STUDIES IN TARGETED RADIOThERAPY.

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ABSTRACT.

Advances in the treatment of patients with cancer, have come from the use of relatively toxic agents. Therapists are constantly searching for ways to reduce the side effects of treatment, whilst maintaining therapeutic efficacy. One route to this objective is to attempt site specific delivery of a cytotoxic agent. This thesis examines the potential of two methods of targeting radiotherapy; monoclonal antibodies, and a small molecular weight radiopharmaceutical $^{131}$I meta-Iodobenzylguanidine (mIBG).

The data presented in this thesis characterises the pharmacokinetics of a monoclonal antibody UJ13A, in children with neuroblastoma. Radiation dose estimates are presented, and a Phase I study of $^{131}$I UJ13A is reported in children with relapsed neuroblastoma.

A number of strategies are explored to improve the therapeutic index of $^{131}$I UJ13A. These include methods to improve quality control of the administered radiopharmaceutical, and attempts to reduce whole body irradiation and immunogenicity by the preparation of radiolabelled antibody, $F(ab')_2$ and Fab fragments.

Changes in the biodistribution of UJ13A and its fragments are explored in the common marmoset, and correlated with changing levels of anti-murine immunoglobulin. Advantages for the antibody fragments are established, although problems are identified in
preparing a stable $F(ab')_2$ digest. Methods for improving the stability and purity of the $F(ab')_2$ digest are explored.

An alternative vector of $^{131}$I is available in neuroblastoma. MIBG accumulates in greater than 90% of neuroblastomas at diagnosis. A direct comparison of the pharmacokinetics of mIBG to whole immunoglobulin is undertaken in both the marmoset and patient population. The data demonstrates the wider tissue distribution of mIBG, and predicts a lower bone marrow dosage at equivalent doses of $^{131}$I. The validity of this result is tested for mIBG of differing specific activity in the marmoset model.

One theoretical method for improving the therapeutic index of $^{131}$I monoclonal antibody therapy, is to restrict its application to a clinical situation where tumour is confined to a body cavity. This is investigated in a study of intrathecal administration of $^{131}$I monoclonal antibody, as treatment for leptomeningeal tumour. The pharmacokinetics and biodistribution of intrathecally administered conjugate is reported for patients and animal models, and a Phase I study of $^{131}$I monoclonal antibody is described for 15 patients with neoplastic meningitis.

Problems with a specific monoclonal antibody UJ181.4, are reported and an alternative monoclonal antibody M340, is described and characterised for clinical use in a new Phase I/II study of intrathecal, radiolabelled antibody in relapsed medulloblastoma.
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CHAPTER 1.

Introduction.
Cure, the ultimate therapeutic goal in cancer, is achieved by a series of cytoreductive steps which lead to the destruction of all clonogenic cells. Three principle treatment modalities are available - surgery, chemotherapy and radiotherapy. If disease is localized, treatment may be directed at the site of tumour growth. Unfortunately, neoplasms are frequently disseminated at the time of clinical presentation, necessitating a systemic approach to therapy. The agents available for widespread tumour control are not tumour specific and are capable of inducing widespread cellular damage. Therefore, the aim of the oncologist is to devise therapy that is both effective and tolerable.

Site specific delivery of cytotoxic agents.
One method of increasing this "therapeutic index" is to selectively localise cytotoxics to tumour cells. This approach to treatment is an area of major research interest. The strategies under investigation include the exploitation of physiological mechanisms that are either highly expressed in tumour tissue, or result from increased metabolic needs at tumour sites. The idea is exemplified by the use of radioactive iodine for the treatment of thyroid carcinoma. Iodine is a normal constituent of the thyroid hormones. As a consequence, thyroid tissue contains a specialised mechanism for
trapping, transporting and incorporating iodine. This mechanism is frequently preserved in metastatic thyroid carcinoma and may be exploited therapeutically by administration of repeated courses of high dose $^{131}$Iodine ($^{131}$I) (1)(3).

A similar philosophy underlines the investigation of the radioactive isotope of Strontium, $^{89}$Sr for therapy of bony metastases of prostatic carcinoma. Irradiation of these metastases by $^{89}$Sr does not rely on incorporation of isotope into tumour tissue, but in the metabolic requirement of osteoblasts for calcium at sites of bony destruction. $^{89}$Sr, like calcium, belongs to the alkali earth grouping and is distributed and metabolized in vivo by mechanisms normally utilized by the calcium ion. (2) Intravenous administration of the $^{89}$Sr results in an increased skeletal uptake of the isotope, with highest concentration at sites of osteoblast activity.

Both elements have been used successfully in patients with metastatic tumours. A combination of surgery and treatment with $^{131}$I has produced a remission rate of 70% in patients with differentiated thyroid carcinoma (3), whilst $^{89}$Sr is reported to palliate bone pain in between 80 and 90% of patients. (4)

Few tumours have such a high affinity for a therapeutic agent per se. Broadening the application of the approach requires modification of the cytotoxic agent to encourage site delivery. Two major areas of investigation are underway. These are the linking of a variety of active agents to a passive, tumour specific
delivery system, or the packaging of cytotoxic agents to alter their distribution characteristics.

**Colloidal delivery systems.**

The latter approach may be achieved by the incorporation of drugs in a colloidal carrier. Various colloidal systems have been investigated, including the use of phospholipid vesicles (liposomes) (5), emulsions (lipid microspheres), (6,7), polymeric microspheres and natural carriers such as cells and low density lipoproteins.

The success of this venture has been limited by the range of potential target sites. After intravenous administration, the fate of the colloidal system is governed by both particle size and surface charge. Above a diameter of 7um, particles are rapidly filtered out by the lung capillaries (8). Consequently, the repertoire of particles above this size is limited to elements of the reticuloendothelial system and lung. Some flexibility within the system may be provided by altering the surface charge of the microsphere. This may be achieved by coating polystyrene microspheres with a surface layer of the polymer Poloxamine 908, which reduces the tissue accumulation of microspheres and prolongs vascular carriage. Coating the same particle with poloxamer 407 targets the particles almost exclusively to bone marrow. (8) This work has resulted in the production of a number of different microspheres with characteristic distribution patterns.
Progress has been made, by exploiting the natural metabolic fate of the liposome. Fidler et al. recognized the limitations of the system and have concentrated on using colloids to target tissue macrophages (9). This is of particular interest as a method for delivering macrophage activating immunomodulators. In vitro, the authors have demonstrated that peripheral blood monocytes are activated by liposomally entrapped lymphokines or the macrophage activators MTP-PE (N-acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanyl-2-(1',2' dipalmitoyl)-sn glycerophosphoryl-ethylamidie, and MDP (N-acetylmuramyl-L-alanyl-D-isoglutamine). The cytotoxic properties of these monocytes have been assessed against tumour and nontumour allogeneic cellular populations. Under conditions of co-cultivation, activated monocytes selectively lysed neoplastic cells with no demonstrable effect on the normal cell population. In further studies, the authors have shown that the monocytes obtained from the blood of patients with colonic carcinoma may be activated in vitro with a variety of liposome encapsulated immunomodulators.

