumour it should be possible to determine the optimal time to administer 'second antibody'.

Repeated administration of radiolabelled antibody, allowing recovery of the bone marrow between courses is an alternative approach that needs to be considered. However, the development of anti-antibodies is likely to be a obstacle to repeated therapy and a potential source of toxicity.

SECTION IV THE IMMUNE RESPONSE TO ANTITUMOUR ANTIBODIES

Chapter 7

THE DETECTION OF HUMAN ANTI-ANTIBODIES IN NORMAL SUBJECTS AND PATIENTS TREATED WITH ANTITUMOUR ANTIBODIES.

7.1 Introduction

Adverse effects of repeated intravenous injection of animal serum and immunoglobulin have been appreciated for nearly ninety years. The allergic reaction described in the patient who received two injections of PK2G illustrates the need to study the frequency of the human immune response to antitumour antibodies. A better understanding of the factors contributing to the development of an anti-antibody response may help to avoid the hazards associated with repeated therapy.

Human antibodies that react with antitumour antibodies have been recognised in some patients who have no history of exposure to animal immunoglobulins (Primus et al. 1980, Schroff et al. 1985, Shawler et al. 1985, Davies et al. 1986, Courtenay-Luck et al. 1986). Little is known about the nature of these anti-antibodies; whether they are present in the serum of normal individuals or if they affect the distribution of the antitumour antibody in the host and

its localisation in the tumour.

The studies in this chapter investigated whether human antibodies to sheep and mouse anti-CEA were present in the serum of laboratory volunteers and patients receiving radioimmunotherapy. To examine the clinical importance of these antibodies the rate of removal of radiolabelled antibody from the circulation was compared in patients with pre-existing antibody, anti-antibody formed as a result of prior exposure to animal immunoglobulins or no detectable anti-antibody. The latter study contained patients who were treated with polyclonal anti-CEA (chapters 5 and 6) and seven patients who were given repeated injections of a mouse monoclonal anti-CEA (A5B7). The clinical details of these patients will be presented in chapter 9.

It is important to establish if the human anti-antibody response is species-specific as it would then be possible to repeat therapy, using antibodies from different species when immune sensitisation occurs. Cross-species reactivity of anti-antibodies was studied in the serum of patients who were treated with polyclonal sheep or mouse monoclonal anti-CEA.

7.2 Binding of IgG and IgM from human subjects to sheep, goat or mouse anti-CEA (PK4S, PK2G or A5B7).

Serum samples from 24 normal human subjects, 12 males and 12 females working in the Department of Medical Oncology at Charing Cross hospital were used to examine whether human IgG and IgM bound to sheep antibody. Samples were tested using the enzyme immunoassay described in chapter 4.2. Wells in microtitre plates were coated with sheep anti-CEA (PK4S) and binding of human IgG or IgM to the plate was detected by incubating with goat antihuman IgG-alkaline phosphatase or rabbit antihuman IgM-horseradish peroxidase. The background absorbance due to binding of the enzyme-labelled antibody to PK4S was on average less than 0.01. Human IgG from all subjects bound to PK4S. The mean absorbance shown in figure 7.1 was 0.444 (median 0.43). Many subjects also had IgM antibodies which reacted with PK4S. The mean absorbance was 0.298 (median 0.192). There was no correlation between the magnitude of the IgG or IgM antisheep response (r= -0.049). A similar distribution of binding to mouse IgG was seen (figure 7.2).

The binding of normal human IgG to goat or sheep IgG appeared specific as it could be inhibited by high concentrations of goat IgG. An example is shown in table 7.1. A sample of pooled normal human serum from laboratory workers was incubated with an excess of goat IgG (Sigma) overnight at room temperature and the binding to PK2G was then measured by the method described above. Included in

table 7.1 is the inhibition of binding by goat IgG from a patient with high pre-existing antigoat antibody and a patient who developed human antigoat antibodies after therapy. Binding is virtually abolished in the presence of goat IgG. Incubation with a high concentration of bovine albumin (nonspecific protein) led only to a small reduction in binding to PK2G.

Table 7.1

INHIBITION OF BINDING OF HUMAN SERUM TO GOAT ANTI-CEA (PK2G) BY EXCESS GOAT IgG

	Buffer	Goat IgG	BSA
NHS	0.259	0.059	0.203
Pt. No.1	1.392	0.069	0.841
Pt. No.3	1.387	0.099	0.198
IP Stand.	1.510	0.025	1.188

mean absorbance of phosphatase substrate at 405 nm. goat IgG 2.5 mg/ml, bovine serum albumin (BSA) 3 mg/ml in diluting buffer. NHS = pooled normal human serum (dilution X 10); patient no.1 post-therapy (dilution X 80); patient no.3 pretherapy (dilution X 160); IP stand = immunopurified human antisheep (see chapter 4.2) (dilution X 80).

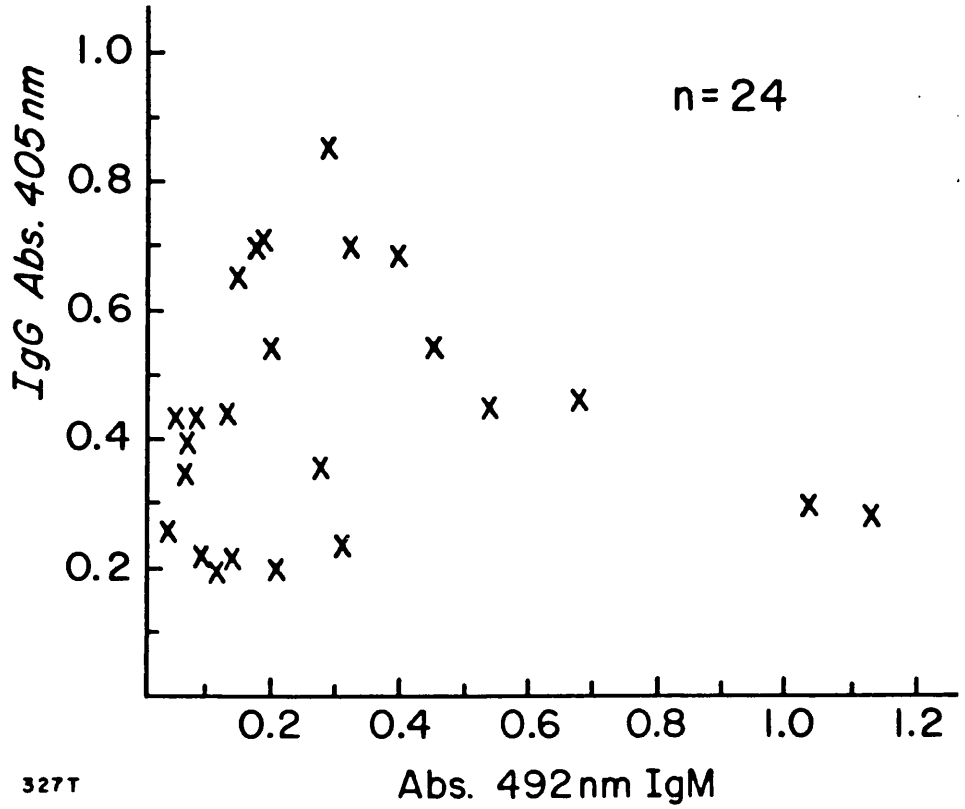


figure 7.1
Binding of normal human IgG and IgM to sheep anti-CEA (PK4S). Absorbance is the mean of duplicates of serum diluted tenfold in diluting buffer.

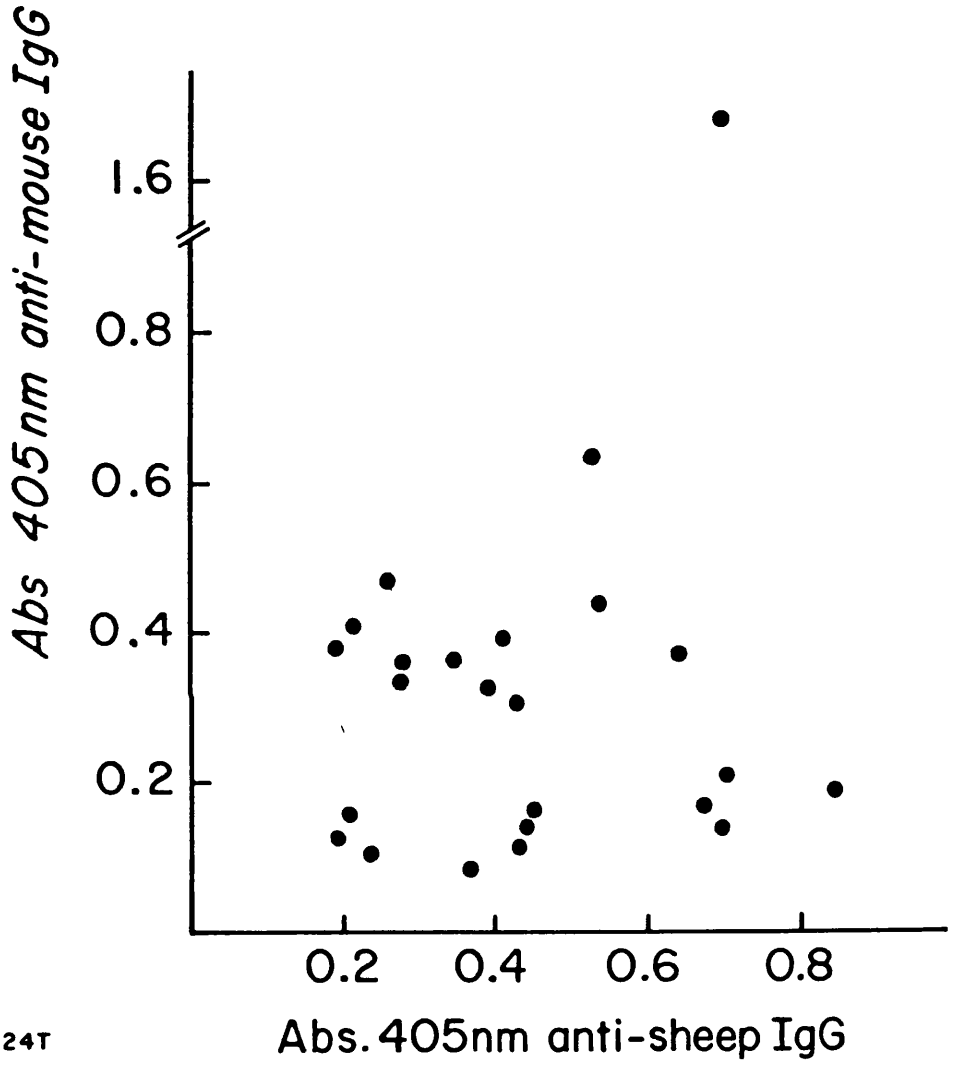


figure 7.2
Binding of human IgG from normal subjects to mouse and sheep anti-CEA. No correlation in binding was seen (r= 0.272). Absorbance results are the mean of duplicates of a tenfold dilution of serum.

An inhibition assay to mouse anti-CEA was used to compare the relative binding of a sample of human IgG antimouse antibody from a normal subject to the constant or variable region of A5B7 (anti-CEA). Serum from the subject with the highest level of antimouse antibody was pre-incubated with A5B7 or a mouse monoclonal antibody of the same isotype, raised against hCG (SB10) and the degree of inhibition of binding of human antimouse antibody to the idiotype (A5B7) was measured as described in 4.2. In the example shown in figure 7.3 the unrelated antibody was able to inhibit binding to A5B7 in a similar manner as an excess of A5B7, suggesting that the antimouse antibody was directed against shared mouse constant regions. A 1 in 10 dilution of this sample of serum was tested for binding to a plate coated with A5B7 and an equimolar concentration of the Fab'_2 fragment of this antibody prepared by pepsin digestion (P. Harwood). The relative optical absorbance, on the same plate at the same time was 1.683 for binding to mouse IgG and 0.218 for binding to the Fab'_2, supporting the notion that much of the binding was to common determinants of mouse IgG rather than to variable regions.

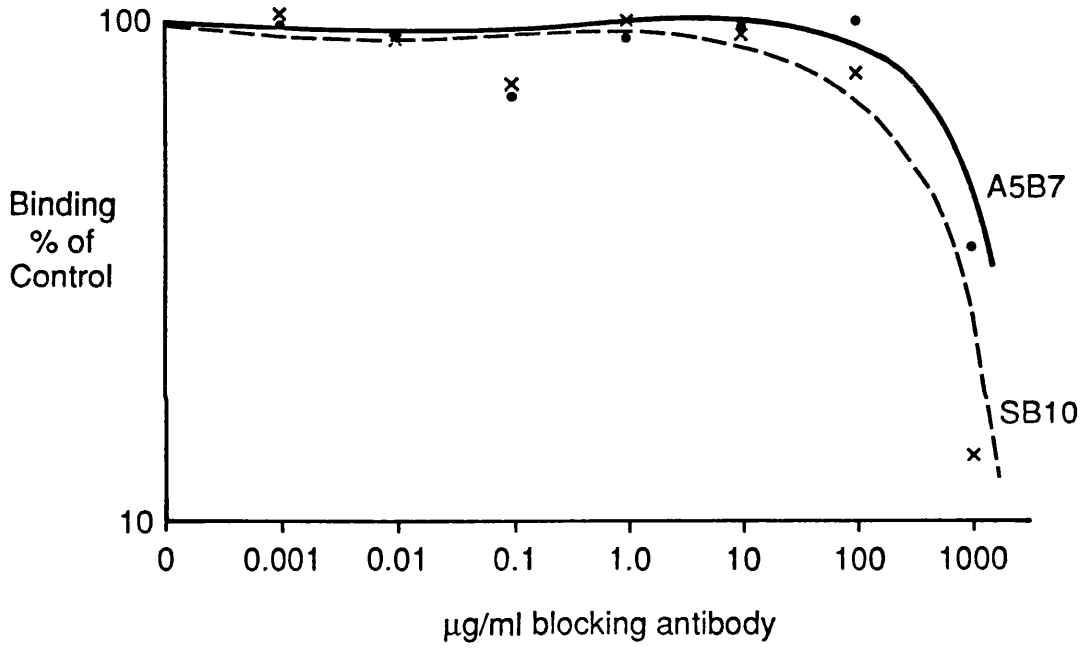


figure 7.3
Inhibition of binding of human IgG antimouse from a normal subject in the presence of mouse anti-CEA (A5B7) and anti-hCG (SB10), an antibody of the same isotype.

7.3 Human anti-goat/sheep antibody response following intravenous ^{131}I goat anti-CEA (PK2G).

Serial samples of sera from four patients who received a single injection of 2.5 mg of PK2G (group 1; chapter 6) were assayed for human IgG and IgM antigoat(figure 7.4) as described in section 4.2. In three patients < 5 μg/ml human IgG anti-sheep/goat was measurable before therapy (figure 7.4 a,c.). An elevation in the level of human IgG anti-antibody was seen in four out of five patients by the 14 th to 20 th day following therapy. An IgM response was detectable in five patients by the 14 th day following therapy. In one patient the concentration of IgG anti-antibody remained above the pretreatment value for more than 200 days after therapy.

The patient in figure 7.4b had elevated human IgG and IgM anti-antibodies before therapy. There was no history of exposure to animal immunoglobulins and an intradermal skin test with 10 μg of PK2G was negative. In this patient there was an initial fall in the serum concentration of IgM and IgG anti-antibody, consistent with the removal of immune complexes formed after the injection of PK2G. This was followed by an elevation in the level of IgM and IgG antisheep, interestingly, over approximately the same time course as in the other 3 patients who had little or no detectable pre-existing anti-antibodies. No hypersensitivity reaction was seen.

The patient in figure 7.4c (no:1) was given a second injection of 2.5 mg PK2G 3 months after the first therapy. He had a negative reaction to an intradermal skin test with 10 µg PK2G and fourteen days before the second injection of anti-CEA he was given an intravenous test dose of 200 µg of PK2G without any adverse effect. Within five minutes of starting the infusion of the second therapeutic dose of radiolabelled PK2G he had an hypersensitivity reaction. He developed chest and loin pain; there was marked peripheral vasoconstriction and a tachycardia. There was no audible wheeze in the chest or fall in blood pressure. The infusion of antibody was stopped; he was given chlorpheniramine and hydrocortisone and the symptoms improved rapidly. A large quantity of human IgG antisheep antibody appeared in the serum following a short latency period of 7 days, compatible with a secondary-type immune response, and reached a peak by 21 days. There was also a less sustained rise in IgM anti-antibody.