The methods have been studied in vivo by investigating the in situ activation of mouse alveolar macrophages. Following intravenous administration of liposomes containing the immunomodulators MDP or MTP-PE, alveolar macrophages (AM) were harvested by pulmonary lavage. Cytotoxicity was assessed against a target cell of syngeneic melanoma. An improvement in AM mediated cytotoxicity of between 30 and 45% was demonstrated in animals receiving the liposome entrapped immunomodulator,
compared with those animals receiving the same liposome suspended in a solution of the immunomodulator. The efficacy of the approach was then tested in the same animal system in which the syngeneic melanoma was allowed to proliferate and metastasize to lung. All control animals were dead within 90 days of injection of the melanoma, whilst 60% of those animals administered liposomally entrapped lymphokines or MDP were alive when the experiment was terminated at 200 days.

These studies suggest that there is potentially a useful role for colloidal delivery systems in a restricted area of oncological therapy. They may be particularly useful in the development of biological response modification.

**Polyclonal antibody conjugates.**
The alternative approach is to link cytotoxic agents to a passive delivery system. The earliest methods of targeting therapeutic agents have come from linking them to antibodies raised against tumour. The work was initiated with the development of polyclonal antisera and has continued with both polyclonal and monoclonal antibody preparations. This has resulted in the production of a large number of potentially therapeutic conjugates, including isotopes of iodine (10), yttrium (11) and astatine (12); drug conjugates of vindesine (13), methotrexate (14), and novel agents such as plant and bacterial toxins (15).
Polyclonal antibodies were the first vectors used for radiation targeting. As early as 1948, Pressman demonstrated that antibodies could be radiolabelled without loss of biological activity (16). In a study that was interested in investigating tissue sites of antibody accumulation, both rabbit nephrotic anti-kidney serum and anti-ovalbumin were successfully radiolabelled with $^{131}$I at a 4:1 molar ratio of iodine to protein.

The concept of using isotopes as tracers for biologically active proteins was extended to the field of oncology in 1953. Pressman showed that the radiolabelled globulin fraction of an antiserum prepared against the Wagner osteosarcoma, localised the tumour in rats bearing the tumour (17). Further work suggested that localization was dependent on the specificity of the antisera (18). In studies with rats bearing the Murphy lymphosarcoma, animals were simultaneously administered $^{131}$I/immune and $^{133}$I/non-immune globulin fractions. By resecting the tumours and counting for both isotopes the authors demonstrated specific accumulation of the $^{131}$I radiolabelled tumour antiserum. In addition to demonstrating specificity, the authors described an uneven deposition of isotope within tumours. Commenting on the relationship between isotope deposition and blood flow the authors suggested that, "Vascularity and blood flow are of prime importance in the localization of antibody, since specific clearance of the localizing
antibody from the blood depends on the passage of the bulk of injected antibody past the antigen".

Investigation and extension of these ideas into clinical practice began with reports that radiolabelled anti-fibrin antibodies successfully localised in human tumours (19). In a study that looked at the ability of anti-glioma antibodies to image recurrent tumour in patients, Mahaley also quantitated tumour uptake of isotope, and attempted to look at the specificity of isotope accrual using the methods described by Pressman (20). In 10 patients, 0.2 to 9.0 \times 10^{-3} \% of the injected dose/g of tumour was measured in resected tissue, 3-7 days after administration of radiolabelled anti-glioma antibody. Unfortunately, the data on specificity was limited. Firstly, the investigators used human serum albumin rather than a gamma globulin fraction as the non-specific control protein. Secondly, although specificity of uptake was reported in 3/5 patients, actual data was provided on one. In this patient the specificity ratio appeared to be of the order of 12:1.

Both of these early studies utilised crude tumour extracts for generating polyclonal sera. With the characterisation of a number of tumour associated antigens such as human chorionic gonadotrophin (HCG), alphafoetoprotein (AFP) and carcinoembryonic antigen (CEA), interest was developed in generating more specific antisera. In 1973, Primus raised antisera in goats against the purified glycoprotein of CEA (21). Though antisera were only partially purified, (prepared by S200
and DEAE cellulose column chromatography), 2.86^+/- 0.34 of the injected dose/g. of tumour specifically localised to human choriocarcinoma xenografted in cheek pouches of Syrian hamsters.

Purity of the antisera was improved by the introduction of affinity chromatography. Using affinity purified, anti-CEA antibody, Mach investigated immunolocalisation in a variety of human colon carcinoma xenografts established in the nude mouse (22). In this model system, he demonstrated both the specificity of isotope localization and achieved levels of 17.3% of the injected dose/g. of tumour.

The affinity purified antisera, raised against tumour associated antigens were extended into clinical trial, initially for imaging and then for therapy. The first major study came from Goldenberg and colleagues. On a background of experimental work, using the GW39, CEA expressing tumour, xenografted into the cheek pouch of Syrian hamsters the group demonstrated; 1) a clear advantage in using affinity purified material, 2) specificity of antibody localization, 3) detection of tumours as small as 0.07g by scintigraphy, and 4) a reciprocal relationship between mass of tumour and specificity (23,24).

Using the same affinity-purified sera, Goldenberg and colleagues reported the results of imaging in a series of patients with tumours expressing CEA (25). These included patients with carcinomas of the colon, breast, uterus and cervix. Using the technique of blood pool
subtraction, Goldenberg successfully identified 10/11 primary sites and 24/29 secondary sites. In addition 4 previously unrecognised tumour sites were detected by this technique. Tumour uptake was quantified as 2.5x background, and localization occurred despite increased circulating levels of CEA.

These promising results formed the background against which the first therapeutic studies of radiolabelled antibodies took place (10,26,27).

The initial trials from the John Hopkins Medical School used affinity-purified polyclonal sera raised in rabbits against purified ferritin and CEA, radiolabelled with $^{131}\text{I}$. The technique was piloted in patients with a variety of tumours (10). In this study a combined treatment strategy was used. 8 adult patients with primary hepatic carcinoma(4), colonic carcinoma(2), lung carcinoma(1), and a primary tumour involving the floor of the mouth(1), received a combination of external beam irradiation, adriamycin, 5-fluouracil, and metronidazole followed by between 58 and 117 mCi of either $^{131}\text{I}$ anti-CEA or ferritin. The study reported no clinical responses in the extra-hepatic tumours but 3/4 responses in the primary hepatic carcinoma group. However, all 3 responders had demonstrated evidence of response, prior to administration of isotopic immunoglobulin.

The larger phase I-II study of the same multiagent regimen for treatment of primary liver tumours recruited 37 patients. Twenty-seven of these were eligible for treatment and 18 had received between 37-157 mCi of $^{131}\text{I}$
immunoglobulin at the time of reporting (27). Of the 18 undergoing therapy only 9 were considered evaluable for tumour response. Six of these patients demonstrated a partial response (> than 50% reduction of tumour volume, by CAT scanning, or a decrease in total liver size by > than 30%) to the treatment regimen.

Major toxicity in both of these early trials was largely haematological. 29% of patients demonstrated a life threatening thrombocytopenia and leukopenia.

A major criticism of these studies was that the radiolabelled immunoglobulin was only one component of a multiagent regimen, making it impossible to be certain of its exact contribution. In addition there was no direct evidence that the accumulation of the radiolabelled antibody within the liver was a specific phenomenon. This latter point was clarified in an experiment that looked at the selective accumulation of anti-ferritin antibody in the rat hepatoma model. In this system, 2.9 x the radiation dose was targeted to the tumour compared with non specific IgG (28). Moreover the specific binding of the anti-ferritin Ig could be inhibited by injecting an excess of unlabelled specific antibody.

Further support for the therapeutic effect of isotopic immunoglobulin was provided by another study from the John Hopkins group (30). A variety of tumours including neuroblastoma and lymphomas contain ferritin, and in 1985 Lenhard R. and colleagues published a study that looked at the effect of 2 to 4 courses of $^{131}$I anti-ferritin immunoglobulin in patients who had advanced
Hodgkin's disease. Patients were eligible for study if they had relapsed after two different therapeutic trials of "standard" combination chemotherapy and had measurable disease at least 4 weeks from last conventional chemotherapy (6 weeks if the last drug regimen included a nitrosourea). Therapy was given in a divided dose of 20 and 30 mCi on day 0 and 5. Tumour response was evaluated at day 56. Patients with evidence of a tumour response or who had static disease were offered an additional 2 courses of $^{131}$I anti-ferritin. Implicit in the study design was recognition of the potential immunogenicity of the immunoglobulin, so 2nd and 3rd cycles of therapy were obtained from animals of different species. 15 of 37 patients demonstrated an objective tumour response. Of these 1 patient achieved a complete remission. The major response was seen following the initial infusion of antibody, with only 2 patients gaining further benefit from a second cycle of treatment. Toxicity was similar to that observed during therapy of liver cancer with 50% of patients developing thrombocytopenia. However, in only 4 of 36 patients did the platelet count fall below 20,000, and no life threatening haemorrhages occurred. The authors commented that this result was comparable or superior to other reported phase II, single agent drug studies in heavily pretreated relapsing Hodgkin’s disease.

These early landmarks in the development of targeted radiotherapy established the methodology for evaluation of different conjugates. In particular, they emphasised
the need to establish specificity of isotope accumulation, documented the impact of vascular flow on tumour uptake, and pioneered the whole area of immunoscintigraphy and immunotherapy.

**Monoclonal antibodies.**

Following the widespread adoption of hybridoma technology (31) to produce monoclonal antibodies, many investigators chose to develop these as potential therapeutic agents.

The production of polyclonal sera results in a composite product that varies in successive preparations. The purified serum contains a number of antibodies derived from different lymphocyte clones, of differing affinities and directed against different epitopes. Whilst these characteristics are not necessarily disadvantageous, an advantage was perceived in selecting monoclonal antibodies of high affinity and tumour specificity. This has resulted in a considerable investment in the production and investigation of monoclonal antibodies for targeting tumours.

Three sources of monoclonal antibody are available: mouse, rat and human. Studies are most advanced with mouse monoclonals and have followed a similar pattern of development to the polyclonal work. The ease of hybridoma technology has resulted in a plethora of antibodies produced against a large number of tumour associated antigens. Selection of antibodies for tumour targeting has been initially based on their *in vitro* and *in vivo* specificity. No antibodies have been identified
which are entirely tumour specific. Instead, antibodies have been selected that detect oncofoetal antigens or with antigens that have a restricted expression on normal tissues.

Most workers have chosen to evaluate the potential efficacy of the monoclonal antibody in a variety of in vitro and in vivo model systems. Cell cultures have proven a particularly useful medium in which to evaluate the cytotoxic effects of unconjugated monoclonals. Eighty-seven murine monoclonal antibodies of differing isotype were studied for their ability to destroy human tumour cells in a large study by Herlyn et al. (32). The selected antibodies were immunoreactive with either melanoma, carcinoma of the gastrointestinal tract, breast or lung carcinoma. The in vitro cytotoxicity of each monoclonal antibody was investigated against human tumour cell lines derived from the appropriate tumours. Both murine and human effector cells were utilised for assessment of antibody dependent cellular cytotoxicity (ADCC) and rabbit and human complement in assays for complement dependent cytotoxicity (CDC). Characteristic patterns of reactivity were identified for antibodies of differing subclasses and were independent of target cell type. Antibodies of all IgG subclasses were able to mediate ADCC, although only IgG2a and IgG3 antibodies were capable of mediating this effect with all the effector cell types studied. The superiority of the IgG2a isotype over antibodies of the IgG2b and IgG1 isotype in inducing ADCC has been confirmed in a variety
of human and murine systems. Using antibodies directed against different epitopes and spontaneous switch variants from the same hybridoma, the relative strength of ADCC reactions has been assessed as IgG_{2\alpha}>IgG_{2\beta}>IgG_{1} (33,34,35). Herlyn et al. found only the IgM class capable of complement fixation but subsequent studies with some IgG_{3} antibodies suggest that this subclass is also capable of CDC (36).

The relevance of this in vivo work has been explored in a variety of murine model systems. Further work from Herlyn's group has indicated that selection of monoclonal antibodies mediating ADCC in vitro, inhibited the growth of tumour in vivo (37). Using the nude mouse model, animals were inoculated with human colorectal carcinoma subcutaneously. This was followed by an intraperitoneal injection of ascitic fluid containing a relevant or irrelevant antibody. Tumour growth was successfully inhibited in the experimental group although not in the control.

Similar effects have been reproduced in vivo in a variety of solid and primary haematological malignancies including the AKR transplantation mouse leukaemia (38), spontaneous ASL.1 and chemically induced EL4 murine leukaemia (39), neuroblastoma (40), and melanoma (41).

These results are fundamental to the assessment of monoclonal antibodies as possible passive delivery systems for other therapeutic modalities. Taken together, the studies suggest that specific antibodies are capable of tumour suppression, especially when
antibodies are administered within hours of innoculation of tumour cells. Delaying administration of the antibody appears to limit the efficacy of treatment, suggesting that tumour volume or other factors related to an established cell population are important in effecting therapy (39,40). Whilst these results would suggest that unlabelled antibodies are unlikely to have a major therapeutic impact in the presence of established disease, they counsel caution in interpreting clinical trials of targeted therapy.

Studies with radiolabelled antibodies have focused on establishing a) the specificity of tumour accumulation of isotope, b) factors influencing isotope accrual and subsequent therapy. Houston et al. demonstrated the specificity of a monoclonal antibody against the lymphocyte Thy 1.1 antigen in vivo, by comparing uptake of radiolabelled antibody in lymphatic tissue of phenotypic variants of the mouse (42). Mice differing in their ability to express the Thy 1.1 phenotype were administered radiolabelled anti-Thy 1.1 monoclonal antibody. By 20 hours after administration of the radionuclide, experimental animals had 9 x the concentration of radiolabel in lymphatic tissue compared with control mice. In a second experiment which matched monoclonal antibody with non-immune murine IgG the ratio of monoclonal antibody to non-specific IgG was 475:1 at 20 hours.

Similar results emphasising the specificity of isotope accumulation has been reproduced in a variety of
human tumour xenograft systems, although the high specificity ratios documented in this early study have not been achieved. Using an immunosuppressed CBA/lac mouse bearing a human malignant teratoma xenograft, Moshakis et al demonstrated specific localization of an IgG\textsubscript{2a} monoclonal antibody LICR-LON/HT13. The "localization index" of specific to non-specific antibody increased with time, from approximately 2:1 at 24 hours to 10:1 at 96 hours. Evidence for the specific nature of antibody/tumour interaction was provided by the ability of excess cold immunoglobulin to inhibit tumour uptake of isotope (43).