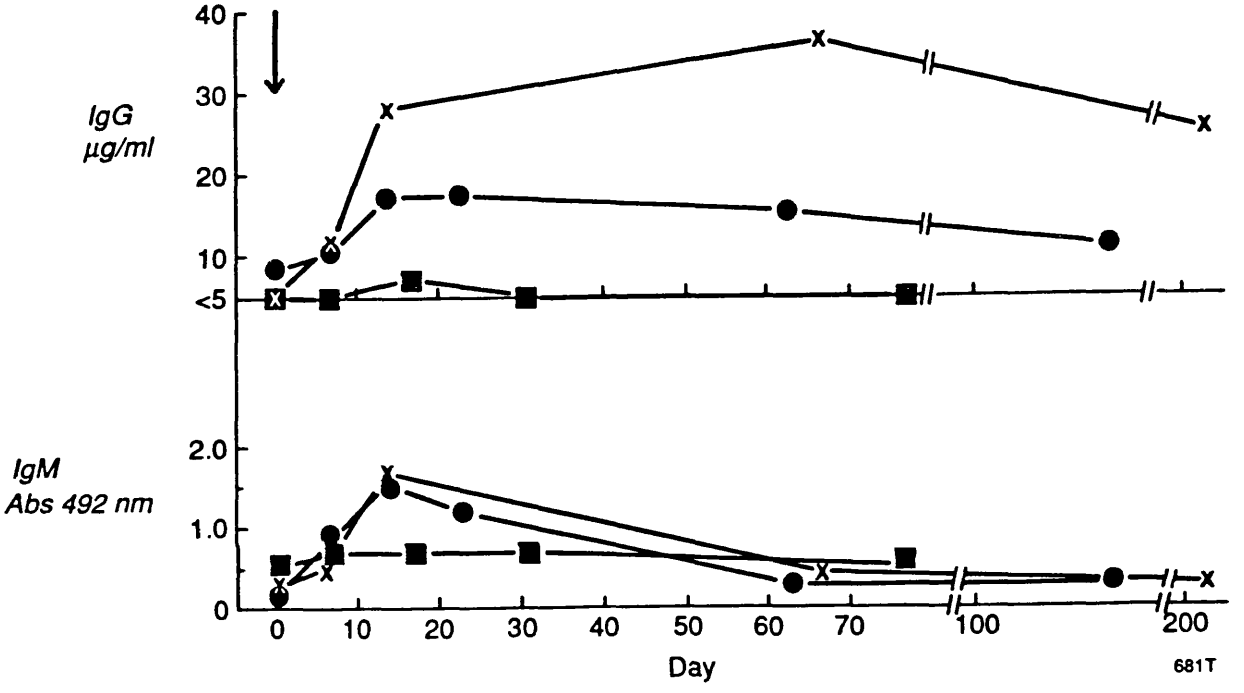


figure 7.4 a
Human IgG and IgM response to goat anti-CEA (PK2G). following a single injection of 2.5 mg of PK2G in three patients.

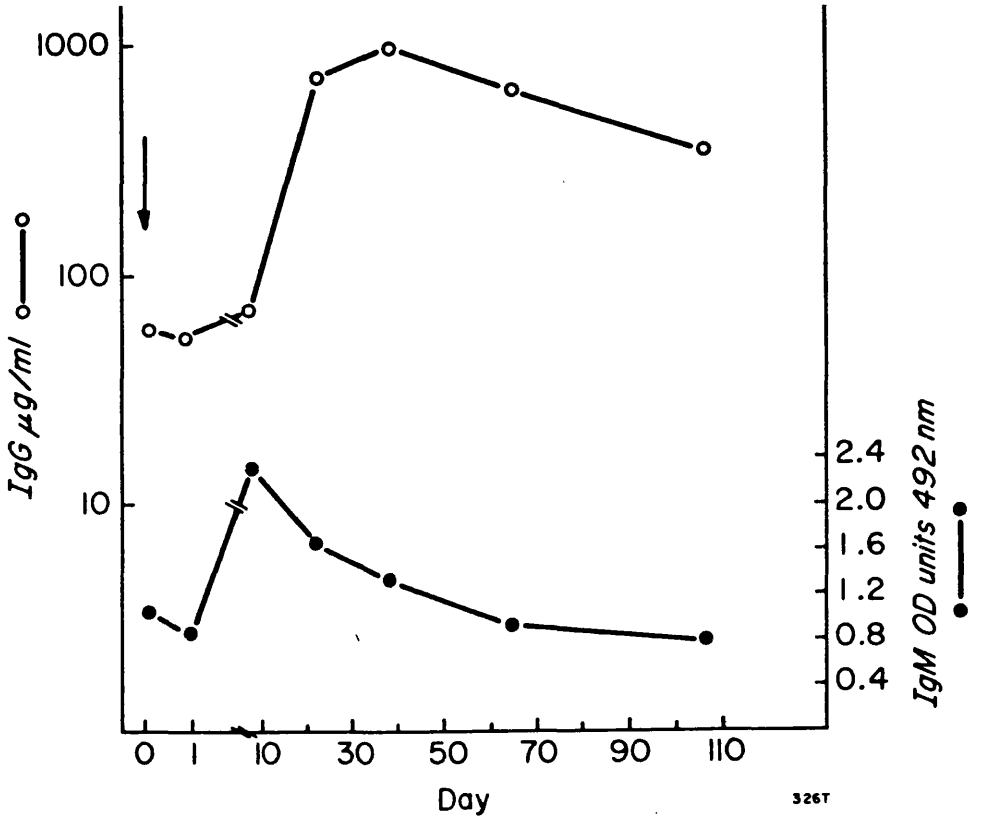


figure 7.4 b.
The human IgG and IgM anti-goat antibody response in a patient with elevated anti-antibodies before therapy.

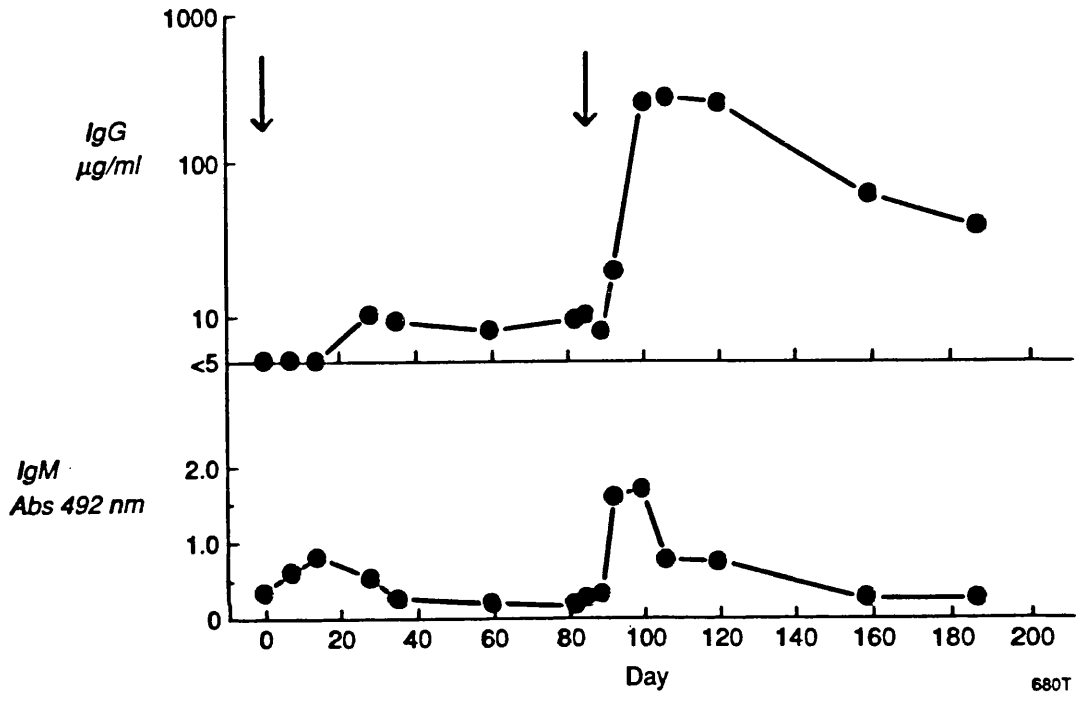


figure 7.4 c
Human anti-antibody response following repeated therapy with PK2G. A small rise in anti-antibody level was seen after the first therapeutic injection of radiolabelled PK2G. Following the second injection there was a rapid rise in both IgG and IgM anti-goat.

7.4 Antisheep antibody response to a single injection of ^{131}I anti-CEA (PK4S).

The immune response was measured in sixteen patients who were given PK4S either alone or followed by 'second antibody' (groups 2,3,4; table 5.1 & 6.1b). The human antisheep antibody enzyme assay was used to compare pretreatment serum anti-antibody levels with those following therapy. It was necessary to develop a working definition of a positive human anti-antibody response to therapy as human IgG from all patients (and normal subjects) exhibited some degree of binding to PK4S. However, values of < 5 μg/ml could not be expressed quantitatively. A human IgG antisheep response was considered to be positive if the anti-antibody concentration after therapy was either more than twice a pretreatment value when this was > 5 μg/ml, or ≥ 10 μg/ml when the pretreatment sample was ≤ 5 μg/ml. The maximum IgG anti-antibody response following therapy is shown in table 7.2.

Using these criteria an elevation in human IgG antisheep was found in 7 out of 15 patients following a single injection of PK4S. In 6 patients the anti-antibody concentration was > 5 μg/ml before exposure to PK4S and the mean serum concentration of anti-antibody in these patients before therapy was 9 μg/ml. None of the patients had positive skin reactions to intradermal PK4S before therapy. One patient (no:8) had received 200 μg of radiolabelled PK4S for a diagnostic localisation scan five weeks before the

therapeutic dose was given. Antisheep antibody became measurable two weeks after the diagnostic scan so that the elevation in anti-antibody in this man was considered to be 'secondary' in type. Raised IgM antisheep antibody, measured by an increase in the absorbance of a sample of serum after therapy was found in all patients who had raised IgG anti-antibodies.

Table 7.2

HUMAN IgG RESPONSE TO PK4S

PK4S mg	Pt.no:	PRE µg/ml	POST µg/ml	Peak day
2.5mg + SA	8	28	198	10
"	10	<5	<5	-
"	11	6	6	-
"	12	<5	<5	-
"	13	10	31	18
2.5mg - SA	6	<5	<5	-
"	7	5	10	-
"	9	<5	<5	-
"	14	12	49	29
"	15	<5	<5	-
5.0mg + SA	16	<5	6	-
"	17	<5	16	14
"	18	11	491	15
7.5mg + SA	19	9	60	21
"	20	8	30	21
"	21	9	9	-

SA = 'second antibody'. Mean of two assays, performed in duplicate.

Overall, 11 (55 %) out of 20 patients who had not previously been immunised against animal immunoglobulin developed human IgG anti-antibody following a single injection of sheep or goat anti-CEA. An increase in anti-antibody was found in 7 out of 9 patients who were defined as having pre-existing anti-antibodies (> 5μg/ml) and in all four patients with pretherapy anti-antibody > 10 μg/ml. The mean serum concentration was 16 μg/ml(range 6 to 74 μg/ml). An anti-antibody response was seen in 4 out of 11 patients with pretreatment levels ≤ 5 μg/ml. There was a tendency for a human IgG anti-antibody response to occur more commonly in patients who had raised human anti-antibody before therapy but the observed difference did not reach statistical significance ($X^2 = 3.43$; $0.1 < p < 0.05$).

The variation in value of the anti-antibody concentration between patients was large. Thus, the mean level of 231 μg/ml of IgG anti-antibody post therapy in the patients with pre-existing antibody was not significantly greater than the mean concentration of 18 μg/ml of anti-antibody found in patients with < 5 μg/ml before therapy ($t = 1.234$; $p > 0.2$). The variation in response and the small number of patients meant that it was not possible to determine whether an incremental rise in antibody concentration was associated with an increased probability of an immune response or a larger anti-antibody response.

7.5 Effect of raised serum anti-antibody on the removal of radioactivity from blood.

The removal of radiolabelled antibody from blood was studied in patients who received a first injection of either sheep, goat or mouse anti-CEA antibody in the presence of raised pre-existing anti-antibodies and a repeated injection of anti-CEA in the presence of anti-antibodies that developed after initial therapy. The results were compared with the findings in a group of patients who had low levels of anti-antibody ($\leq$ 5 μg/ml) before the first therapy. The results of 31 therapeutic injections in 28 patients (21 polyclonal and 7 monoclonal anti-CEA) presented in figure 7.5 are expressed as the percent of the injected activity per kg blood remaining 24 hours after the injection of radiolabelled antibody. Patient no: 8 is excluded from the group with raised pretreatment antibodies and included in the group receiving repeated therapy.

The rate of removal of radiolabelled antibody in the patients receiving the first injection of anti-CEA in the presence of a pretreatment anti-antibody concentration > 5 μg/ml was not significantly faster than that seen in the patients with anti-antibodies $\leq$ 5μg/ml. Tumour was identified by gamma camera scanning in 8 out of 10 patients with > 5 μg/ml pretreatment anti-antibody. The two patients in whom tumour was not visualised had numerous small lung metastases.

The removal of radiolabelled antibody was accelerated in the patients retreated in the presence of raised anti-antibodies ($t= 3.348$; $p < 0.01$) and in these patients tumour localisation was not seen following a repeated injection of radiolabelled anti-CEA (for an example see figure 9.8.)

7.6 Anti-antibody response to 'second antibody'.

Sera from all ten patients given 2.5 mg of the antitumour antibody and then 5 to 12.5 mg of horse or donkey anti-sheep antibody were tested for human anti-horse/donkey antibodies. An increase in antidonkey antibody was seen in 8 out of 10 patients. Seven of these patients had also formed antisheep antibodies. Anti-donkey/horse antibodies were not seen in any of the five patients who did not receive the 'second antibody'.

7.7 Cross-reactive human IgG antisheep and antimouse.

Sera from five patients treated with polyclonal sheep or goat anti-CEA who had high levels of IgG antisheep were tested for the presence of human IgG antimouse, and blood samples from five patients treated with mouse anti-CEA were tested for IgG antisheep antibody to determine whether anti-antibodies cross-reacted with IgG from another species.

An increase in IgG antimouse antibodies was seen in all patients following therapy with polyclonal anti-CEA and in 4 out of 5 patients the level of IgG anti-antibody after

therapy was > 10 µg/ml (figure 7.6). Two patients treated with A5B7 developed an increase in the concentration of antisheep antibody and in one case the level was > 10 µg/ml.

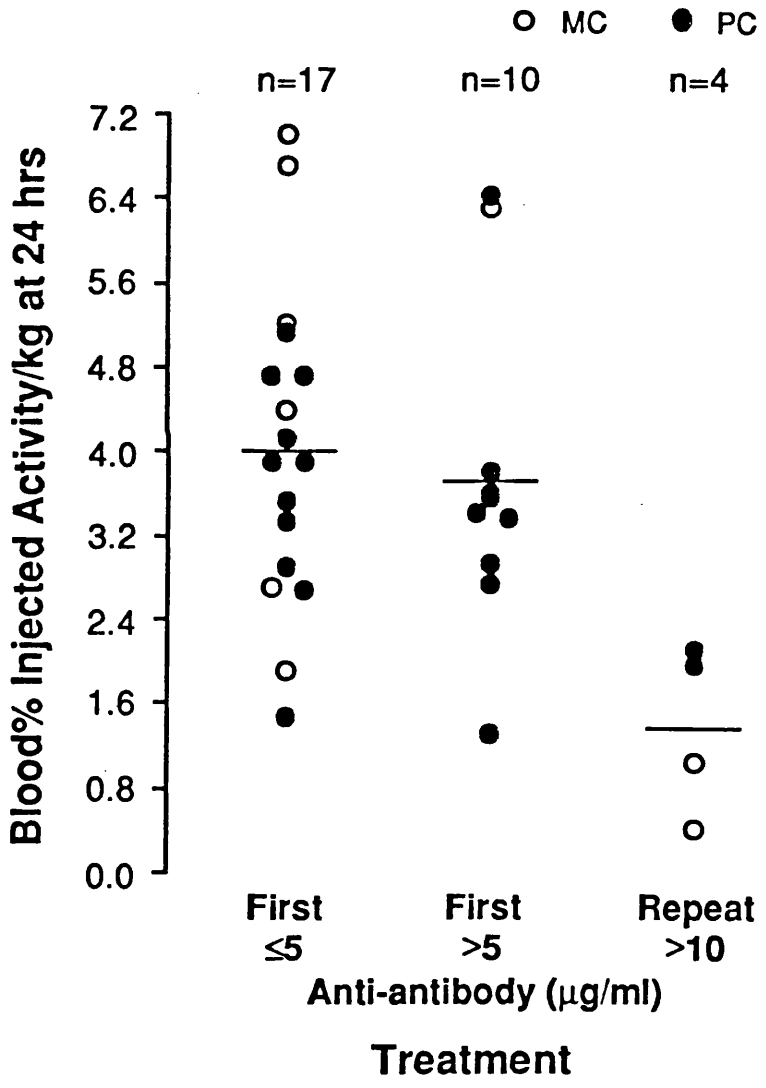


figure 7.5

Removal of radiolabelled antibodies in the presence of low or raised anti-antibodies; pre-existing therapy or formed after previous injections of radiolabelled antibody.