Similar studies have been undertaken for a variety of other monoclonal antibodies (44,45,46). They point to a relatively high but variable tumour uptake of isotope, lying between 1 and 9% of the injected dose. These studies have been extended to investigate the effect of a number of variables on tumour accumulation of isotope, including the selective accumulation of antibody "fragments", i.e F(\text{ab'})\textsubscript{2} and Fab protease digestion products (47,48), new radionuclides (49, ), protein dose (51), tumour size and antigen expression (52).

Whilst these studies are particularly useful for assessing the stability of the labelled conjugate they are poor predictors of therapeutic effect in vivo. Tumour resection studies in patients, have demonstrated that whilst it is possible to reproduce the specificity ratios observed in animal model systems, it has not been possible to obtain the absolute levels of isotope.
accumulation (53,54,55). Estimates of the percentage of injected dose gaining access to tumour, irrespective of the monoclonal antibody, is similar in several human studies. All studies have indicated a disappointing level of uptake of approximately $10^{-3}\%$ of tumour (53,56,57,58). This factor coupled with the antigenic heterogeneity of many tumours (59,60) argues against successful targeted radiotherapy with $^{131}\text{I}$ (61).

As a result progress towards clinical trials of targeted radiotherapy has been cautious (62,63,64). Toxicity in these studies have fallen into two categories; radiation effects and problems associated with administration of the antibody itself. Myelosupression in 3/6 patients receiving between 145 and 150 mCi of $^{131}\text{I}$ T101 (64) and 2/10 patients receiving 1 and 3 treatments of 137-192 mCi of the $^{131}\text{I}$ Fab fragment of MoAB anti-p97 (63), was probably a result of radiation exposure. However the occurrence of urticaria, rash, (63) fever, pruritis and dyspnoea (64) are likely to represent hypersensitivity.

Insight into the probable frequency of side effects is provided by studying the results of studies that have concentrated on antibodies as potential biological response modifiers. The majority of these investigations are in patients with haematological malignancy (65,66,67), although a small number have reported results with selected solid tumours (68,69).

Dillman et al. reported the toxicity of intravenous monoclonal antibodies in 82 patients with 10 different
malignancies (70). The vast majority of antibody infusions were performed with antibodies of the IgG\textsubscript{2a} isotype, in doses ranging from 2.5mg to 500mg of antibody. Reactions of some degree were documented in 27 patients (33%), and in 57 (31%) of infusions. Most side effects occurred when antibodies were administered that reacted with circulating cells. Thus, toxicity was seen in 20/82 of the first infusions where antibodies detected an antigen expressed on circulating cells, compared with 0/55 for patients without this characteristic. Consequently, no toxicity was observed in patients administered antibody for therapy of solid tumours, except in the use of the anti CEA MoAb 35, which was also weakly reactive with granulocytes.

The numerically significant toxicities were fever, diaphoresis, rigors and pruritis, which occurred in 12-16% of administered courses. Severe reactions of anaphylaxis (1 patient) and bronchospasm (2 patients) were seen infrequently. The author noted that toxicity occurred in all patients administered antibody at a rate greater than 0.5-1mg of protein/min, suggesting that dose rate may also be an important factor in inducing toxicity.

The range of side effects is similar to that reported in a variety of other studies (66,71,72). However, experience with the antibody 3F8 in patients with melanoma and neuroblastoma suggests that these toxicities may occur without circulating target cells (68).
A second feature highlighted first in clinical trials with unlabelled antibody, and then with the use of radiolabelled protein, has been the induction of an anti-mouse immunoglobulin (Ig) in the host (73,74,75). Schroff et al. reported on the emergence of anti-mouse Ig response in the completed National Cancer Institute of America phase 1 clinical trials of MoAb T101 in patients with chronic lymphocytic leukaemia and cutaneous T cell leukaemia (CTCL) (71), and the MoAb 9.2.27 in patients with malignant melanoma. This report documented the presence of pre-existing mouse IgG reactive antibody in the serum of a proportion of patients and healthy controls. Following administration of the monoclonal antibody, all patients with CTCL and 30% of patients with melanoma developed elevated titres of anti-mouse Ig. The responses occurred rapidly and were principally directed against the Fc component of the monoclonal antibody. In only one patient was the response directed against the F(ab')2 portion of the molecule.

In an expanded analysis of this data, Shawler et al looked specifically at the anti-murine response of those patients receiving MoAB T101 (74). This work suggested that the anti-idiotypic component of the anti-murine Ig response rose steadily with the number of antibody exposures. A similar result has been obtained in the use of radiolabelled antibodies (75). The significance of these results depend on the strength of the anti-idiotypic response for different monoclonals. It is likely that a variety of strategies will be required to circumvent this
problem, depending on the immunodominance of the monoclonal antibody.

There is but scanty clinical data on the efficacy of radiolabelled monoclonal antibodies. Setting aside the specialised use of intra-cavity administration, reports were limited to the use of radiolabelled fragments in melanoma and radiolabelled T101 in lymphoma. The 3/6 responses to $^{131}$I/T101 have been partial and of short duration (1-2 months). As the monoclonal antibody has also been used in unconjugated form, the results are disappointing. Of the 10 patients selected for therapy in the series from Carasquillo (63), 2 had any objective tumour response. Of these, only 1 achieved a true partial response with a reduction of $50\%$ in tumour volume. The major problems encountered were rapid emergence of human anti-mouse Ig response, variability in tumour uptake of antibody, hepatic accumulation of isotope, complexing of antibody with tumour shed antigen and in vivo deiodination of the radiolabelled protein.

These results do not suggest any special advantage of monoclonal antibodies over affinity-purified polyclonal sera. This probably reflects the importance of the vascular barrier in limiting access of conjugate to tumour as well as host interaction with biologically active proteins i.e immune response, complexing with tumour shed antigens. With respect to the problem of immune response, cycling of polyclonal sera from different species may "weigh the balance" in favour of polyclonal antibodies.
A variety of strategies are available to circumvent these problems. They include both manipulation of the delivery system, of the therapeutic agent and identification of appropriate clinical situations where targeting may result in some advantage.

**Rationale for targeting radiotherapy in neural tumours.**

**Monoclonal antibodies and neuroblastoma:**

Interest in using monoclonal antibodies to target therapy in neural tumours stem partially from frustration with conventional methods of treatment. The paediatric tumour neuroblastoma, is a neoplasm that is frequently disseminated (Stage IV) at presentation. Whilst the tumour is highly chemo- and radiosensitive, only 25% of children with Stage IV disease survive beyond 2 years from diagnosis (76,77). A variety of strategies are being investigated to improve survival, including consolidation of clinical remission with "megatherapy" (78), and maintenance of clinical remission with new agents such as retinoic acid. To date, pilot studies of these approaches do not suggest any major breakthroughs.

The apparent radiosensitivity of the tumour (79,80), has led a number of groups to include total body irradiation (TBI) in the treatment regimen. The short and long term toxicity of TBI in heavily pretreated children has encouraged the development of experimental approaches to radiation delivery.

The use of monoclonal antibodies to target treatment in paediatric tumours has been of interest to several
research groups. Two of these have concentrated on exploring this therapy in neuroblastoma, using antibodies designated UJ13A and 3F8. The monoclonal antibody UJ13A was first described by Allan et al. in 1983 (81), and was raised following immunisation of Balb/c mice with 16 week human foetal brain. Fusion of splenocytes with the murine myeloma resulted in the production of a monoclonal antibody of IgG\textsubscript{1} isotype. The potential of the antibody for radiation targeting was indicated by the restricted expression of the UJ13A antigen in normal tissue. The antigen was most strongly expressed in central and peripheral nervous tissue, including retina. In contrast more than 90% of neuroblastoma specimens obtained at either diagnosis or after chemotherapy were shown to express the antigen.

In 1984, Goldman reported from this institution that radiolabelled UJ13A identified tumour deposits in children with neuroblastoma (82). Nine patients received between 0.9 and 2.8mCi of $^{131}$I UJ13A or 2.0 to 2.5 mCi of $^{123}$I UJ13A and were then imaged sequentially over a time period of 7 days. No uptake of isotope was seen in the brain or eye despite the high normal expression of the UJ13A antigen in these tissues. This was explained by the exclusion of the antibody from these sites by an intact blood-brain or blood-retinal barrier. The six primary tumour sites detected by conventional means (ultrasound, CAT scan and plain X-Ray), were all demonstrated by immunoscintigraphy. Isotope accrual was demonstrated in 10 other sites, 7 of which were known to
be sites of disease. Of the remaining 3 abnormal areas on immunoscintigraphy, 2 proved positive by further "conventional" investigation. These promising results led the authors to continue investigation of the antibody as a radiation targeting agent.

Using the nude mouse model, xenografts of the human neuroblastoma cell line TR14 were established. Dual label studies were undertaken in this model system with $^{131}$I UJ13A and the irrelevant monoclonal antibody $^{125}$I FD44 (83). Whilst there was considerable variation in the accumulation of antibodies in different tumours, the median ratio of UJ13A to FD44 was 7:1. This translated into a median of 6% of the injected dose per gram of tumour at 24 hours after injection.

Using increased quantities of $^{131}$I conjugated to UJ13A (150uCi and 10ug of protein), regression of xenografts in nude mice was seen. Over a 21 day period, tumours of approximately 1cm$^3$ regressed to 10% or less of their original volume. Repeat injections of conjugate resulted in the apparent ablation of the tumours, although regrowth of the tumour at the primary site always occurred. Recurrent tumours still expressed the UJ13A antigen. These results could not be obtained with a 10 fold excess of antibody or $^{131}$I alone. A 50 fold excess of cold antibody successfully blocked the anti-tumour effect of radiolabelled antibody, and suggested a specific effect of the conjugate.

Similar studies were undertaken using the antibody 3F8. This monoclonal antibody was raised against human
neuroblastoma by hyperimmunisation of Balb/c mice and subsequent fusion of splenic cells with the SP-2 mouse myeloma (84). The antibody has been characterised as an IgG₃ monoclonal recognizing the ganglioside GD2. This antigen, like UJ13A is pan-neuroectodermal in distribution and has a similar frequency of expression on human neuroblastoma (85). It differs significantly from UJ13A in its ability to fix human complement (36).

In a similar study to that reported by Jones et al, Cheung reported that ¹³¹I radiolabelled 3F8 consistently produced tumour shrinkage in the nude mouse model, at doses of 1mCi of isotope (86). The effect was dose dependent and did not occur when antibody was administered alone. Recurrence of tumour by 3 weeks after injection of conjugate was also documented. On the basis of immunoscintigraphy, it was felt that this was not due to the emergence of 3F8 negative clones. Nor did there appear to be selection for radioresistant clones as repeat injections resulted in further tumour shrinkage.

Clinical studies with radiolabelled 3F8 parallel the experience of other investigators in the field. Six patients with neuroblastoma, pretreated with chemotherapy were imaged with ¹³¹I 3F8. Using a higher administered dose of radiolabel than reported by Goldman et al, 3F8 localised 1/2 bulky abdominal deposits (87). The remaining data is not presented by site and so it is difficult to evaluate the sensitivity and specificity of ¹³¹I 3F8, however the data suggests immunoscintigraphy broadly reflected the expected pattern of disease.
Cheung et al. also hoped that 3F8 might be useful for therapy, and in this context several important observations were made. Like UJ13A no uptake in brain was demonstrated. Unlike UJ13A rapid dehalogenation of the conjugate was observed with rapid appearance of isotope in stomach; however, no significant reticuloendothelial uptake was seen. In 2 patients who had undergone biopsy of their tumour 3 days after administration of isotope, only 0.019 and 0.0045% of the injected dose/gm of tumour was measured. Autoradiography of the tumour suggested that the distribution of isotope in tumour was patchy.

To date, reported clinical use of 3F8 has centred on its use as a biological response modifier (68). Relying on the ability of 3F8 to fix human complement the authors have administered between 5 and 100mg/m² of unlabelled protein in patients with recurrent or progressive melanoma and neuroblastoma. The major problems following this treatment has been the induction of hypertension, focal urticaria and severe pain which has not been dose dependent. Of the 8 patients with neuroblastoma, 1 patient with disease limited to bone marrow, and 1 patient with bone marrow and bony disease underwent a complete response to therapy.

This clinical experience with antibodies 3F8 and UJ13A reflects the outcome of work in other tumours and suggests that neuroblastoma is unlikely to be a "special case" so far as radiation targeting is concerned. This thesis concerns itself with the development of radiation
targeting in neuroblastoma and extends the work initiated by Goldman, Jones and others.

An area of particular interest is the use of antibody fragments. Methods have been established for the enzymatic digestion of whole antibody to produce both F(\text{ab}')_2 and Fab species. Several possible advantages are perceived in their use. The Fc portion of the antibody governs several important functions of the protein. It is responsible for immunoglobulin interaction with the reticuloendothelial system, for secondary biological functions (such as complement fixation) and plays an important part in defining an antibody’s rate of clearance. Removal of the Fc component by fragment preparation results in a more rapid whole body clearance of isotope. This may be clinically advantageous by reducing the whole body radiation dose. In addition, legitimate concerns regarding the contribution of ADCC and complement fixation to therapeutic response are diminished. Further benefit may be derived from a reduction in the immunogenicity of the monoclonal antibody. Several authors have demonstrated that during initial exposures, the immunodominant portion of the antibody is the Fc component. A reduction in the host response to murine protein should allow a more flexible approach to dose scheduling.

**Meta-Iodobenzylguanidine and neuroblastoma.**

A new dimension to the targeting of radiotherapy in neuroblastoma has been added by the introduction of meta-
iodobenzylguanidine (mIBG) as an imaging and therapeutic agent (88,89). This compound is an iodinated aromatic analogue of the hypotensive drug guanethidine (90). The compound is stored in sympathetic tissue and a variety of chromaffin tumours (91,92,93). Work with the human neuroblastoma cell line, SK-N-SH, and 16 human phaeochromocytoma cell lines indicate that intracellular accumulation of mIBG may take place via an active transport mechanism (94,95). The relevance of these findings for patients with chromaffin tumours has been the subject of intense investigation. A variety of authors have documented the value of the $^{123}$I or $^{131}$I radiolabelled compound as sensitive and specific agents for detection of phaeochromocytoma and neuroblastoma (96,97).