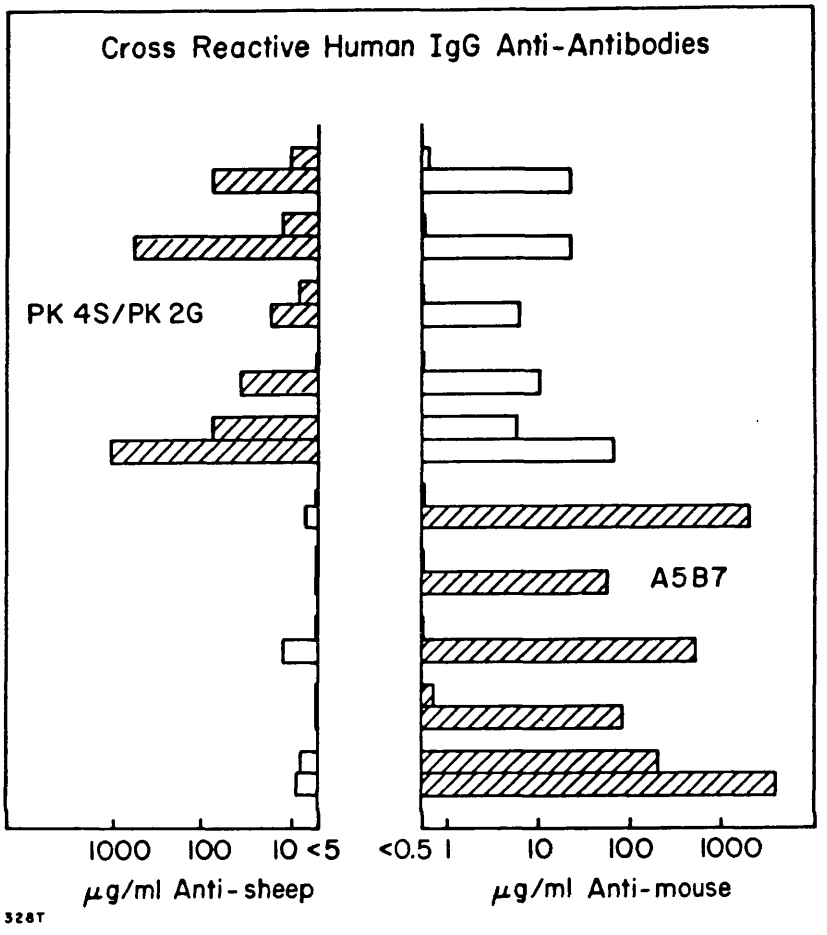


figure 7.6

The concentration of IgG anti-antibody in serum before and after therapy; The hatched bars indicate the concentration of anti-antibody to the species antitumour antibody before and after therapy. The open bars show the concentration of anti-antibody cross-reacting to either mouse or sheep before and after therapy.

7.8 Serum rheumatoid factor before and after radioimmunotherapy.

Rheumatoid factor was assayed in 28 patients treated with polyclonal or monoclonal anti-CEA. 4 patients had a positive RA latex test prior to antibody therapy and 2 had a positive RAHA and RA latex test. Of these 6 patients 4 had marginally elevated IgG anti-antibodies, and 3 out of 5 IgM

anti-antibodies. IgM anti-antibodies were was considered to be positive as the optical absorbance value was > 0.4. Using latex or haemagglutination assays rheumatoid factor was not found more commonly in patients with raised pre-existing anti-antibodies ($X^2 = 3.18$).

Following therapy the RA latex test became positive in a further 4 patients, but reverted to negative in 2 following therapy with anti-CEA. Thus, a total of 8 patients had a positive RA latex test after therapy and in 7 of these there was an elevation in the level of serum anti-antibody after anti-CEA. However, there was no association between these observations as 5 patients developed anti-sheep/goat antibody without the development of rheumatoid factor.

No chronic changes in serum immunoglobulins or complement (C3 or C4), monitored regularly over a two to three month period after therapy were seen and none of the patients developed clinical features of type III hypersensitivity or 'serum sickness' following therapy with radiolabelled antibodies.

7.9 Discussion

The demonstration that normal human IgG and IgM bind to sheep and mouse IgG (anti-CEA) supports the evidence that anti-antibodies are a constituent of normal human serum (Hay et al. 1975, 1976). The binding of these anti-antibodies to anti-CEA appeared to be specific as it was inhibited by animal immunoglobulin. It has been shown that the site of

binding is a function of the Fab' portion of the molecule and not due to Fc-Fc interaction (Hay et al. 1976, Courtenay-Luck et al. 1986). No correlation was found between the amount of IgM and IgG anti-antibodies in healthy subjects.

No association was seen between the development of rheumatoid factor and IgG anti-antibodies in this study. Courtenay-Luck et al. (1987), using a monoclonal antibody assay, have shown that the serum level of IgM rheumatoid factor is raised in patients with cancer and often increases further following therapy with a mouse monoclonal antibody.

9 (42.8%) out of 21 cancer patients previously untreated with polyclonal goat or sheep anti-CEA had raised anti-antibodies before therapy. The difficulty in defining a raised pretreatment value of anti-antibody means that the prevalence of raised anti-antibodies in cancer patients is unknown. Two studies have reported a mean level of 4 μg/ml of human IgG antimouse antibody in control subjects and patients with melanoma or lymphoproliferative disorders undergoing antibody therapy (Schroff et al. 1985, Shawler et al. 1985).

The significance of a raised pretreatment anti-antibody level is related to its effect on subsequent therapy. This study has shown that pre-existing anti-antibody did not impair tumour localisation or accelerate the clearance of the radiolabelled antibody from the circulation. These findings are in keeping with the evidence presented by Primus et al. (1980) who showed that radiolabelled goat

anti-CEA formed complexes with human immunoglobulin and that the development of these immune complexes in serum did not prevent localisation of the radiolabelled antibody in the tumour. The difference in behaviour of these complexes and those formed after repeated therapy may be due to a lower binding affinity of the pretreatment anti-antibody for the antitumour antibody than for tissue-bound antigen. In support of this notion a large quantity of normal IgG was required to inhibit the binding of anti-antibody to the animal immunoglobulin on the solid phase.

The rapid blood clearance of radiolabelled antibody and absence of tumour localisation seen in patients retreated in the presence of anti-antibody formed as a result of previous exposure to diagnostic or therapeutic quantities anti-CEA underlines the need to prevent the development of an immune response to antitumour antibodies. The time-course of the severe reaction seen in the patient retreated in the presence of a raised anti-antibody concentration was consistent with a type I hypersensitivity reaction but the cardinal features of anaphylaxis were absent. The symptoms of loin, back and chest pain are more in keeping with the symptoms of visceral ischaemia due to deposition of immune complexes in the microvascular circulation. It is interesting that the intradermal skin test failed to predict the development of an hypersensivity reaction.

The likelihood of developing an immune response to antitumour antibody following therapy was not significantly increased in the patients who had detectable anti-antibody

before exposure to sheep or goat immunoglobulins. Also, the number of days before peak antibody production was seen in the group without pre-existing antibodies was not longer than in those patients with raised anti-antibodies before treatment. It is therefore unclear whether the immune response in either group was of a primary- or secondary-type.

The absence of an immune response may reflect a differential sensitivity to the immunogen between patients. It is unlikely to be due to the dose of protein as the administration of one tenth of the amount of antibody used in this study commonly leads to an anti-antibody response (Pimm et al. 1985). No dose-response relationship was apparent in this or any other study with antitumour antibody therapy although it has been suggested by Carrasquillo et al. (1984) that repeated therapy with radiolabelled Fab' fragments increases the proportion of patients developing antimouse antibodies.

A large proportion of patients in this study were found to have antibodies cross-reacting with sheep and mouse immunoglobulin. This result differs from that of Klein et al. (1986) who found, using an autoradiographic Ouchterlony gel diffusion technique that only 2 out of 14 patients treated with radiolabelled antiferritin had antibodies that cross reacted with antiferritin from six species. More recently the same group have used a radioimmunoassay this showed that cross-reactive antibodies in 36 % of patients who developed anti-antibodies (Klein et al. 1988). Schroff

et al. (1985) reported that 3 out of 3 patients treated with the mouse monoclonal antibody, 9.2.27 had anti-antibodies which reacted with rabbit IgG. But, anti-rabbit antibodies were not detected following therapy with T101, an anti-T cell antibody.

The immune response to mouse immunoglobulin following polyclonal sheep anti-CEA was greater than the antisheep response following monoclonal antibody therapy. The amount of pre-existing anti-antibody to these species did not differ so that it is possible that therapy with a polyclonal antitumour antibody lead to a 'broader' immune response than that with a monoclonal antibody. Further studies could help to distinguish whether the sites of cross-reaction are on shared constant domains or the idiotypic domains of the immunoglobulin molecule.

From these experiments one can conclude that localisation of radiolabelled antibody is not impaired by pre-existing anti-antibody but that repeated therapy is ineffective and hazardous in the presence of anti-antibodies that have arisen following prior exposure to the antitumour antibody. While it may be possible to give a second injection of antitumour antibody using antibody antibodies derived from different species (Order et al. 1985) or immunological Fab' fragments (Carrasquillo et al. 1984) host sensitisation is still likely to prevent multiple courses of therapy. An alternative strategy to prevent host sensitisation is required and this will be considered in the next two chapters.

Chapter 8

SUPPRESSION OF THE IMMUNE RESPONSE TO A MOUSE MONOCLONAL ANTIBODY IN RABBITS WITH CYCLOSPORIN A.

8.1 Introduction

If an improvement in the therapeutic effect of radioimmunotherapy depends upon the ability to give repeated injections of radiolabelled antibody then there is a need to develop ways of circumventing the anti-antibody response. Potential methods of preventing the anti-antibody response have been reviewed in chapter 1. This chapter will examine whether immunosuppression with cyclosporin A is able to prevent the anti-antibody response to repeated injections of the mouse monoclonal antibody (W14) in rabbits.

This animal was chosen as it has previously been used as a model to study the action of cyclosporin A on the humoral immune response to single injections of antigen (Lindsey et al. 1982, Smith 1982).

The toxic effects of cyclosporin A in patients are most marked following prolonged therapy (Cohen et al. 1984). In these experiments a short course of a large dose will be used as the maximum immunosuppressive effect of cyclosporin

A occurs when the drug is given around the time of antigen administration (Borel et al. 1977).

Previous studies have indicated that the effect of immunosuppressive agents is improved by using ultracentrifuged antibody (Rossen et al. 1971). However, it is unclear whether ultracentrifugation of a monoclonal antibody reduces its immunogenicity. This study will compare the effect of cyclosporin A on the anti-antibody response following a combination of the drug with either ultracentrifuged or non-ultracentrifuged W14.

8.2 Treatment protocol

There were 4 animals in each of four treatment groups. A further 16 animals, also four per group, were used to repeat the experiments. Experiments were performed with 3 groups at any one time as it was technically difficult to work with more than 12 rabbits. Groups 1 and 2 received 20 mg/kg cyclosporin A daily for 6 days by intramuscular injection into the hind quarter. Ultracentrifuged W14 was given to group 1 and group 3. Groups 2 and 4 received non-ultracentrifuged antibody (see figure 8.1). Five of the thirty-two rabbits were male; three in group 2 and one in group 3 and 4.

The course of cyclosporin A was restarted on day 15 in groups 1 and 2 and all four groups received a second injection of W14 and tracer dose of ^{125}I W14 on day 16.

Antibody produced in supernatant culture was used for the second set of experiments in groups 1, 2, and 3, as ascites-derived W14 was unavailable.

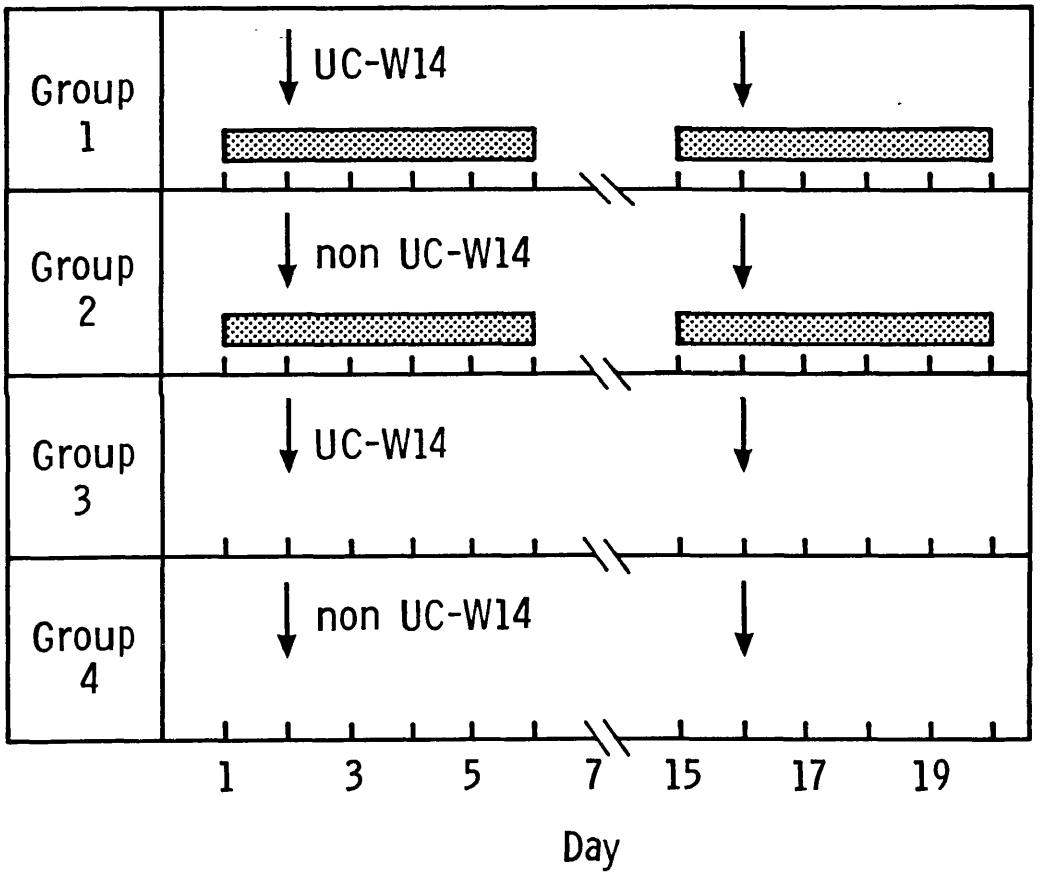


figure 8.1
Treatment schema. UC-W14 (ultracentrifuged W14); non UC-W14 (uncentrifuged W14); ▒▒▒▒▒▒ cyclosporin A.

8.3 Results

1. Clearance of ^{125}I monoclonal antibody from blood

The clearance of ^{125}I W14 is shown in figure 8.2. A biphasic pattern of clearance of ^{125}I was seen in the animals that did not receive cyclosporin A (groups 3 and 4). The mean antibody half-life, taken after an initial equilibration period, was 1.9 days (standard deviation 0.41). An acceleration in the elimination rate of ^{125}I occurred after the sixth day. This coincided with the appearance of rabbit antimouse antibody (*vide infra*). In contrast, the rate of elimination of ^{125}I remained constant in the animals that received cyclosporin A (groups 1 and 2).

A similar pattern of clearance was observed when the experiments were repeated with the second batch of four animals. A comparison of the amount of ^{125}I remaining showed that at fourteen days after the first injection, group 1 had significantly more radiolabel in the circulation than group 3 ($p < 0.01$; $t = 3.93$). (Figure 8.2a).

A second injection of W14 was given on day 16. The elimination half-life of antibody in groups 1 and 2 did not differ significantly from the first injection (figure 8.2b). However, within thirty minutes of injection of W14 a rapid clearance of the radioisotope was seen in the animals that had developed an immune response to mouse antibody following the first injection. The amount of ^{125}I per gram of blood was about one fifth to one tenth of that found in the animals given cyclosporin A.