In patients with neuroblastoma it would appear that approximately 95% of tumour deposits may be detected with $^{123}$I mIBG at disease presentation (98). Factors influencing tumour accrual of isotope may be complex, as imaging sensitivity appears to fall to around 70% after patients have been treated with chemotherapy.

Lack of appropriate tumour models has made it difficult to investigate experimentally aspects of tumour accrual and persistence of mIBG. Of the human neuroblastoma cell lines, only SK-N-SH has been reported to accumulate mIBG in vitro (94), but unfortunately, the cell line has not xenografted efficiently. The result has been a rapid evaluation of the radionuclide in clinical trials.
A number of investigators have piloted the use of this method of targeting $^{131}$I. The studies have been variable in their construction and execution, with the result that clinical response and reported toxicity has also varied. At one end of the scale, Hartmann et al. could not demonstrate any objective tumour response in 8 children receiving $^{131}$I mIBG (99). All children had been extensively pretreated and had relapsed after intensive chemotherapy or had chemoresistant disease. At administered doses lying between 1.1 to 4 GBq all patients suffered marrow toxicity.

Schwabe et al. have employed a different strategy, and have administered sufficient radiolabel to administer a whole body dose of 200 rads or a liver dose of 500 rads. This has resulted in the administration of multiple courses of up to 280mCi of isotope at any one course. Of 11 patients, 1 patient with marrow disease entered a complete remission. This was sustained with further therapy and bone marrow transplantation. A further 6/11 patients had a partial response (PR) to treatment, where PR was defined as regression of tumour by 1/3 of its original volume (100). Similar results have been reported from Voute et al (101). In the Dutch series of 18 patients 2 patients had a complete response to multiple high doses of mIBG. In addition a further 5 patients had a 50% reduction in tumour volume. The associated toxicity has been largely haematological with thrombocytopenia dominating the clinical picture. A similar pattern of toxicity was documented in the series
from Schwabe et al. These results have been supported from a number of small series from additional centres (Klingebiel Th. et al., 6/8 responses (102), Claudiani F. et al., 4/4 responses (103), Bestagno M. et al., 1/4 responses (104), Troncone L. et al., 2/4 responses (105). These encouraging results in patients with relapsed neuroblastoma suggest that iodine 131 delivered appropriately may result in an objective tumour response.

Several directions are suggested by these studies. Further work is required to define the place of mIBG clinically. Optimizing the dose scheduling of $^{131}$I mIBG requires a more detailed understanding of the behaviour of the compound in vivo. Larger phase I and II clinical trials may then be constructed in rational way. A continuing research effort is required to identify suitable model systems in which to investigate tumour factors influencing mIBG accumulation. Lastly, the introduction of mIBG raise the question as to whether monoclonal antibodies are ever likely to have any role to play in radiation delivery in neuroblastoma.

This thesis is directed at the first and last of the issues raised. The biodistribution of mIBG is both defined and compared with the available monoclonal antibody UJ13A.

**Targeted radiation therapy and other neural tumours.**

The introduction of mIBG offers a realistic alternative to monoclonal antibodies as a vector for targeted radiotherapy in neuroblastoma. However, for the majority
of tumours no such option exists and monoclonal antibodies remain the sole existing vectors for tumour targeting. An alternative approach to antibody modification, is to select out specific clinical situations which lend themselves to enhanced tumour uptake of antibody. These may be viewed in two distinct categories. Firstly, where there is loss of the usual "tight vascular junctions" in tumour and secondly, where tumour is limited to an accessible body cavity.

The first set of circumstances occurs where arteriovenous connections are made through sinusoids rather than through a capillary network. Thus it is possibly easier to target tumour in liver, bone marrow and the vascular compartment, than at other sites. The early success with isotopic immunoglobulin in hepatoma may reflect such a situation (10), as may the clearance of neuroblastoma from bone marrow in the series reported by Cheung (68).

The second approach was pioneered by the Hammersmith Hospital Oncology Group (106). The authors rationalised that administration of antibodies into a restricted space would result in a higher tumour to normal tissue ratio. The specificity of the targeting approach would ensure that therapeutic results would be superior to those obtained with non-specific agents such as colloidal gold. The first three patients treated in this way had a malignant effusion in 3 different body cavities. The first patient had a pleural effusion secondary to an undifferentiated adenocarcinoma, the second a pericardial effusion due to extension of a primary squamous cell
tumour of lung, and the third a malignant ascites from ovarian carcinoma. Each patient was given 20 mCi of $^{131}$I radiolabelled monoclonal antibody HMFG2 into the relevant serous cavity. No adverse reactions were documented and all three patients had some evidence of response.

The group has subsequently concentrated on the development of this form of therapy for treatment of ovarian carcinoma (107). A series of 24 patients with persistent epithelial ovarian cancer after surgery and chemotherapy, were reported in 1987. These patients had varying levels of disease, and were categorised as having "minimal residual disease", defined as less than 2 cm in diameter, and "macroscopic disease", greater than 2 cm in diameter. Patients received a variety of monoclonal antibodies, as the vector of $^{131}$I. Doses were escalated from 20 mCi of radioactivity to 205 mCi. At these dose levels, toxicity was reported as acceptable, with pyrexia (7/24 patients) and nausea and vomiting (9/24) being the chief side effects. No bone marrow suppression was reported.

Assessment of response was complicated by a reluctance to perform repeat "second look" laparotomy or laparoscopy. This was crucial, as 2/3 of the patients entered into the study were assessed as having "minimal residual disease" at the time of treatment. However, it was clear that all of the patients with macroscopic residual disease, were dead within 9 months. Nine of those patients with "minimal residual disease", made an initial clinical response to treatment, which was
sustained in 4, 6 months to 3 years from treatment. The authors felt that these results compared well with results from other series in which patients had drug resistant ovarian cancer.

**Targeting in the central nervous system.**

The central nervous system occupies a body cavity with unique features that make it suitable for investigating targeted radiotherapy. Unlike the serous cavities, the CNS has a well regulated internal circulation. The internal and external surfaces of the neural tissue are bathed in circulating cerebrospinal fluid (CSF). The volume of the fluid filled cavity is approximately 150 mls in the normal adult and CSF is constantly in flux with a turnover of approximately 20mls/hour (108).

The introduction of antibodies directly into CSF would appear a more fruitful approach to "targeting" than using the vascular route. Although the CNS is well vascularised, an operational barrier exists to exclude entry of circulating macromolecules. This property is manifest histologically as a continuous endothelium with many "tight junctions" (109). However, in the presence of tumour, there is frequently a regional disruption in the blood brain barrier. These changes in neovasculature have been studied extensively and are characterised by an increased capillary network, an increase in vessel diameter, endothelial fenestration and excessive numbers
of plump endothelial nuclei per circumference of the vessel wall.