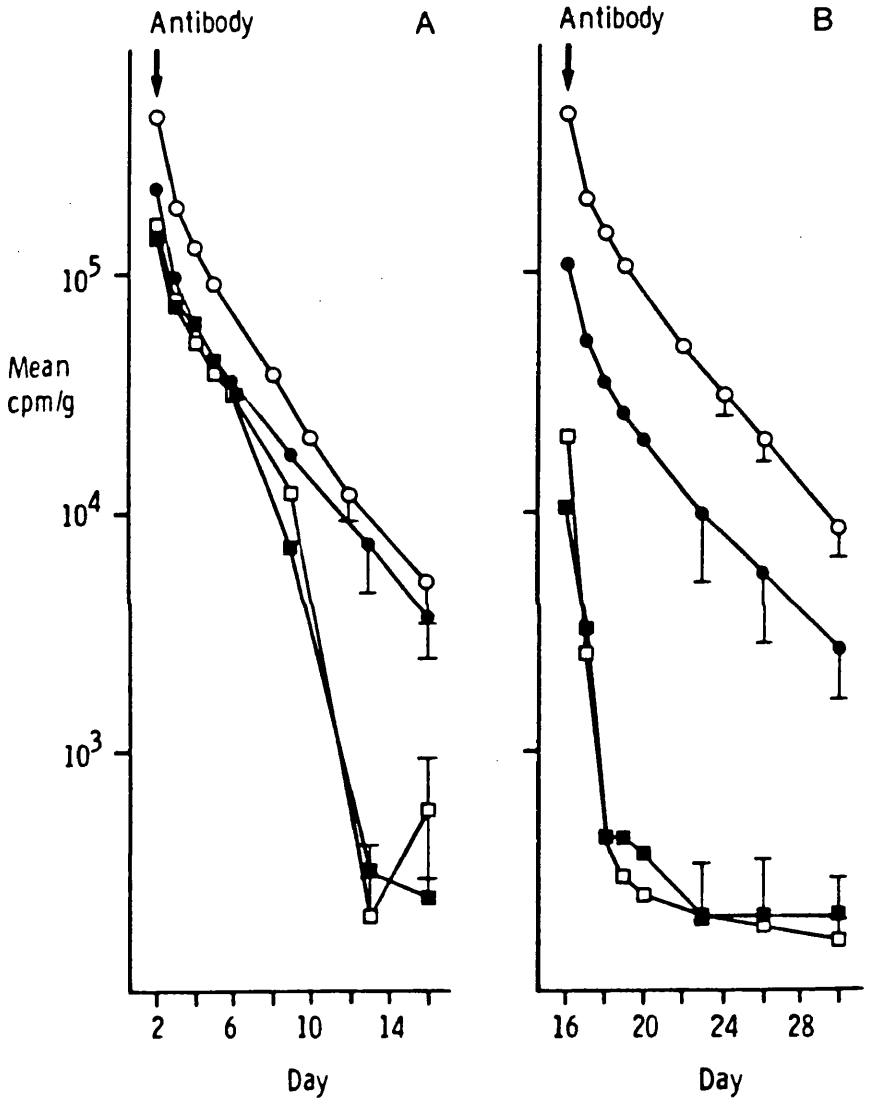


figure 8.2

The clearance of ^{125}I mouse anti-hCG (W14) in four groups of four rabbits. CsA = cyclosporin A. ● Group 1 (UC-W14 and CsA); ○ Group 2 (non UC W14 and CsA); □ Group 3 (UC-W14); ■ Group 4 (non-UC W14). Results are the mean counts per minute (cpm) per gram of blood in four animals per group. In a.) the first sample was taken 30 minutes after injection on day 2 and in b.) it was 30 minutes after the injection on day 16. Group 2 received approximately 1.4 times the amount of radioactivity given to the other groups. For clarity standard deviations for early time-points are not shown.

At the end of the second fourteen day period significantly more antibody remained in group 1 compared with group 3 ($p < 0.01$; $t = 5.19$). The clearance of ^{125}I W14 in the group receiving ultracentrifuged antibody alone did not differ significantly from the animals given non-ultracentrifuged antibody. Also, ultracentrifugation did not alter the clearance rate of ^{125}I in the two groups of rabbits given cyclosporin A.

2. Rabbit antimouse antibody response

None of the rabbits had detectable antimouse antibody before immunisation. The enzyme immunoassay for rabbit IgG antimouse was able to detect 0.1 µg/ml or more of antibody in the serum. The results of both sets of experiments have been combined and are represented by the graphs in figure 8.3. Rabbit IgG antimouse antibody was not detectable in any of the 8 animals that were given cyclosporin A in combination with ultracentrifuged antibody (figure 8.3a). However, antimouse antibody developed in 2 out of 8 animals given cyclosporin A and uncentrifuged antibody, in both cases following the first injection of W14 (figure 8.3b). In one animal, the amount of antimouse antibody was similar to that seen in the rabbits that did not receive cyclosporin A but no further elevation in antibody level occurred after the second injection. All 16 animals that were not given cyclosporin A made antimouse antibody by four weeks.

There was no evidence of immunological tolerance in the animals given ultracentrifuged antibody alone as all the animals formed antimouse antibody and displayed a pattern of clearance of ^{125}I similar to that seen in animals given non-ultracentrifuged W14. However, the quantity of anti-antibody detected in group 3 varied greatly (figure 8.3c). Less anti-mouse antibody was measurable in the four animals given W14 prepared from supernatant culture than ascites-derived antibody ($p < 0.02$; $t = 4.87$). The four animals that responded poorly to W14 produced in supernatant cell culture (figure 8.3c) were given 200 µg of ascites-derived W14 intravenously eight weeks later. It was possible to boost the anti-mouse antibody response in 3 out of 4 animals. The one poor responder to W14 was able to produce only a small amount of antibody to bovine albumin, 200 µg, given intravenously at the same time to test the immune competence of the animals. The other 3 animals developed a high titre of antibody to bovine albumin. All the control animals (non-ultracentrifuged W14; group 4) received antibody prepared from ascites and they made a good immune response after the first and second injections (figure 8.3d).

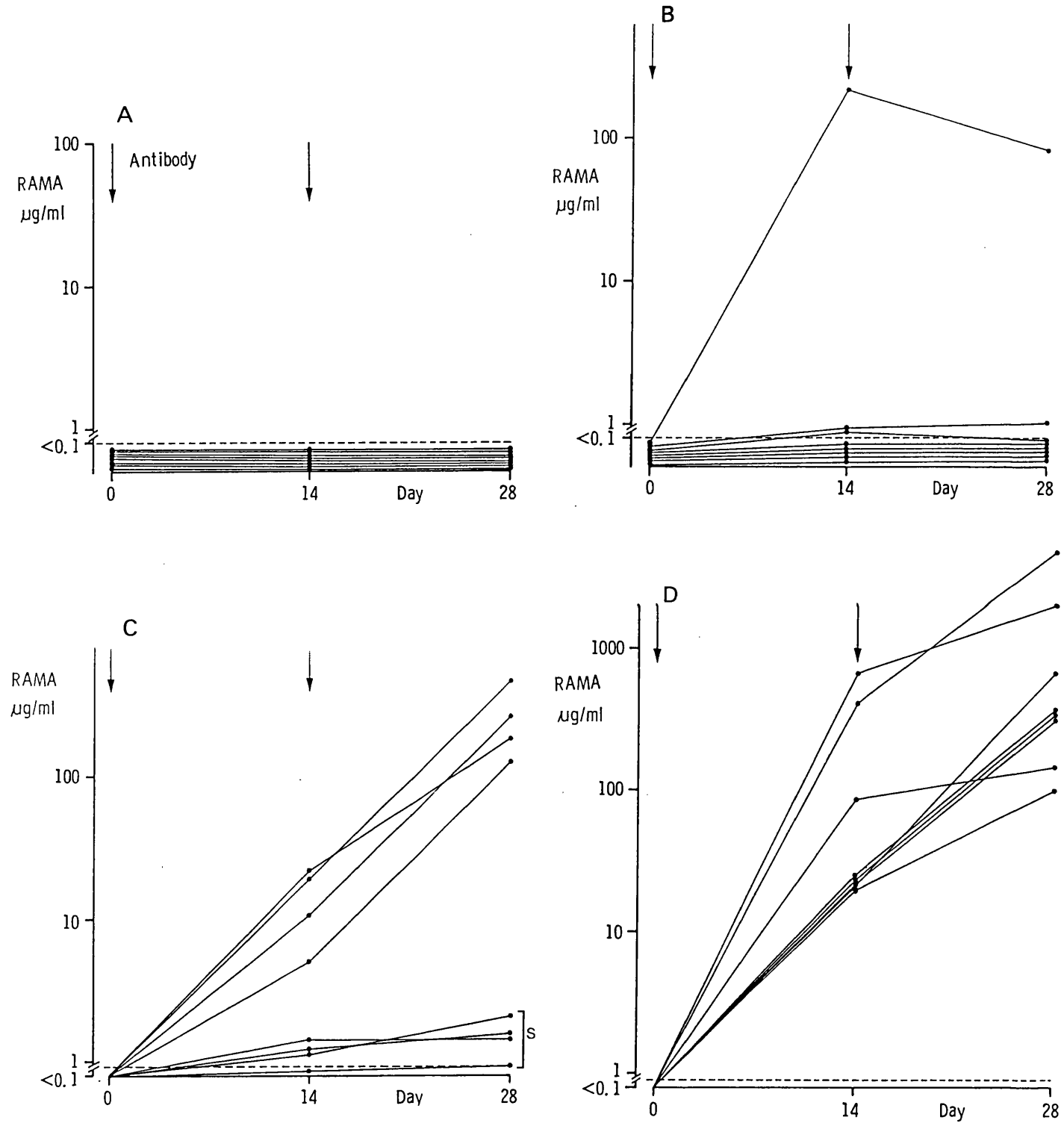


figure 8.3

Rabbit IgG antimouse antibody response (RAMA) in a.) group 1 (CsA and UC-W14); b.) group 2 (CsA and non UC-W14); c.) group 3 (UC-W14); d.) group 4 (non UC-W14). Results < 0.1 µg/ml are shown below the dotted line. In c.) s = W14 produced in supernatant culture. CsA = cyclosporin A.

3. Recovery of Immune response after cyclosporin A

Eleven of the fourteen rabbits that failed to make antimouse antibody while on cyclosporin A (eight from group 1 and three from group 2) were kept to examine whether they were able to respond to W14 eight weeks after the completion of cyclosporin A therapy. They were challenged with a third intravenous injection of 200 μg of W14, without cyclosporin A. Four animals from group 1 rechallenged with uncentrifuged W14 developed antimouse antibodies (figure 8.4). The amount of antimouse antibody was similar to the level detected in the control animals (group 4) after primary challenge with W14 (see figure 8.3d). Two of the animals from group 4 were included as positive controls; in both a further elevation in the blood level of rabbit antimouse antibody was observed.

In 6 out of 7 rabbits rechallenged with 200 μg of ultracentrifuged W14 (four from group 1; and three from group 2) the concentration of rabbit IgG antimouse antibody remained less than 10 μg/ml. This was significantly less than in the animals rechallenged with the uncentrifuged preparation (X^2 = 4.48 (with Yates' correction) $0.02 < p < 0.05$). In 3 rabbits (one from group 1; two from group 2) no antibody response was detectable. Two of these animals were then challenged with 400 μg of uncentrifuged W14, intravenously, two weeks later and both animals remained unresponsive.

A single intravenous injection of 200 μg of bovine serum albumin (Sigma) in 0.9% saline was given to these two

animals as well as two animals that had made a good response against W14. An antibovine serum albumin antibody response was detected in one of the animals unresponsive to W14 and both control animals.

The elimination pattern of ^{125}I in the seven animals re-challenged with ultracentrifuged W14 was similar to the that seen following the first injection of W14 and consistent with a 'primary-type' immune response (figure 8.5). The antibody half-life in the unresponsive animals remained prolonged and was not significantly different from the non-responders in figure 8.2.

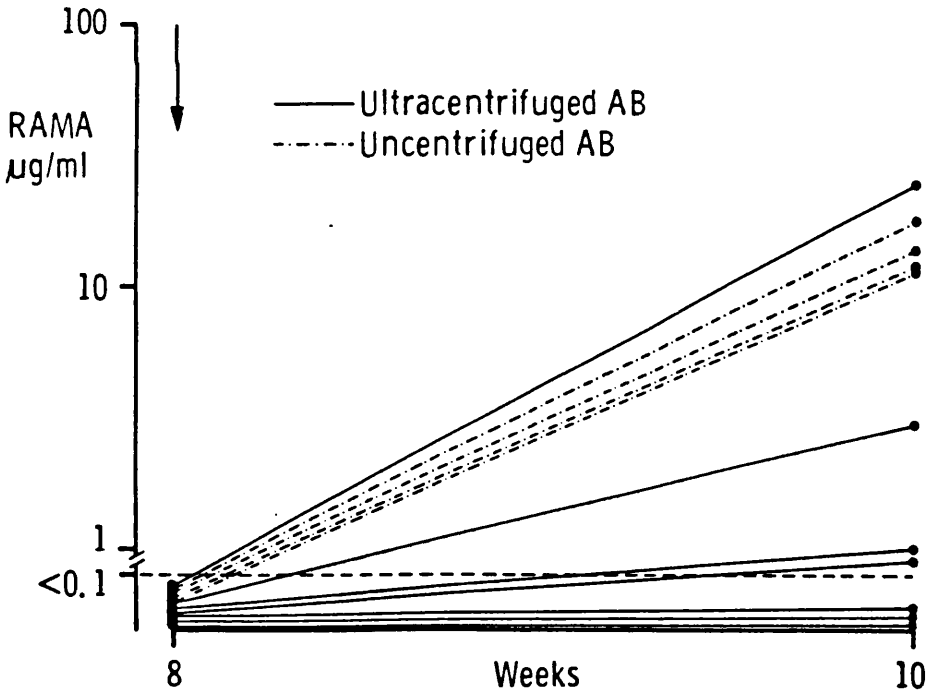


figure 8.4
The rabbit IgG antimouse antibody response (RAMA) 8 weeks after completing therapy with cyclosporin A.

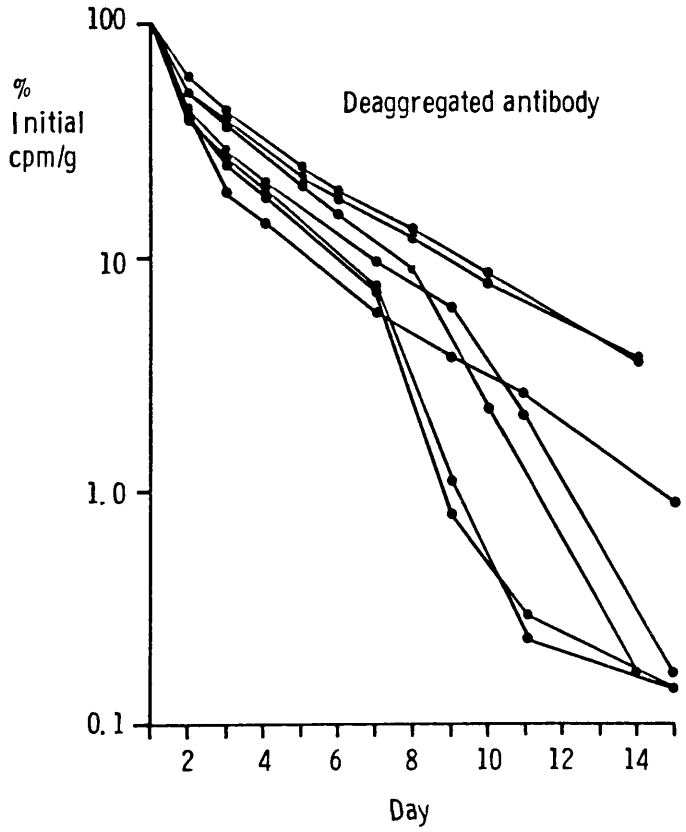


figure 8.5
Clearance of ^{125}I W14 in the seven animals challenged with UC-W14 8 weeks after completing therapy with cyclosporin A. An acceleration in the elimination of ^{125}I W14 is seen between 7 to 9 days in the animals that developed antimouse antibody. The results are expressed as a percentage of the injected dose as the data are compiled from experiments carried out at different times.

4. Toxicity.

The mean growth rate over the 10 week period of study was constant in the animals that did not receive cyclosporin A. During therapy with cyclosporin A there was virtually no increase in the mean weight of the rabbits. Thereafter, the growth rate increased and by 10 weeks there was no significant difference between the weight of the rabbits in the cyclosporin A and control groups (figure 8.6).

There were no deaths during the experimental period. One rabbit died five months after the completion of the experiments. This animal had been given ultracentrifuged antibody, produced in supernatant culture, without cyclosporin A and had exhibited a poor immune response to both W14 and bovine serum albumin.

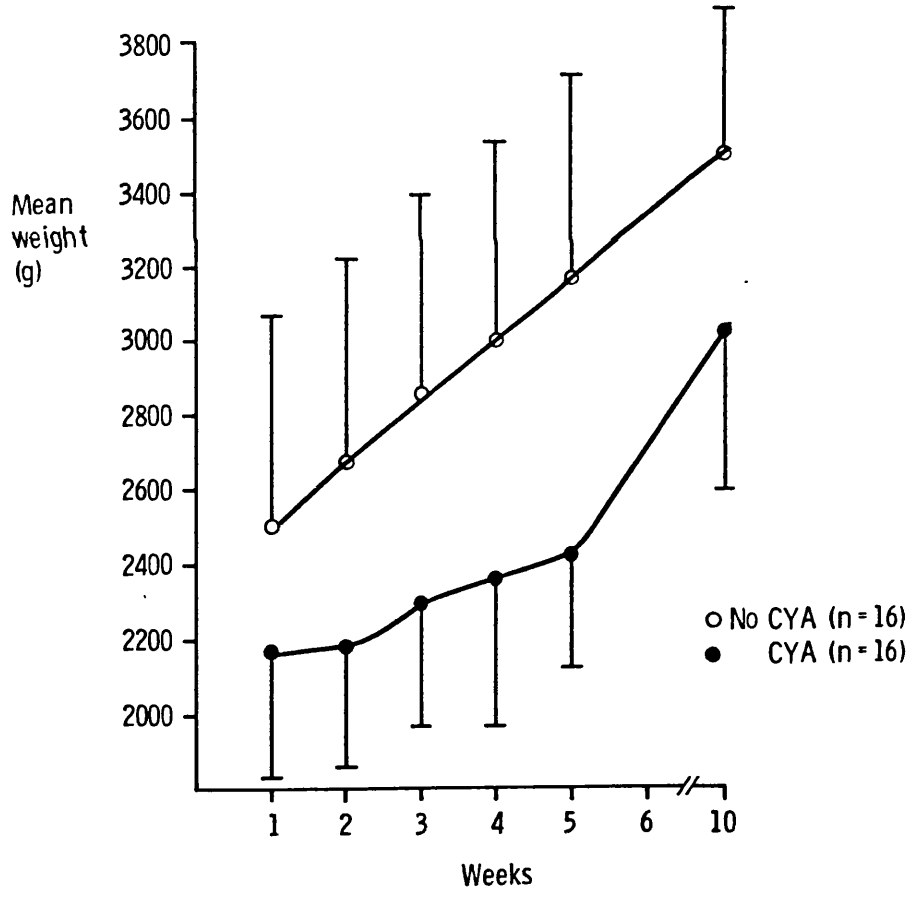


figure 8.6
Growth of rabbits before and after treatment with cyclosporin A (CYA). Mean weight and standard deviation.