To investigate the possibility that regional disruption of the blood brain barrier conferred an advantage for radiation targeting in solid brain tumours, Richardson et al. investigated the uptake of monoclonal antibody UJ13A in human gliomas (110). Eight patients proceeding to surgery because of a clinical diagnosis of glioma underwent a $^{99}$Tc glucoheptonate scan followed by between 0.8 and 2.5 mCi of $^{131}$I UJ13A. The purpose of the glucoheptonate scan was to define disruption in the blood brain barrier. Patients were imaged sequentially over a period of 24 hours for $^{131}$I accumulation at the tumour site. Operative samples of tumour and where possible, normal brain and temporalis muscle were obtained 3-16 days after antibody administration. Immunohistology confirmed that all the tumours expressed UJ13A antigen. Results of the scintigrams following administration of $^{131}$I UJ13A, correlated with the observed regional blood brain barrier disruption. The tumour to brain ratio of $^{131}$I varied between 3.1-12.8. However detailed studies of samples of cyst fluid and necrotic tumour suggested that the isotope was just as likely to be associated with these areas as with viable tumour. The % of injected dose present in areas of histologically viable tumour ranged from $4.3 \times 10^{-3}$ to $3.6 \times 10^{-4}$ %/g of tumour. These results do not differ ostensibly from estimates of antibody uptake following intravenous administration of other conjugates in
patients with solid tumours outside the CNS, and certainly do not suggest that tumour targeting will be enhanced by regional permeability in the blood brain barrier.

Further work, following a similar strategy, but investigating specificity of uptake has been undertaken. Using the "paired label" approach of Pressman and Day, the "irrelevant" antibody HMFG2 was compared with UJ13A. The results confirmed the absolute % uptake of isotope by tumour and documented that antibody accumulation was non-specific (110).

An alternative approach is to select out a sub-population of brain tumours that are both radiosensitive and bathed in circulating CSF. This would permit administration of the radionuclide directly into the thecal space. This technique is theoretically applicable to a significant group of tumours and include primary neural tumours with a propensity to spread along the leptomeninges, such as medulloblastoma and pineoblastoma, and secondary tumour deposits such as leukaemia, Non Hodgkin’s Lymphoma (NHL) and various carcinomas.

These tumours reflect a spectrum of clinical problems associated with their control which make the issue of targeted radiotherapy an interesting proposition. Medulloblastoma is a highly radiosensitive tumour with a tendency to seed along the spinal canal (111). Advances in neurosurgery, post operative care and radiotherapy have resulted in continuing improved survival. A 50% five year survival is now expected but only after high
dose irradiation to the primary site and spinal irradiation (112,113). Despite radiation doses close to tissue tolerance the major cause of treatment failure is lack of local tumour control (114,115). It is unlikely that targeted radiotherapy could make a significant contribution to this aspect of patient care. However, it may be a method of gaining control of spinal metastases either at presentation or relapse.

The involvement of the spinal meninges in leukaemia is almost universal, and is quite frequent in Non-Hodgkin's Lymphoma (NHL). The introduction of first, cranio-spinal irradiation and, later, cranial irradiation and intrathecal methotrexate into treatment programmes resulted in improved survival (116). Radiation alone, and the combined treatment strategy of radiation and chemotherapy have a high short and long term toxicity. Spinal irradiation may result in impaired spinal growth and cranial irradiation with or without methotrexate may result in endocrine, neural and neuropsychological sequelae.

Histopathological studies of both CNS leukaemia and carcinomatous meningitis suggest that the disease is lepto-meningeal until well advanced (117,118).

As many as 10% of carcinomas metastasise to the CNS, of which the largest groups are formed by carcinomas of lung and breast (119). Whilst the majority of these common tumours metastasise as solid, parenchymal tumours, a proportion diffusely infiltrate the meninges (120). Malignant meningitis is both symptomatically unpleasant...
and difficult to treat successfully. Reported median survival with this condition is highly variable lying between 1-6 months (121,122,123). Targeted therapy might offer both palliation and a mechanism for more effective treatment of this site.

The success of the approach is dependent on many factors, some of them outlined in the previous discussion. The specificity of the tumour targeting is particularly important. Intrathecal colloidal gold fell from favour for leptomeningeal therapy due to the high amount of non specific pooling of isotope in the cauda equina. High radiation doses were received by the sacral nerve roots, with resultant late radiation necrosis (124). Using monoclonal antibodies, it might be possible to alter the biodistribution of radionuclide and avoid these effects.

In summary, the main objectives of this thesis are to explore and extend the clinical use of targeted radiotherapy in neural tumours. The study documents the problems associated with the use of intravenously administered monoclonal antibody UJ13A in treatment of neuroblastoma. Strategies for improving targeted radiotherapy in childhood neuroblastoma are explored. These include the preparation of antibody fragments and use of the small molecular weight radiopharmaceutical mIBG. Lastly, in recognition of the problems associated with vascular access to tumour by monoclonal antibody,
radiation targeting is explored in the specialised clinical context of leptomeningeal spread of tumour.
CHAPTER 2.
Materials and Methods.

MATERIALS.

Chemicals reagents.

<table>
<thead>
<tr>
<th>Chemical</th>
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<tbody>
<tr>
<td>Acetic acid</td>
<td>Fisons PLC</td>
</tr>
<tr>
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</tr>
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<td>Difco</td>
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<td>Ficoll</td>
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<td>Worthington Biochemicals</td>
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<td>Sodium tartrate</td>
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Sorbitol (−D)  
Sulphosalicylic acid  
Trichloroacetic acid  
TEMED (N,N,N’-Tetramethyl ethylene diamine)  
Tris (Tris(hydroxy methyl methylamine)  
Trypan Blue

Sigma  
Hopkin and Williams Ltd  
Fisons PLC  
Bio-Rad  
Fisons PLC  
Sigma

Buffers and solutions.

Acrylamide (30% stock solution)  
0.1M Citrate buffer, pH 3  
Ficoll-hypaque  
IEF Polyacrylamide stock solution  
Ouchterlony Coomassie Brilliant Blue stain  
Ouchterlony destain  
Phosphate Buffered Saline (PBS)

30g acrylamide  
0.8g bisacrylamide  
to 100mls with water.

0.1M citric acid  
titrated to pH 3 with  
0.1M Na₂HPO₄

63.53g Ficoll in 600ml  
distilled water (DW)  
133.7ml isopaque  
made to 1 litre in DW.

24.25g acrylamide  
0.75g bisacrylamide  
to 100mls with DW

5g Coomassie Brilliant Blue  
450ml ethanol  
100ml acetic acid  
450ml DW

250ml ethanol  
100ml acetic acid  
450ml DW

8g/l NaCl  
0.2g/l KCl  
0.15g/l Na₂HPO₄  
0.2g/l KH₂PO₄  
pH 7.2
0.2M Phosphate Buffered Saline
pH 7.4

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Polyacrylamide gel Brilliant
Blue stain

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SDS PAGE Running Buffer

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<tr>
<td>Glycine</td>
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<tr>
<td>SDS</td>
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SDS Sample buffer

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<td>Tris pH 6.8</td>
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<tr>
<td>DW</td>
<td>3.1ml</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.01%</td>
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Immunological Reagents

Fluorescein conjugated rabbit
anti-mouse F(ab')2 fragment

Dakopatts

Fluorescein conjugated goat
Ig to mouse IgG1/IgG2a/IgG2b
IgG3/IgM(Fc)

Nordic Immunological
Laboratories Ltd

Goat Ig to mouse IgG1/IgG2a
IgG2b/IgG3/IgGM

Nordic Immunological
Laboratories Ltd

Monoclonal antibodies:

2D1

Dr. P. Beverly, Imperial
Cancer Research Fund.

F8-11-13

Dr. J. Fabre, Blonde
McIndoe Institute.