8.4. Discussion

These experiments have shown that cyclosporin A prevents the formation of rabbit IgG antimouse following repeated administration of a mouse monoclonal antibody and delays the clearance of radiolabelled antibody from blood. The clearance of a repeated injection of W14 was unchanged in the absence of rabbit antimouse antibody. This is important as tumour localisation is reduced if clearance of the antitumour antibody is rapid. Pedley et al. (1989) showed that early removal of radiolabelled antibody by administration of an anti-antibody leads to a reduction in the uptake of radioactivity by the tumour.

The results support the previous observation that cyclosporin A inhibits the primary humoral immune response (Lindsey et al. 1982) and show that a subsequent injection of immunogen fails to lead to the development of an anti-antibody response as long as therapy with cyclosporin A is continued.

These *in vivo* findings are in keeping with the results of adoptive transfer experiments in mice (Klaus and Kunkl 1983) and suggest that in rabbits, as in mice, T helper cell effector function remains suppressed as long as cyclosporin A is given. The mechanism of this action is unclear but it appears that in the presence of cyclosporin A 'memory cells' fail to develop. In support of this hypothesis there was a lag period before 'immune elimination' of ^{125}I W14 occurred after rechallenging the animals with W14 eight weeks after

cyclosporin A. This lag phase may represent the time required for T helper cells to act on previously naive antibody-forming cells. Also, the amount of antimouse antibody formed after this challenge was of the same order of magnitude as that following a primary antibody response in virgin animals.

A dose of 20 mg/kg/day was selected as it has previously been shown that the effective dose of cyclosporin A needed to suppress the immune response in rabbits lies between 10 to 25 mg/kg/day (Harris et al. 1982). A six day course produced adequate immunosuppression but led to a reversible delay in growth of the animals.

The variation in concentration of rabbit antimouse antibody after immunisation with ultracentrifuged W14 was greater than after uncentrifuged antibody. A low concentration of rabbit antimouse antibody in figure 8.3c was seen in animals given ultracentrifuged W14 derived from supernatant culture. As group 4 were given uncentrifuged antibody derived from ascites one cannot conclude that the observed difference in antibody response was due to ultracentrifugation or supernatant derived antibody. From this study it is unclear whether ultracentrifugation of W14 leads to a reduction in immunogenicity similar to that previously observed with polyclonal immunoglobulin (Dresser 1962). No qualitative differences in composition of W14 were measurable when centrifuged and uncentrifuged fractions were examined by high-pressure liquid chromatography (HPLC) (see figure 3.2). Nevertheless, a white precipitate was seen at

the bottom of the centrifuge tube after the first centrifugation and it is possible that this contained aggregated IgG.

Furthermore, the dose of ultracentrifuged W14 used in this study did not lead to the development of immunological tolerance. In previous studies immunological tolerance was induced in rabbits with much larger doses of ultra-centrifuged polyclonal immunoglobulin (Dresser and Gowland 1964, Biro and Garcia 1965).

It is difficult to draw any firm conclusion about the value of ultracentrifugation of W14 in these experiments. The presence of antimouse antibody in 2 out of 8 animals given uncentrifuged W14 does not refute the hypothesis that ultracentrifugation has no effect on the action of cyclosporin A. It would be interesting to examine the effect of cyclosporin A on the immune response to heat aggregated monoclonal antibody.

The observation that rabbits were able to produce antimouse antibody when challenged with uncentrifuged W14 eight weeks after the completion of cyclosporin A therapy is consistent with the findings Borel et al. (1977) and Denham et al. (1980) who demonstrated a rapid recovery of humoral immunity in mice and rats following cyclosporin A. It was interesting to find that antimouse antibody levels were significantly lower in the animals that were rechallenged with ultracentrifuged antibody after therapy with cyclosporin A.

Immunological tolerance to allografts has been observed in some animals treated with cyclosporin A (Green and Allison 1978, Calne et al. 1978, Nagao et al. 1982). Prolonged unresponsiveness to red cell alloantigens in rabbits is the only report of tolerance to humoral immunity following a short course of therapy with cyclosporin A (Smith 1982). The lack of response to W14 eight weeks after cyclosporin A was due to immunological tolerance in at least one animal as it made antibodies to bovine serum albumin given at the same time as W14. The mechanism of immunological tolerance following cyclosporin A appears complex. Experiments in rats have shown that the continued presence of a heart allograft is required for the maintenance of donor-specific tolerance to skin grafts (White and Lim 1988). The demonstration *in vitro* that cyclosporin A suppresses cytolytic T cells to a greater extent than suppressor T cells has led to the proposal that a sparing of suppressor T cells might be the mechanism responsible for immunological tolerance after cyclosporin A (Hess and Tutscka 1980). Immunological tolerance has not been reported following cyclosporin A therapy in man.

In summary the evidence that cyclosporin A abrogates the immune response to repeated injections of a monoclonal antibody in rabbits is sufficient to examine the effect of this drug in patients treated with repeated injections of radiolabelled antibody.

Chapter 9

SUPPRESSION OF THE HUMAN ANTIMOUSE ANTIBODY RESPONSE BY CYCLOSPORIN A

9.1 Introduction

There are two potential benefits from repeated therapy with antitumour antibodies. The first is an extension of the overall time that the tumour is exposed to the antitumour antibody and the second is the provision of a period between treatment that allows normal tissues to recover from the toxic effects of the immunoconjugate. In chapter 7 it was shown that the presence of human anti-antibodies is associated with an acceleration in the clearance of radiolabelled antibody from the circulation. This chapter will examine whether the immune response to an antitumour antibody can be suppressed by cyclosporin A and whether, in the absence of human anti-antibodies, the distribution of radiolabelled antibody and localisation in the tumour are altered by repeated therapy.

Cyclosporin A was first administered to patients undergoing renal or bone marrow transplantation (Calne et al. 1978, Powles et al. 1978). Doses of up to 24 mg/kg/ day

of cyclosporin A have been given to patients after bone marrow transplantation. Although toxic, this regimen demonstrated the effectiveness of the drug in controlling graft-versus-host disease. A short course with a large dose of cyclosporin A was used in the study described in this chapter as this regimen was effective in suppressing the humoral immune response to an antitumour antibody in rabbits. The patients in this study all had CEA-producing tumours and were treated with a mouse monoclonal antibody raised against CEA (A5B7).

The distribution of radiolabelled antibody was assessed by measurement of the radioactivity in blood and by gamma camera imaging.

9.2 Treatment outline

Repeated injections of ^{131}I A5B7 were given to six patients, aged 41-76 years (median 59) who had locally advanced or metastatic CEA-producing tumours and less than 6 µg/ml of human IgG antimouse antibody in the serum. All patients received at least two courses of ^{131}I A5B7; three were given cyclosporin A, 24 mg/kg/day by mouth with each injection of A5B7. Details of the patients are described in table 9.1.

The treatment schedule is outlined in figure 9.1. Approximately 50 mCi of ^{131}I A5B7 were given with each course of therapy, at two-week intervals, and the cumulative activity did not exceed 200 mCi ^{131}I. Treatment was stopped

if there was tumour progression or unacceptable toxicity. The dose of cyclosporin A was reduced if either the patient experienced severe vomiting or the serum creatinine increased above the upper limit of normal (> 130 µmol/l) during treatment. Potassium perchlorate was given with each course of antibody therapy and potassium iodide was continued until four weeks after the last course. An intradermal injection of 10 µg A5B7 was given before each therapy.

The distribution of the radiolabel in normal organs and tumour was examined by serial gamma camera imaging at approximately 5, 24, 48, 72 and 144 hours and then twice a week during the second week. Human antimouse antibody levels were measured before therapy and at 10 to 14 days after each treatment.

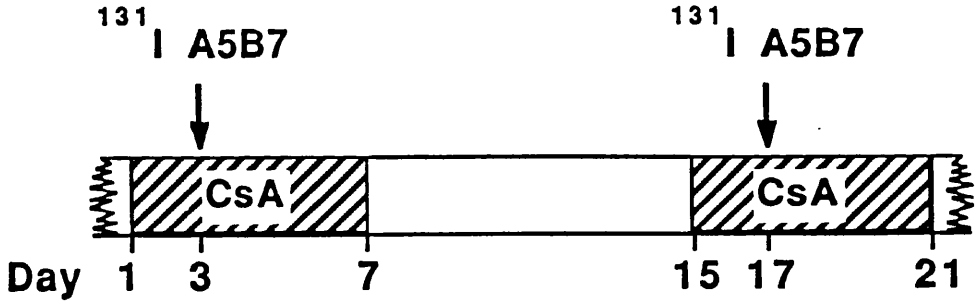


figure 9.1
Schema for repeated therapy with radiolabelled antibody and cyclosporin A.

Table 9.1 Clinical details of patients treated with ^{131}I A5B7

No:	AGE	SEX	TUMOUR DATE.DIAG	PRIOR TREATMENT	DISEASE SITES	PREV RIL ?	A5B7 DATE	mg	^{131}I mCi	TUMOUR LOCALISATION
					CYCLOSPORIN A					
22(FL)	44	male	rectum 4/85	ant.resection Mit.C/5FU	liver	no	3/87	4.3 3.4 4.1 3.5	45 46 43 39	liver " " "
23(RC)	51	male	stomach 11/85	EMA/CO,gastrectomy, oesophagectomy, Mit.C/ Mit.C/5FU, radiotherapy, Adria.	liver, lung mediastinum	yes	5/87	4.9 7.8 5.8	41 67 80	liver, lung " " " "
24(HM)	39	female	colon 11/85	colectomy,pelvic surgery, 5FU/CCNU, 5FU/Mit.C	liver, pelvis	no	4/87	3.8 4.1	45 31	liver,pelvis " "
					NO CYCLOSPORIN A					
17(AG)	66	male	rectum 4/85	ant. resection, radiotherapy, AP resection	pelvis, lung	yes	1/87	4.4 4.5	50 42	pelvis "
25(BP)	73	female	rectum 3/85	ant. resection, radiotherapy, CCNU/ Mit.C	sacrum, pelvis lung	yes	5/87	4.2 5.1	43 15	sacrum, pelvis no localisation
26(CG)	75	female	small cell lung 12/86	Carboplt,VP16,Vcr/CA, radiotherapy	lung, liver, thor. & lumb. spine	no	7/87	5.0 4.7	43 55	lung, thoracic spine " " "

MitC= Mitomycin C; 5FU= 5 fluorouracil; CCNU= lomustine; carboplt= carboplatin; VCR= vinristine; Adria= adriamycin; CA= cyclophosphamide and adriamycin.
ant. resection= anterior resection; thor & lumb= thorax and lumbar; AP resection= abdomino-perineal resection

9.3 Results

1. Human antimouse antibody

The concentration of human IgG antimouse before and after treatment with ^{131}I A5B7 is plotted in figure 9.2. In the 3 patients who were given cyclosporin A the concentration of human IgG antimouse 14 days after the second injection of A5B7 was not significantly changed from the pretreatment values (mean 3.5 µg/ml sd. 2.7). In contrast a rise in the antimouse antibody level was seen after the first injection of ^{131}I A5B7 in the patients who were not given cyclosporin A and this continued reaching a mean of 1998 µg/ml (sd. 387) 14 days after the second injection.

Progressive disease in one patient in the cyclosporin A group (no: 24) prevented further therapy. The other two continued treatment; patient no: 23 received three and patient no: 22 four courses of ^{131}I A5B7. The level of human antimouse antibody increased 2 weeks after the third and fourth course in these patients. The maximum concentration of anti-antibody, seen fifteen days after the fourth course was 493 µg/ml. This was less than 25% of the concentration of anti-antibody detected in patients treated without cyclosporin A. Treatment of the latter group stopped after two courses because of a greatly raised concentration of human antimouse antibody. In one patient (no: 25) a second injection of A5B7 was given in the presence of 39 µg/ml of human antimouse antibody. Within six minutes of starting the

infusion of radiolabelled antibody the patient became flushed and developed severe bilateral loin pain followed by central chest pain. There was no hypotension or bronchospasm. The infusion was stopped and she was given 10 mg chlorpheniramine and 100 mg hydrocortisone intravenously. The symptoms subsided within 30 minutes.

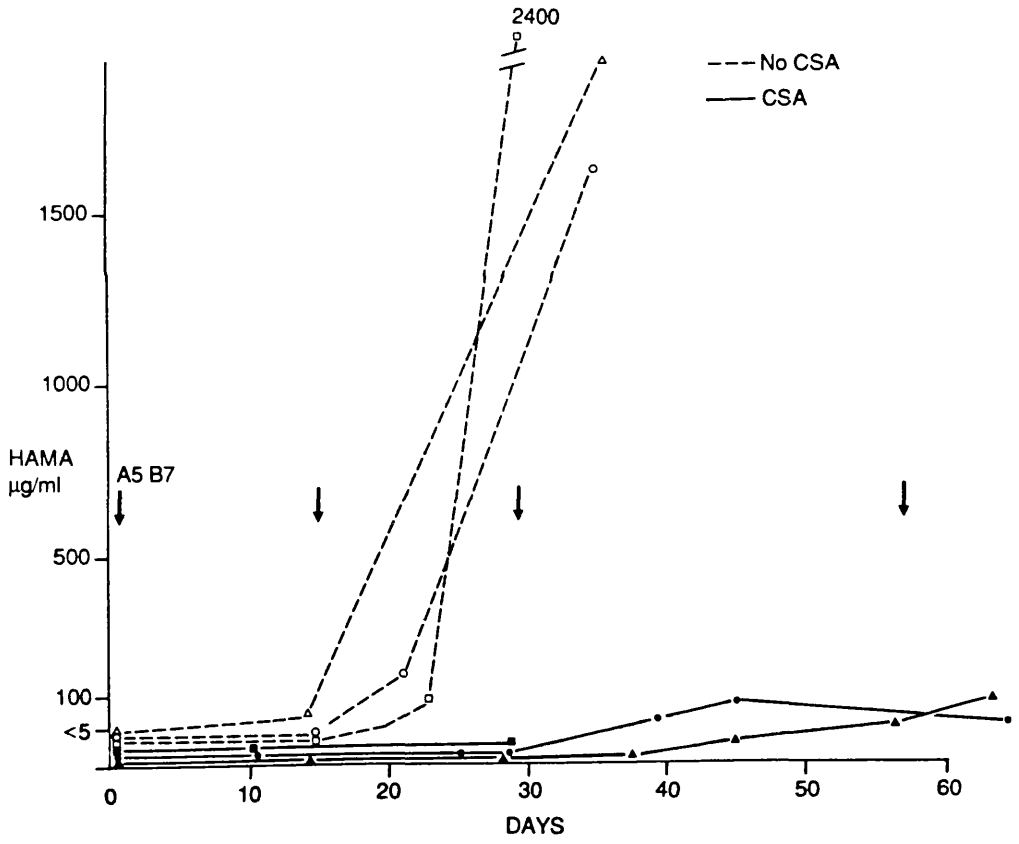


figure 9.2

The human IgG antimouse antibody response following repeated therapy with ^{131}I A5B7 (arrows). ------ patients treated with or _____ without cyclosporin A. Patients indicated by ● had 3 and ▲ 4 courses of ^{131}I A5B7.

Cyclosporin A was given to an additional patient (no: 26), who had 225 μg/ml of human antimouse antibody in the serum before the administration of radiolabelled antibody to examine the effect of the drug on 'pre-existing' human antimouse antibody levels. The patient was 41 years old and had inoperable rectal carcinoma and a past history of carcinoma of cervix. There was no previous history of exposure to murine proteins. Cyclosporin A was unable to prevent a rise in human antimouse antibody. The concentration of human antimouse antibody ten days after treatment was 480 μg/ml. A second course of ^{131}I A5B7 resulted in an acute reaction at the end of the infusion (about 30 minutes from start) characterised by epigastric pain, flushing, faintness and vomiting. There was no tachycardia or hypotension. The peak flow fell from 530 l/min to 430 l/min at the time of reaction. Antihistamine and hydrocortisone were given intravenously and the symptoms subsided within five minutes.

The inhibition assay described in chapter 4 was used to measure human IgG directed against the idiotype. It was possible to demonstrate anti-idiotypic antibody in all patients at some time during therapy (figure 9.3). The pattern of the IgG response to the constant and variable regions of the A5B7 molecule in the patients who received cyclosporin A did not appear to differ from that seen in the patients who were given ^{131}I A5B7 alone.

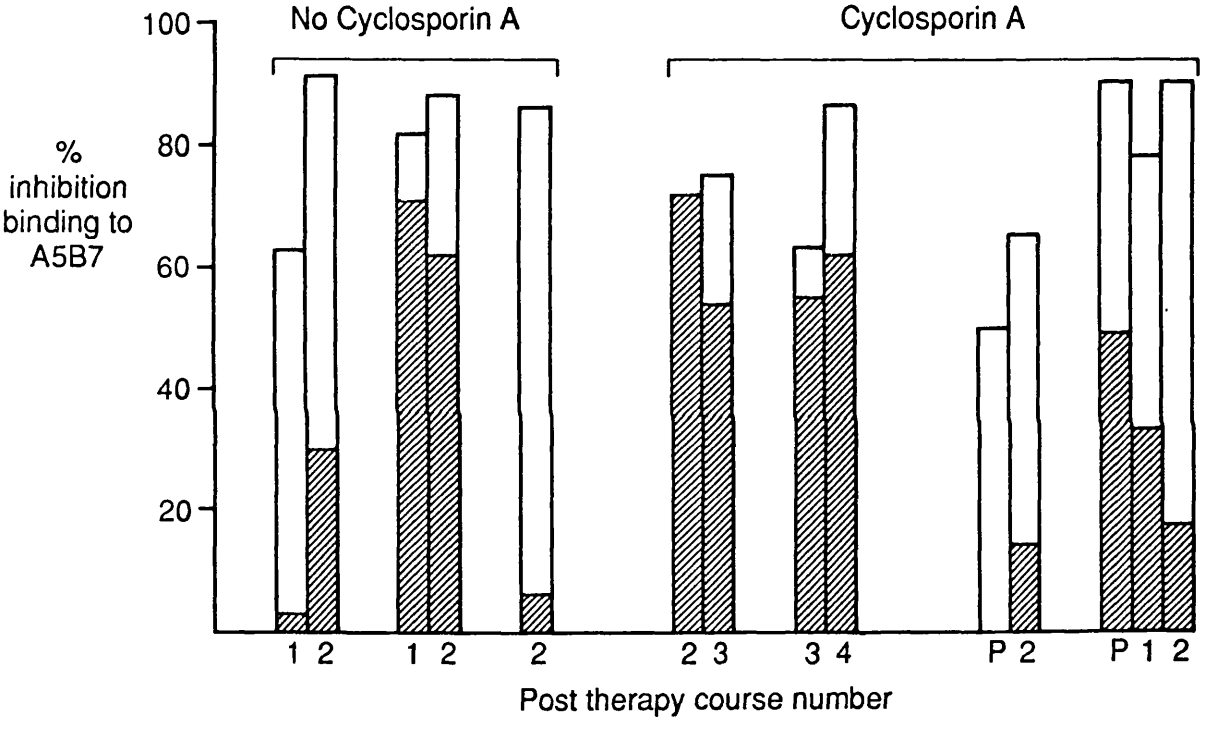


figure 9.3
The anti-idiotypic response to A5B7. Samples of serum containing human IgG antimouse were diluted to approximately 150 ng/ml and incubated with 1 mg of A5B7 ☐ or 1 mg of SB10 ▨ .

2. Clearance of ^{131}I A5B7 from the circulation

The clearance of ^{131}I from blood was measured after each injection of ^{131}I A5B7. When the serum concentration of human IgG antimouse was less than 6 μg/ml repeated therapy did not lead to any significant alteration in the rate of clearance of the radiolabel from blood. This is illustrated by figure 9.4 which shows the pattern of elimination of successive injections of ^{131}I A5B7 in two patients. The elimination half-life in the other patients is shown in table 9.2.

The rate of elimination of ^{131}I was not affected by pre-existing anti-antibody (patient no:26) but a second injection of A5B7 given to two patients who developed an increase in the concentration of antimouse antibody after the first course of ^{131}I A5B7 led to an acceleration in the clearance of radiolabel (figure 9.5).

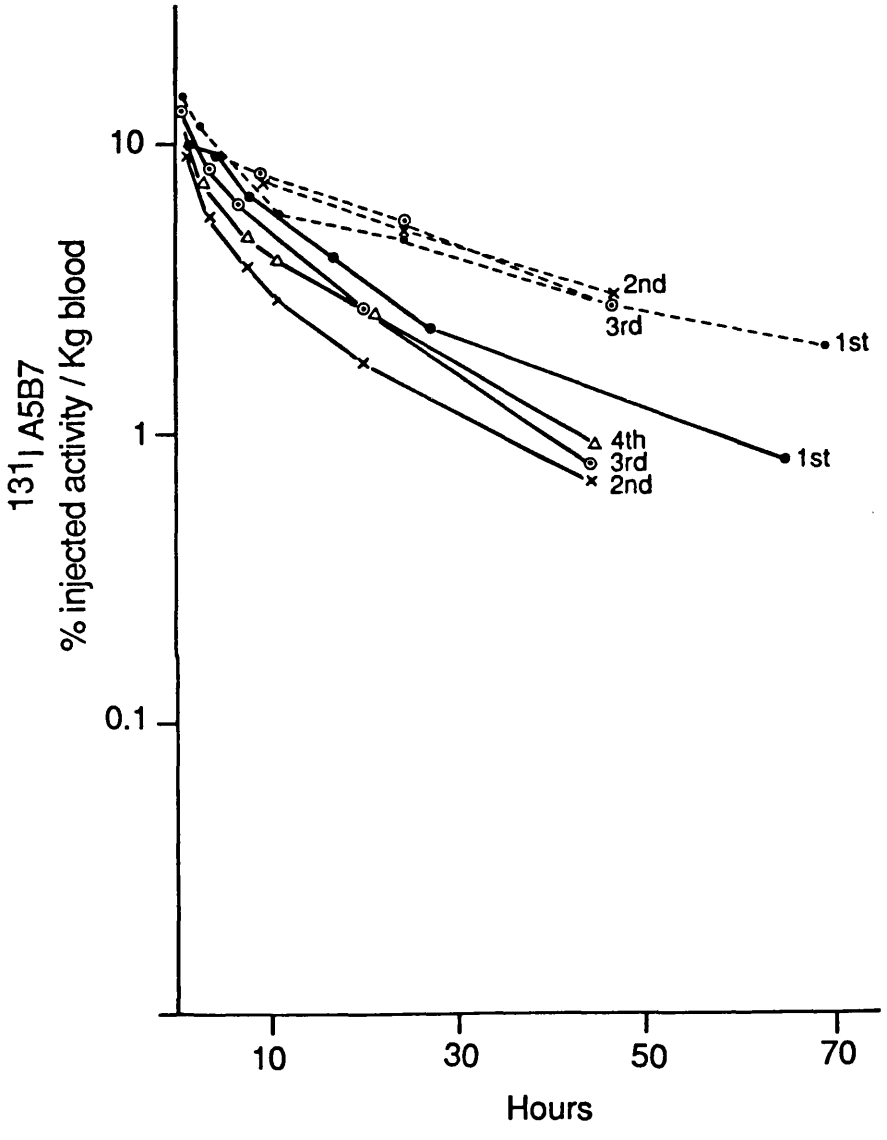


figure 9.4
Elimination of repeated injections of ^{131}I A5B7 from blood. The concentration of human antimouse antibody before each injection of A5B7 was less than 6 µg/ml. No significant difference between the rate of clearance of 4 injections of ^{131}I A5B7 in patient no: 22 _____ is seen, nor between 3 injections in patient no: 23 ------ . The radioactivity in the samples was corrected for decay of ^{131}I.

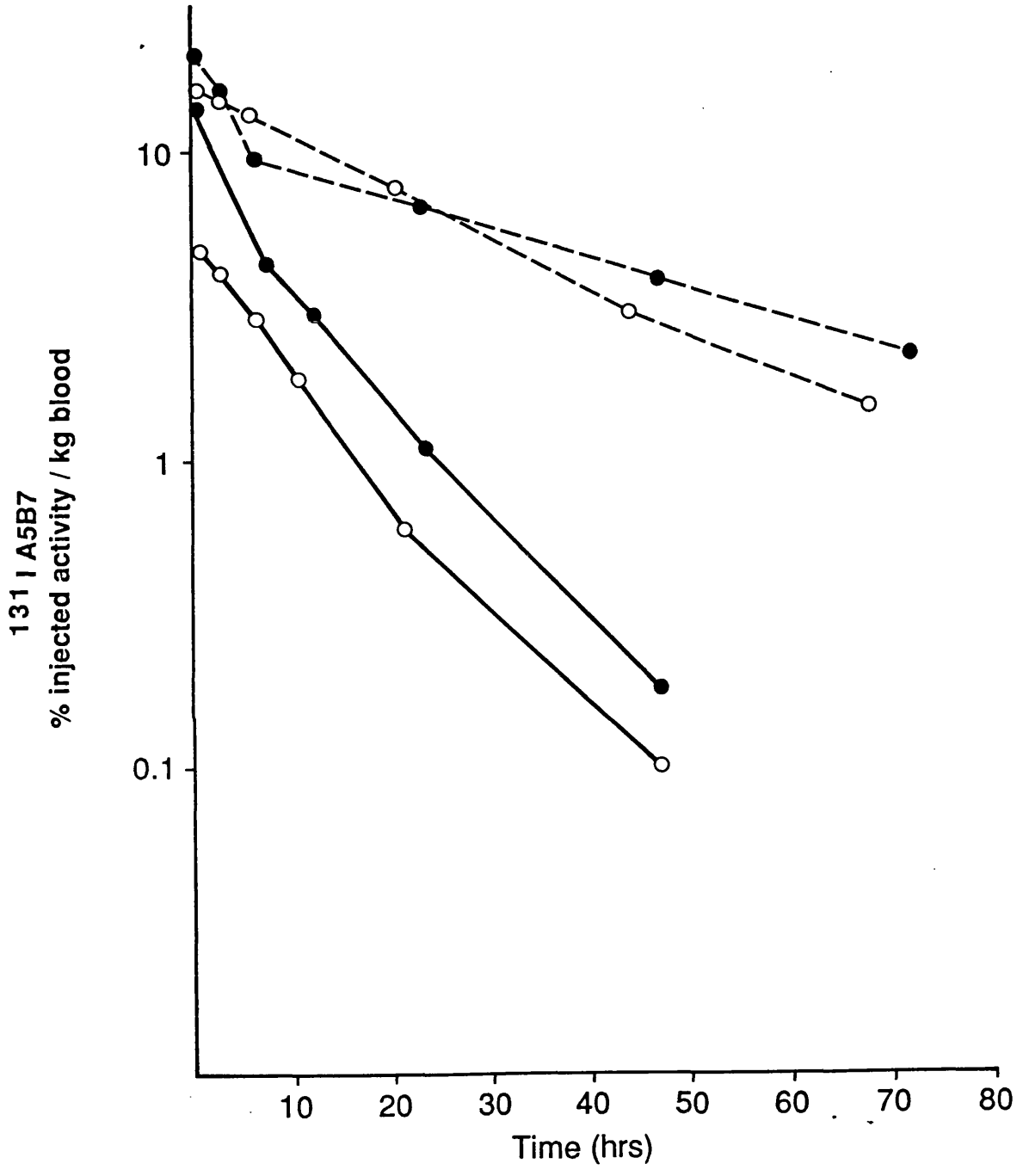


figure 9.5
Clearance of ^{131}I A5B7 from blood in the presence of an elevated concentration of HAMA (human antimouse antibody). The rate of elimination of radiolabelled antibody after the second injection of ^{131}I A5B7 _____ in patient no: 25 (39 µg/ml HAMA) ● and no: 26 (480 µg/ml HAMA) o is more rapid than after the first injection ------ . Radioactivity is corrected for decay of ^{131}I.

The half-life of antibody in the circulation did not appear to be related to the serum concentration of CEA. In the absence of human antimouse antibody clearance of the radiolabelled antibody was remarkably consistent within patients although there was considerable variation between individuals (table 9.2).

Table 9.2

CLEARANCE OF ^{131}I A5B7 FROM BLOOD

Patient	half-life hr	(mean)	CEA µg/l
no:22	11*,14,12,11	(12)	622
no:23	26,26,23	(25)	566
no:24	10,12	(11)	94
no:17	20,17	(18.5)	66
no:25	32	(32)	141
no:27	43,26	(34.5)	35

* half-life following each course was determined from the clearance of radioactivity from blood. The initial point of measurement was 6 hours after injection of A5B7.

3. Tumour localisation following repeated therapy with ^{131}I A5B7

The distribution of radioactivity following a single injection of ^{131}I A5B7 is shown in figure 9.6. The early gamma camera image showed radioactivity in the heart, great vessels, liver and bladder. Uptake by the spleen was unpredictable and tended to be seen in later images.

Visualisation of radiolabelled antibody in the tumour became clearer with late images as the background radioactivity diminished. In some cases SPET imaging was helpful in identifying tumour deposits that were not seen by planar imaging. An example is shown in figure 9.7a. The SPET image demonstrated a tumour deposit that corresponded to a filling defect seen on a SPET image of a ^{99m}Tc colloid liver scan. With time the filling defect became clearer as the amount of radiolabelled antibody in the tumour relative to normal tissues increased (figure 9.7b).

Tumour was visualised by gamma camera imaging in all patients after the first dose of ^{131}I A5B7. The images acquired following repeated administration of ^{131}I A5B7 showed that when human antimouse antibody levels were below 6 μg/ml, each injection of radiolabelled antibody led to further accumulation of radioactivity in the tumour. Examples from two patients are shown in figure 9.8. No difference in the general distribution of radiolabelled antibody was seen in either patient.

Clearance of the radioisotope from the urine after each course of therapy was fairly constant with 65 % (sd 17.8) of the administered activity excreted by 48 hours.

In contrast, a very different distribution of the isotope was seen following the second injection of radiolabelled antibody in the presence of human antimouse antibody (patient no: 25)(figure 9.9). The gamma camera image 3 hours after the second injection of ^{131}I A5B7 demonstrated radioactivity only in the liver and bladder.

Localisation of radioactivity in the tumour was seen after the first but not second injection of radiolabelled antibody.

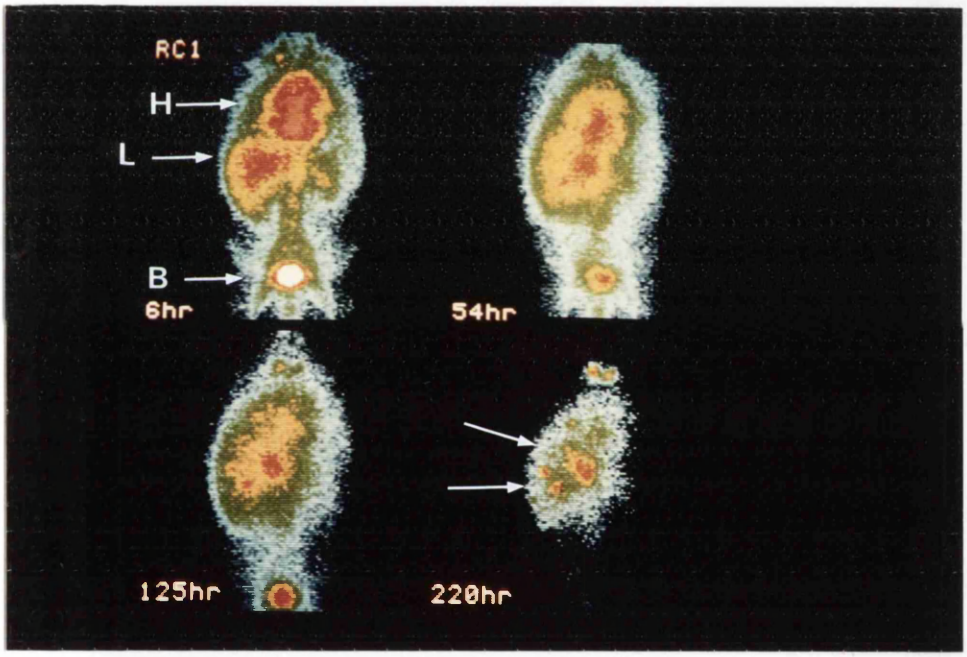


figure 9.6

Anterior planar gamma camera scan showing the distribution of radioactivity from 6 to 220 hours after a single injection of ^{131}I A5B7. Activity is seen in the heart (H), liver (L), and bladder (B). As the background radioactivity in the liver diminished visualisation of the deposits of tumour (arrows) became clearer.

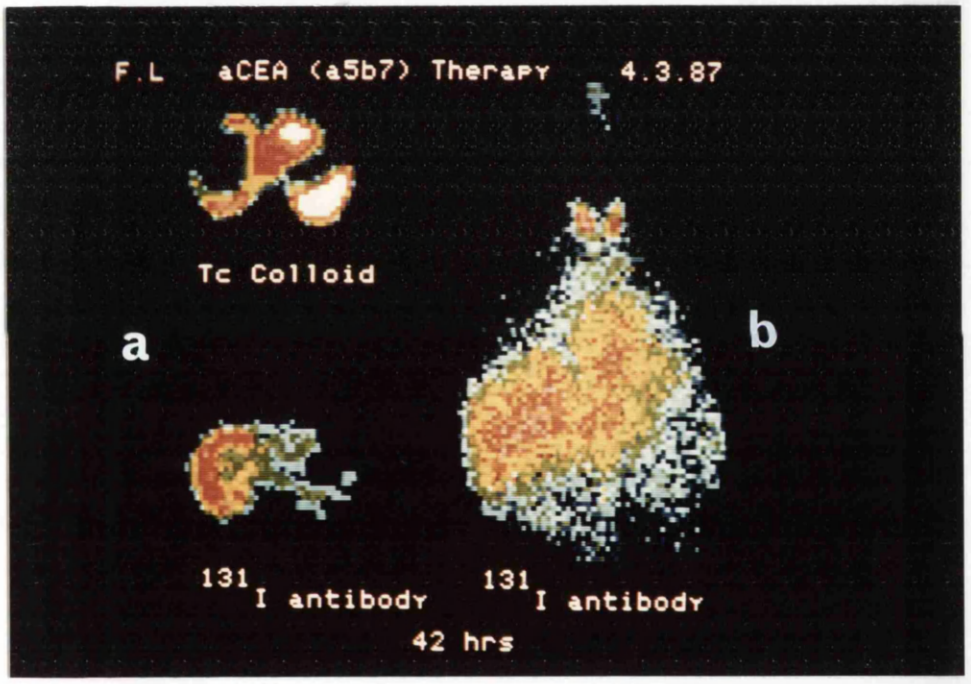


figure 9.7
Detection of a deposit of tumour in the liver using SPET imaging. a.) A SPET scan at 42 hours shows an abnormal filling defect in the liver due to tumour (patient no:22) that corresponds to a defect seen on a SPET image of a ^{99m} Tc colloid scan. b.) Planar imaging fails to identify the tumour.

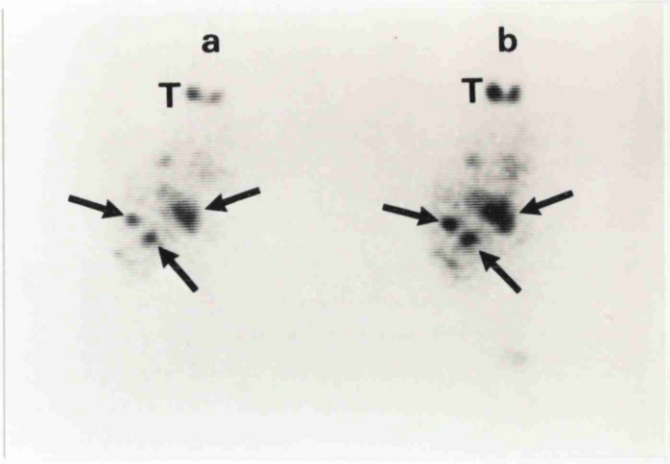


figure 9.8

Localisation of ^{131}I A5B7 in tumour deposits following repeated therapy with a HAMA concentration < 6 μg/ml. The anterior planar images show tumour (arrows) in the liver a.) 220 hours after the first injection of ^{131}I A5B7 and b.) 213 hours after the third course. Some radioactivity is seen in the thyroid (T).

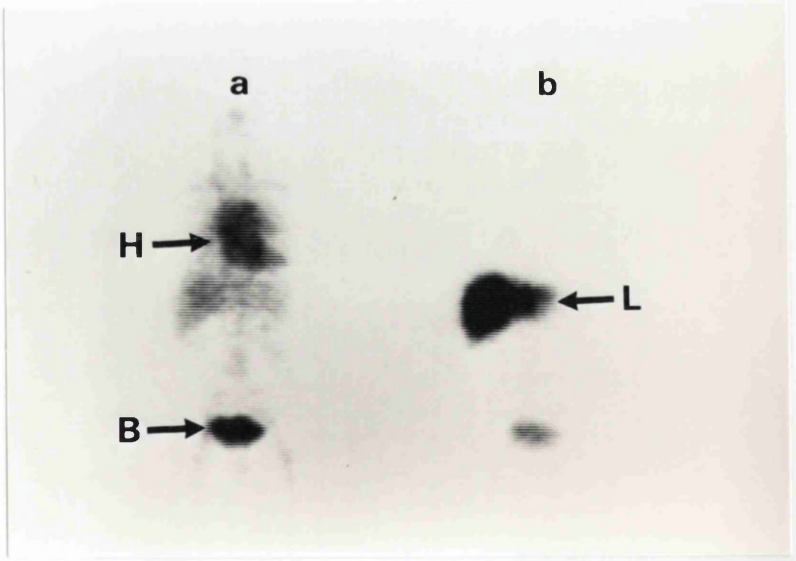


figure 9.9

Accelerated clearance of radiolabelled antibody following a second injection ^{131}I A5B7 in the presence of HAMA. a.) An anterior planar scan of the trunk shows ^{131}I in the heart (H), great vessels and bladder (B) 6 hours after the first injection. b.) By 3 hours after the second injection the scan shows that ^{131}I has cleared from the circulation into the liver (L).

4. Tumour response

Known tumour sites, demonstrated by radiological imaging were measured before and one month after the completion of therapy. There was no response to therapy in any of the patients nor improvement in abnormal liver function tests, present in 3 out of 7 patients.

Serum CA19-9 was raised in 5 patients. Serial estimations of serum CEA and CA19-9 were performed after decay of the isotope. There was no significant change in the serum level of either tumour marker during the month following radioimmunotherapy.

5. Toxicity of radioimmunotherapy and cyclosporin A

Nausea or vomiting were experienced by all four patients and were attributed to cyclosporin A. The symptoms tended to worsen with successive therapy. The fourth treatment in patient no: 22 was delayed (to day 56) because of severe vomiting during and after the third course. He was unable to complete the last course of cyclosporin A because of intractable vomiting. The dose of cyclosporin A was reduced in 2 patients because of symptomatic toxicity. The mean concentration of cyclosporin A in blood between day 3 to 5 of therapy was maintained above 1000 ng/l in all but two patients. The development of human antimouse antibody did not appear to be related to a reduction in the serum concentration of cyclosporin A (see table 9.3). A rise in the concentration of plasma creatinine was seen in all patients but the level of plasma creatinine returned to

normal after the end of therapy. Headaches and hiccups occurred in one patient. Liver function tests did not become abnormal or worsen during therapy. There were no deaths during the treatment nor episodes of infection in the patients who received cyclosporin A. Cyclosporin A did not appear to adversely affect survival although a formal comparison of the two groups is not possible because of differences in the type of tumour and stage of the disease at the time of treatment.

Myelotoxicity was the principal side-effect attributed to ^{131}I. The maximum cumulative activity administered was 188 mCi. Changes in the haemoglobin, total white cell and platelet count are plotted in figure 9.10a.,b. In 4 out of 6 patients the platelet count fell to $<100 \times 10^9/l$. In two it was $< 50 \times 10^9/l$. The platelet count in patient no:23, who received a cumulative activity of 188 mCi was still falling more than 141 days after the first therapy. However, the prolonged period of thrombocytopenia in this patient was not accompanied by leucopenia. Three other patients had platelet counts of $< 100 \times 10^9/l$; occurring 27 days after a cumulative activity of 92 mCi, 8 days after 76 mCi and 8 days after 98 mCi. Only one (no: 27) became both thrombocytopenic and leucopenic. Platelet transfusions were given but she died from progressive tumour before recovery of the blood count had occurred. No other unexpected toxicities were recorded in the two groups of patients.

table 9.3

The human antimouse antibody response and Cyclosporin A therapy

Pt.	course	mean dose CsA mg/kg	(range)	*mean blood CsA ng/l	(sd)	creatinine µmol/l +pre	max	**post	HAMA µg/ml
22	1	20	(24-18)	1509	(68)	58	101		0.5
	2	18		1359	(253)		102		0.6
	3	18		1489	(288)		146		3
	4	11	(18-8)	<800			122	74	493
23	1	24		1034	(227)	64	86		0.7
	2	18	(24-12)	1479	(126)		96		6
	3	15	(18-6)	1357	(448)		108	55	77
24	1	24		910	(126)	49	103		4
	2	24		1188	(141)		55	82	4

* CsA (cyclosporin A) measured on day 3, 4 and 5 of therapy; plasma creatinine measured before start of radioimmunotherapy (+) and (**) lowest value post therapy.

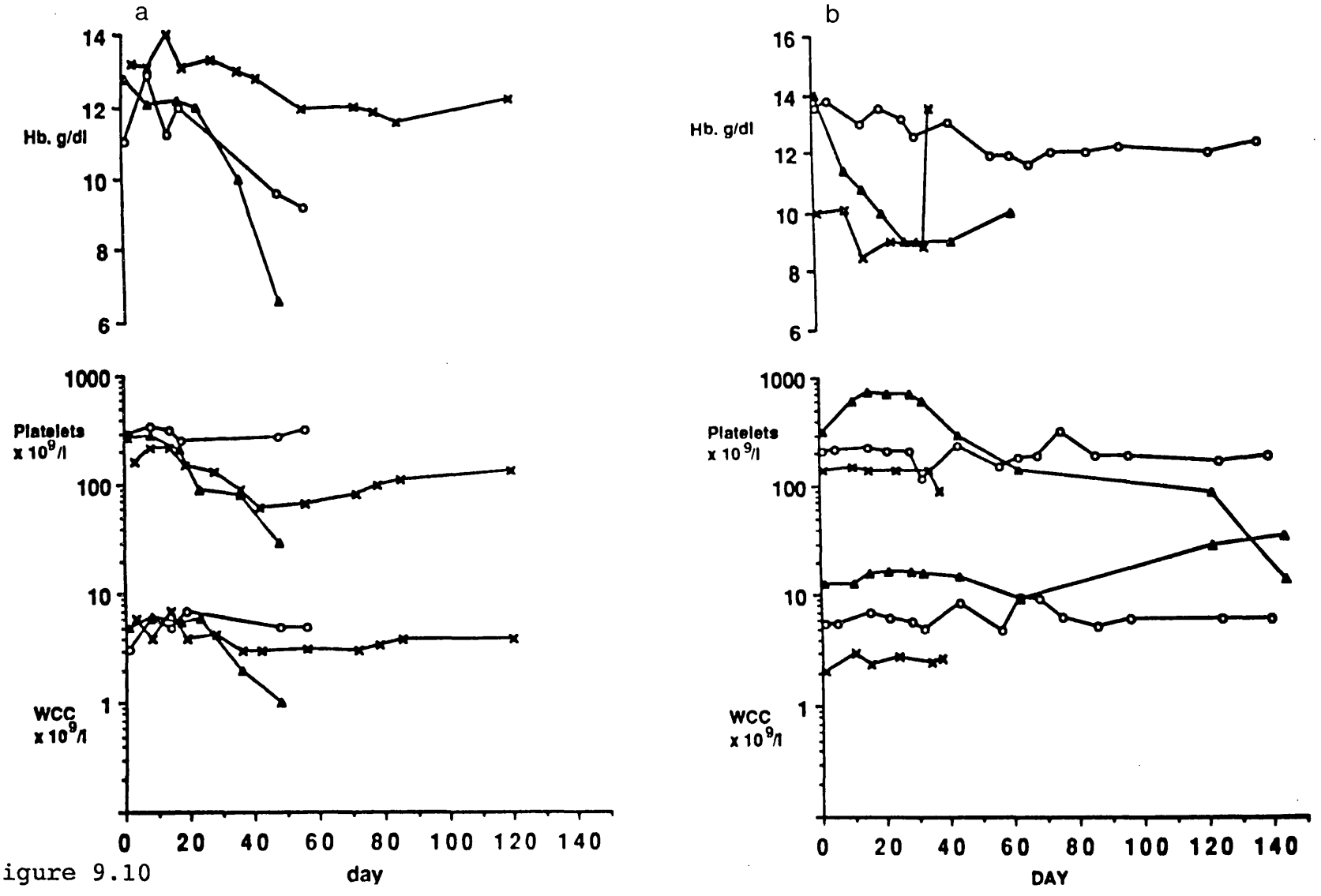


figure 9.10

Changes in haemoglobin concentration, total white cell and platelet count after therapy with a.) ^{131}I A5B7; patients no: 17 × ; no:25 ○ ; no: 27 ▲ . and b.) ^{131}I A5B7 and cyclosporin A; patients no: 22 ○ ; no: 23 ▲ ;no: 24 × .

9.4 Discussion

This study has demonstrated that cyclosporin A can inhibit the human antimouse antibody response to a mouse monoclonal antibody. Only a minority of patients with cancer fail to develop an immune response to antitumour antibodies and the eventual production of human anti-antibodies in 2 out of 3 patients despite cyclosporin A suggested that this group of patients was immunocompetent. The ability to suppress the anti-antibody response during four courses of antitumour antibody, administered over an eight-week period, has identified a new method of giving repeated therapy with immunoconjugates.

Direct comparison of the results in this study with immunosuppressive manoeuvres performed by other groups has to be made with caution as the type of tumour, and dose and schedule of antibody administration differ. However, the degree of immunosuppression appears superior to the results presented in a preliminary report by LoBuglio et al.(1988). They observed incomplete suppression of the human anti-antibody response following one or more injections of a ricin antibody conjugate together with low or high-dose cyclophosphamide and prednisone, or azathioprine and prednisone. In another study a combination of all three drugs reduced the formation of human antimouse antibody following 10 days of therapy with OKT3 from 73% to 38% (Thistlethwaite et al. 1986).

Two other approaches have been used to circumvent the

problems associated with the human anti-antibody response. The first, discussed in chapter 7, involved changing the species from which the antitumour antibody was derived (Order et al. 1985). In the second, plasmapharesis was used to remove human antimouse antibody. However, the reduction in antimouse antibody concentration in patients with a high titre of antibody was not sufficient to prevent accelerated clearance of the antitumour antibody upon retreatment of the patients (Zimmer et al. 1988).

One needs to consider why eventual 'escape' from the effects of cyclosporin A occurred in two patients. It is unlikely to be due to subtherapeutic amounts of cyclosporin A in serum as the level of human antimouse antibody increased in the presence of a high concentration of the drug (table 9.3). It is possible that the duration of cyclosporin A therapy was too short. The drug was stopped 96 hours after the infusion of antibody and at this time the amount of circulating A5B7, predicted from the blood clearance curve in figure 9.4, was 15 ng per ml of blood. Persistence of A5B7 in the reticuloendothelial system could have led to late sensitisation to the antigen. Alternatively, a rebound exaggeration in T helper cell function might have been responsible. This phenomenon has been shown to exist in mice following a short course of cyclosporin A. Only partial suppression of the immune response occurred after rechallenge with the immunogen in the presence of cyclosporin A (Klaus and Kunkl 1983).

Prolongation of therapy with cyclosporin A would not

have been possible without a substantial reduction in the dose. The effect of continuous administration of the drug, using a lower dose, needs to be tested as it may lead to more effective immunosuppression and it is likely to be less toxic. However, the hazards of long-term treatment with cyclosporin A will need to be considered if continuous therapy is a more satisfactory way of preventing the human anti-antibody response.

The failure of cyclosporin A to prevent the rise in anti-antibody level in patient no: 26 suggested that in this patient priming of T helper cells had occurred prior to antibody therapy. The presence of memory T cells in other patients could also have accounted for the eventual appearance of antimouse antibodies despite therapy with cyclosporin A. It is unknown whether memory T cells exist in patients who have low levels of antimouse antibody before therapy or whether they develop in the absence of a detectable antimouse antibody response in patients given small quantities of radiolabelled antibody for tumour localisation studies.

There was no evidence to suggest that the quality of the immune response was affected by cyclosporin A as anti-idiotypic antibodies were detected in both groups of patients. These have been found in most patients treated with monoclonal antibodies (Koprowski et al. 1984, Shawler et al. 1985, Jaffers et al. 1986 Traub et al. 1988). It remains to be seen whether an anti-idiotypic response will also be found after therapy with human monoclonal

antibodies.

Thrombocytopenia (< 100 x 10^9/l) was observed in 4 of the six patients given two or more injections of ^{131}I A5B7. As discussed in chapter 6, many factors are likely to be responsible for bone marrow suppression following radioimmunotherapy. In three of the four patients the fall in platelet count occurred after a cumulative activity of less than 100 mCi ^{131}I. In one of these patients, who was given 98 mCi the mean half-life of ^{131}I in the blood was 34 hours. It is interesting to contrast this with a mean blood half-life of 12 hours in the patient who received 173 mCi of ^{131}I and who did not develop thrombocytopenia. Also, the degree of bone marrow toxicity could be worsened by repeating treatment at a time when myeloid precursor cells are recovering from a previous therapy.

The reduction in the concentration of haemoglobin at the same time as the fall in the white cell count (patient no: 27) suggested that other factors, such as bone marrow infiltration by tumour may have contributed to the changes in the peripheral blood count. In one patient the platelet count continued to fall 85 days after the third dose of radiolabelled A5B7 but the granulocyte count was high throughout this period again suggesting other factors might have been responsible for the thrombocytopenia. The patient died from progressive disease nearly 2 months after the last platelet count was obtained. However, prolonged pancytopenia has previously been observed as long as two and a half years after radioimmunotherapy with ^{131}I antiferritin for hepatoma

(Order et al. 1985).

The contribution of ultracentrifugation of antibody to the prevention of the human anti-antibody response remains unclear. Recently, it has been found that cyclosporin A is able to prevent the human immune response to injections of uncentrifuged antibody conjugated to ricin A chain (P. Scannon personal communication).

The value of preventing the anti-antibody response during targeted tumour therapy with immunoconjugates was underscored by the observation that radiolabelled antibody was rapidly excreted from the circulation and failed to localise in the tumour of patients who had developed anti-antibodies. In the absence of human antimouse antibody the distribution of radiolabelled antibody in the circulation, tissues and tumour remained unchanged with repeated therapy.

The use of SPET to image the tumour and normal organs has aided the development of more accurate methods of dosimetry (Riggs et al. 1988) and these should help to determine whether successive courses of ^{131}I A5B7 are likely to increase the therapeutic potential of radioimmunotherapy. Study of tumour dosimetry is part of a separate investigation that will be reported elsewhere.

In summary, the attenuation and delay in onset of the human anti-antibody response by cyclosporin A facilitates the investigation repeated therapy with immunoconjugates. However, the formation of anti-idiotypic antibody in these patients suggests that further efforts to suppress the immune response are needed.

SECTION V CONCLUSIONS

Chapter 10
CONCLUSIONS AND FUTURE DIRECTIONS

Ten years have elapsed since Ettinger et al. (1979) first published the results of intravenous therapy with a radiolabelled antibody to CEA. Some patients with melanoma, hepatoma, Hodgkin's disease, and lymphoma have responded to radioimmunotherapy (Carrasquillo et al. 1984, Order et al.1985, Lenhard et al. 1985, Rosen et al 1987, DeNardo et al. 1988). There is also evidence to suggest that therapy with radiolabelled antibodies has contributed to an improvement in the survival of some patients with hepatoma by making the tumour surgically resectable (Sitzmann et al. 1987). However, in most cases the response to radioimmunotherapy is at best transient. The ability to give repeated courses without the development of a host immune response may lead to an improvement in therapeutic effect. But it is unlikely that major advances in therapy will occur unless a larger amount of radioactivity is delivered to the tumour.

Experiments reported in this thesis have demonstrated that suppression of the human immune response with cyclosporin A leads to uptake of radioactivity in the tumour with each course of treatment. The eventual appearance of

anti-antibodies despite high-dose intermittent therapy with cyclosporin A has led to a study which is examining the effect of continuous administration of a lower dose of the drug. The preliminary results indicate that 15 mg/kg/day is as effective as intermittent therapy and less toxic (Ledermann et al. 1989). Although small amounts of antimouse antibody were formed during the continuous administration of cyclosporin A further injections of ^{131}I A5B7 and cyclosporin A, in the presence of < 50 μg/ml human IgG antimouse, did not result in any adverse effects or alteration in the blood clearance of the antitumour antibody. This observation requires further study as cyclosporin A may have other effects such as altering the affinity of the anti-antibody or reducing the clearance of immune complexes. For practical purposes the level of anti-antibody above which therapy with antitumour antibody is likely to be hazardous needs to be defined.

The appearance of anti-idiotypic antibodies in patients treated with cyclosporin A suggests that other measures to prevent the human immune response to antitumour antibodies need to be examined. For instance one could use cyclosporin A and cyclophosphamide in combination. However, it would be preferable to avoid the hazards of generalised immunosuppression either by developing a mechanism of specific immunosuppression or immunological tolerance.

Various approaches are being investigated in animals. Specific inactivation of antibody-forming cells has been shown to occur *in vitro* and *in vivo* with antibody-daunomycin

conjugates. Daunomycin conjugated to antibody via an acid sensitive cis-aconityl group is activated by cleavage of the spacer in the acidic environment of the lysosomal compartment of the target cell (Diener et al. 1986, Durrant et al. 1989). It has also been possible to induce tolerance to heat-aggregated human IgG in mice by giving the antigen with an anti-L3T4 (CD4) rat monoclonal antibody directed against T helper/inducer cells (Benjamin and Waldmann 1986).

Alternatively, tolerance has been induced following chemical modification of the antibody. Mice injected with human monoclonal antibody conjugated to mono-methoxypolyethylene glycol became tolerant to the native antibody and it was possible to transfer tolerance to syngeneic animals by T cells and cell extracts (Wilkinson et al. 1987).

Human monoclonal antibodies are likely to be the least immunogenic but their large-scale production has been hindered by instability of the hybridomas a low yield of protein. Recent developments in molecular engineering have made possible the production of chimeric antibodies containing human constant region genes and mouse variable region genes spliced together and transfected in an expression vector to a hybridoma (Morrison et al. 1984). Recently, a genetically reshaped human IgG monoclonal antibody (CAMPATH-1H) has been given to two patients with non-Hodgkin lymphoma. A tumour response was seen and neither patient made an antiglobulin response (Hale et al. 1988).

As more effective methods of overcoming the human anti-antibody response become available it should become easier to give repeated injections of radiolabelled antibodies. In this situation myelotoxicity of radiolabelled antibodies will become dose-limiting.

'Second antibody' reduces the bone marrow radiation dose after it is given but the overall reduction in dose is small and not significantly different from patients treated without 'second antibody'. However, it is important not to dismiss the role of 'second antibody' therapy. Analysis of the gamma camera data obtained following treatment of patients with PK4S (chapters 5 & 6) showed that maximum uptake of radioactivity in the tumour occurred within 8 hours of administration of the antibody (Begent et al. 1989b). This is earlier than in mice (Pedley et al. 1989) and the difference may be due to the faster blood clearance of ^{131}I PK4S in man. If 'second antibody' were given at the time of peak radioactivity in the tumour there might be a greater reduction in bone marrow radiation leading to an improvement in therapeutic ratio.

Alternative measures of reducing bone marrow toxicity such as autologous bone marrow 'rescue' has been investigated by Buchegger et al. (1988b). Preliminary results have shown that syngeneic marrow can be used to replenish the bone marrow of mice bearing human colon cancer xenografts following treatment with repeated injections of radiolabelled antibody. In many of the animals there was disappearance of the tumour and prolongation of the disease-

free survival.

The response of xenograft tumours to radioimmunotherapy has been better than that seen in man. A greater understanding of the factors responsible for this difference may help to improve targeted therapy in man. The uptake of radioactivity in a xenograft tumour, expressed as a percent of the injected activity is typically 1000 times that found in the tumours of patients. The difference is most probably due to the size of tumour. Moshakis et al. (1981) showed that no selective uptake of radiolabelled antibodies occurred in tumours weighing more than 200 to 250 mg. For small tumours, less than 100 mg, the uptake of radioactivity was inversely related to tumour size (Pedley et al. 1987). In most reports prolonged tumour regression following radioimmunotherapy was found in tumours weighing less than 200 mg (Cheung et al. 1986, Sharkey et al. 1987, Buchegger et al. 1988a, Ceriani and Blank 1988, Chiou et al. 1988, Pedley et al. unpublished observations). Lee et al. (1988) showed that the effectiveness of radioimmunotherapy was correlated with tumour size.

Although in proportion to body mass a 100 mg tumour in a mouse is approximately equivalent to a tumour of 300 g in man one cannot assume that the vascularity of a tumour of increases with its size. The vasculature of large tumours is often poor and the tumour cell mass and geometric arrangement of cells lead to a high interstitial pressure that prevents diffusion of molecules (for review see Jain 1988). Rostock et al. (1985) have some evidence to support

this; a decrease in the localisation ratio with increasing tumour size was commensurate with a fall in the vascularity measured by 51-chromium labelled red cell uptake. Also, an increase in uptake of radiolabelled antibodies by tumours has been seen following treatment with β adrenergic neurone blocking agents or external beam radiation. This has been attributed to an improvement in vascular access or permeability (Smyth et al. 1987, Msirikale et al. 1987).

Fab' immunological fragments have been shown to penetrate deeper into multicellular human colon spheroids than intact IgG (Sutherland et al. 1987). While access to the tumour might be improved Fab' fragments are removed more rapidly from the body. Covell et al. (1986) have devised a mathematical model to compare the pharmacokinetics of intact IgG and immunological fragments in mice. Further investigations are needed to examine the relative benefits of therapy with intact IgG or immunological fragments in an animal xenograft model system.

It seems likely that radioimmunotherapy will be most successfully applied to the treatment of a small tumour burden and a better understanding of the factors which influence the uptake of radiolabelled antibodies by tumours are likely to improve the prospects of therapy of larger tumours.

Can the potency of the immunoconjugate be increased? It has been estimated that radioiodine therapy for thyroid cancer delivers several thousand cGy to the tumour (Scott et al. 1970). Two factors may contribute to the lower tumour

dose following ^{131}I radiolabelled antibody therapy. Uptake of ^{131}I in the thyroid is about ten times that of other tumours and any prolongation of the residence of ^{131}I in the thyroid cell would lead to a further increase dose-rate. Dykes et al. (1987) have calculated that a therapeutic advantage of radiolabelled antibodies is only likely to be seen if there is more than a 10 fold increase in the uptake of radioactivity by the tumour. However, this model assumes a uniform distribution of antibody in the tumour and it does not consider the effects of an antibody clearance system such as 'second antibody'. Autoradioagraphy of human tumours shows a heterogeneous distribution of radioactivity (Esteban et al. 1987), making dosimetry of the tumour microenvironment difficult. The model of Dykes et al. (1987) may underestimate the radiation dose delivered to areas of the tumour nodule associated with a high concentration of radioactivity.

The use of radioisotopes other than ^{131}I, such as those with greater energy, longer residence in the tumour or the administration of radiation sensitisers can best be made when the accuracy of tumour dosimetry improves. Notwithstanding, therapeutic trials with 90-yttrium labelled antibody, a pure β emitter, have begun in animals and patients (Anderson-Berg et al. 1987, Sharkey et al. 1988b, Order et al. 1986,1988). The effect of antibodies conjugated to α emitters such as 211-astatine or 212-bismuth have also been studied in animals (Harrison and Royle 1987, Macklis et al. 1988).

This chapter has highlighted some areas of current research that could lead to more effective therapy with radiolabelled antibodies. New methods of therapy are being developed, such as a two-stage amplification system in which an enzyme immunoconjugate is administered followed by a prodrug that is activated at the tumour site (Bagshawe 1988). This form of treatment may benefit from incorporation of antibody clearance systems, such as 'second antibody'. Similarly, suppression of the host immune response by cyclosporin A increases the number of courses of antitumour antibody that can be given and provides a framework for testing immunoconjugates of the future.

APPENDIX A BUFFERS & REAGENTS

1. 0.02 M PHOSPHATE BUFFERED SALINE (Dilution buffer)

sodium chloride	8.0g
potassium phosphate	0.2g
di-sodium phosphate	2.9g
potassium chloride	0.2g
Tween 20	0.5ml
sodium azide	0.2g
distilled water	1000ml

bovine albumin (Sigma) added to make a 0.1% solution
Wash buffer for ELISA did not contain BSA

2. 10% DIETHANOLAMINE HYDROCHLORIDE BUFFER pH 9.8

diethanolamine	97ml
distilled water	800ml
magnesium chloride	0.4g
sodium azide	0.2g

add 1M hydrochloric acid to pH 9.8 and make up to 1000ml with distilled water.

3. 0.01M SODIUM CARBONATE/BICARBONATE BUFFER (Coating buffer) pH 9.5

0.1M sodium carbonate	30ml
0.1M sodium bicarbonate	70ml

The buffer was diluted ten fold with distilled water before use.

4. 0.05M PHOSPHATE BUFFERED SALINE (gel filtration buffer)

sodium chloride	9.0g
di-sodium phosphate	7.25g
sodium di-phosphate	0.74g
sodium azide	0.2g
distilled water	1000ml

5. O-PHENYLENEDIAMINE SUBSTRATE (OPD) (substrate for horseradish peroxidase)

0.01M CITRATE BUFFER pH 4.8

citric acid	8.4g
tri-sodium citrate	17.64g
distilled water	1000ml

OPD substrate 12.5mg O-phenylenediamine (Sigma) added to 50ml citrate buffer. 10µl hydrogen peroxide (30% w/v) was added and the solution was stored in the dark until use. The colour was developed by adding 4M hydrochloric acid.

6. COOMASSIE BLUE 0.1 %

Coomassie blue	1 g
methanol	300ml
acetic acid	100ml
distilled water	600ml

For the 'destain' solution Coomassie blue was omitted.

7. PHOSPHATE BUFFER	0.05M	0.2M
di-sodium phosphate	14.3g	57.2g
sodium phosphate	0.95g	3.8g
distilled water	1000ml	1000ml

8. BARBITONE BUFFER pH8.6 (for immunoelectrophoresis)

Tris	88.6g
barbitone	44.8g
calcium lactate	1.0g
distilled water	2000ml

This buffer was diluted 1 in 4 before use.

9. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

8.5 % SDS polyacrylamide gel

Acrylamide	12.75 ml

30 g acrylamide and 0.8 g bis-acrylamide per 100ml

1M Tris HCl pH 8.8	16.8 ml
20 % SDS	0.225ml
distilled water	15.3 ml

5% SDS polyacrylamide stacking gel

Acrylamide	1.67 ml
1M Tris HCl pH 6.8	1.25 ml
20 % SDS	0.05 ml
distilled water	7.03 ml

NNN'N' tetramethylethylenediamine (TEMED) 30 µl and 150 µl of 10% ammonium persulphate were added to initiate and terminate polymerization.

ELECTROLYTE RUNNING BUFFER

Tris base	0.025 M
Glycine	0.192 M
SDS	0.1% w/vol

SAMPLE BUFFER

10% glyerol
2 % SDS
0.08 M Tris HCl pH 6.8
0.02 % bromophenol blue
(5 % mercaptoethanol- for reduced samples)
Samples were boiled for 10 minutes before application to the gel.

MOLECULAR WEIGHT MARKERS (SIGMA)

200	Kd	Myosin
116	Kd	β galactosidase
94	Kd	phosphorylase B
68	Kd	bovine serum albumin
43	Kd	ovalbumin
30	Kd	carbonic anhydrase

APPENDIX B

HAEMATOLOGICAL TOXICITY

	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Haemoglobin (g/ dl)	≥ 11.0	9.5-10.9	8.0-9.4	6.5-7.9	< 6.5
Leucocytes (x 10^9/l)	≥ 4.0	3.0-3.9	2.0-2.9	1.0-1.9	< 1.0
Granulocytes (x 10^9/l)	≥ 2.0	1.5-1.9	1.0-1.4	0.5-0.9	< 0.5
Platelets (x 10^9/l)	> 100	75-99	50-74	25-49	< 25

WHO Handbook for reporting results of cancer treatment.

WHO offset publication no:48. World Health Organisation, Geneva 1979.

APPENDIX C

Publications in support of the thesis

1. BEGENT, R.H.J., LEDERMANN, J.A., GREEN, A.J., BAGSHAWE, K.D., RIGGS,S.J., SEARLE, F., KEEP, P.A., ADAM, T., DALE, R.G., GLASER, M.G. 1989. Antibody distribution and dosimetry in patients receiving radiolabelled antibody therapy for colorectal cancer. Br. J. Cancer (in press).

2. LEDERMANN, J.A., BEGENT, R.H.J., KELLY, A.M.B, WOOD, S.R., SMITH, D.B., BAGSHAWE, K.D. 1989. Repeated antitumour antibody therapy in man: use of cyclosporin A to suppress the host response. abstr. Br. J. Cancer (in press).

3. LEDERMANN, J.A. Radiolabelled antibodies for cancer therapy. 1989 Hosp. Update 56-66.

4. LEDERMANN, J.A., BEGENT, R.H.J., BAGSHAWE, K.D., RIGGS, S.J., SEARLE, F., GLASER, M.G., GREEN, A.J., DALE, R.G. 1988. Repeated antibody therapy in man with suppression of the host response by cyclosporin A. Br J Cancer 58 654-657.

5. LEDERMANN, J.A., BEGENT, R.H.J., BAGSHAWE, K.D. 1988. Cyclosporin A prevents the anti-murine antibody response to a monoclonal antibody in rabbits. Br J Cancer 58 562-666.

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APPENDIX D

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