FD32

Dr. J. Kemshead, Imperial
Cancer Research Fund.

HMFG1

Dr. J. Taylor-Papadimitrou, Imperial
Cancer Research Fund.

HMFG2

Dr. J. Taylor-Papadimitrou, Imperial
Cancer Research Fund.

M340

Dr. J. Kemshead, Imperial
Cancer Research Fund.
Mel.14 Dr. S. Carrel, Ludwig Institute, Lausanne.

UJ13A Dr. J. Kemshead, Imperial Cancer Research Fund.

UJ181.4 Dr. J. Kemshead, Imperial Cancer Research Fund.

81.C6 Dr. D. Bigner, Duke University, North Carolina.

Chromatography materials.

Protein A Sepharose CL-4B Pharmacia Fine Chemicals

Sephadex G25, G50, G75 and G150 Pharmacia Fine Chemicals.

Silica gel TLC Plates Merck.

Radionuclides.

Iodine 123 Harwell Atomic Energy Research (Chemistry Division).

Iodine 125 Amersham International

Iodine 131 Amersham International

meta-Iodobenzylguanidine Amersham International

Culture Media.

Complete Dulbecco’s Modification of Eagles Medium. (DMEM) Flow Laboratories supplemented with 20% heat inactivated FCS, 2mM glutamine 100IU/ml penicillin-streptomycin.

Complete RPMI 1640 medium Gibco laboratories supplemented with 10% heat inactivated FCS, 2mM glutamine and 100IU/ml penicillin-streptomycin
METHODS.


1a. Recloning of monoclonal antibodies by limiting dilution.

A vial of frozen cells was thawed rapidly, diluted and washed in an excess of complete DMEM. The population of cells was expanded in 60mm tissue culture dishes containing complete DMEM supplemented with mouse macrophages. (Obtained from the peritoneal washings of Balb/c mice, at a ratio of 1 washing per 50ml of medium). The proportion of live cells in any volume was calculated by Trypan Blue exclusion, and the expanded population was diluted to give an estimated 1, 3, 5, 10 and 30 live cells per 100ul of tissue culture medium. A 96 well plate was seeded with 100ul of cell suspension at each dilution, and incubated at 37°C. Wells were examined daily for growth, and antibody production (by indirect immunofluorescence). Wells containing antibody producing cells at lowest dilution were chosen for expansion.

1b. BM-cycline treatment of hybridomas.

Mycoplasma positive hybridomas were grown in 60mm dishes containing complete DMEM, supplemented with macrophages and 40ul of BM cycline stock solution 1 or 2, per 10ml of growth medium. Cells were treated with 3 cycles, consisting of 3 days growth in BM cycline 1 and 4 days growth in BM cycline 2.
1c. Monoclonal antibody production in ascites.
Hybridomas were grown in macrophage supplemented DMEM. 5x10^6 cells per mouse were prepared by repeated washing and centrifuging in PBS. Cells were resuspended in 100μl of PBS and injected peritoneally into Pristane primed Balb/c mice (200μl of pristane oil I.P, 10 days before administration of hybridomas). Ascites was collected from the peritoneal cavity and centrifuged at 1,500 x g for 10 minutes.

1d. Freezing of cells.
Cells were washed in complete DMEM, centrifuged at 400 x g for 5 minutes, and resuspended in 1ml of complete DMEM containing 10% heat inactivated FCS and 10% DMSO. After transfer to a sterile freezing vial, cells were placed on top of tissue paper, lying on a bed of solid carbon dioxide. After 2-4 hours, cells were transferred to liquid nitrogen storage facilities.

1e. Trypan Blue exclusion for quantifying the proportion of viable cells.
20μl of a cellular suspension was mixed with 20μl of Trypan Blue vital dye, and loaded into the counting chamber of a haemocytometer. All cells excluding blue dye were counted as viable.
2) Protein purification and analysis.

2a. Preparation of an IgG fraction by ammonium sulphate precipitation.

Saturated ammonium sulphate is prepared by adding 1000g of salt to 1000mls of distilled water at 50°C. The solution was cooled to room temperature, decanted and the pH adjusted to 7.2 with 5M NaOH.

Sufficient ammonium sulphate solution was added to protein solution to give a 45% v/v mixture. The resulting precipitate was stirred at room temperature for 30 minutes, and separated from the residue by centrifugation at 1000 x g for 15 minutes. The precipitate was washed twice in a 45% v/v solution of saturated ammonium sulphate, and re-dissolved in phosphate buffered saline (PBS).

The procedure was repeated using a 40% solution saturated ammonium solution, and the final solution dialysed against PBS at 4°C.

2b. Protein A affinity chromatography for isolation of IgG from a protein mixture (125).

Staphylococcal Protein A, covalently linked to Sepharose CL-4B, was swollen in PBS, for a period of 30 minutes, and packed in a 10 ml glass column. The column was equilibrated in binding buffer*, and a maximum of 20 mg of IgG/ml of gel was loaded onto the column. The column was washed with the binding buffer until no further
protein was detected by optical density at 280nm, in the effluent. Protein A bound IgG was eluted from the column by adding 0.1M Citrate buffer, pH 3.0. The pH of the eluted protein was rapidly adjusted to >6.5 by addition of 0.75M Tris.HCl buffer, pH 8.8.

*The binding buffer for Monoclonal antibody UJ13A was 0.15M PBS, pH 7.2, whilst for UJ181.4 and M340, 1.5M glycine/NaOH.3M NaCl, pH 8.9 was required.

3. Characterisation of the protein product.
3a. SDS Polyacrylamide gel electrophoresis.
A running gel was prepared according to the method of Laemmelli (126), by polymerisation of a 30% stock solution of acrylamide/bis-acrylamide in 0.75M Tris.HCl buffer, 0.2% SDS, pH 8.8, with 15 ul of TEMED and 1ml of a 1mg/ml solution of ammonium persulphate. The concentration of acrylamide in the gel was varied depending on the desired separating conditions. Air was excluded from the gel apparatus during polymerisation, by an overlay of 50% isobutanol. This was removed along with unpolymerised gel at the end of the reaction.

A 3% stacking gel was prepared in 0.25M Tris.HCl buffer, pH 6.8, containing 0.2% SDS 10ul of TEMED and 0.5ml of a 1mg/ml solution of ammonium persulphate. This was poured onto the surface of the separating gel. Immediately after pouring the stacking gel, a 2mm well forming comb was inserted into the unpolymerised liquid. This remained in situ until polymerisation was completed.
The gel slab was placed in an electrophoresis tank, fixed vertically to the electrophoresis apparatus and running buffer was placed in the upper and lower reservoirs. Following loading of protein samples, the gel was run at a constant voltage of 50V through the stacking gel, and 100V through the separating gel.

3b. Preparation of samples for SDS gel electrophoresis. Non-reduced samples were prepared by adding protein to the desired volume of SDS sample buffer and boiling for 1 minute. The protein mixture was centrifuged at 10,000 x g and loaded into sample wells. Reducing conditions were achieved by the addition of sufficient 1M dithiothreitol to the sample buffer, to produce a final concentration of 0.1M.

3c. Fixing and staining of proteins following SDS PAGE. Where the quantity of protein per well was > than 5ug, gels were fixed and stained by overnight immersion in Coomassie Brilliant Blue stain, and then destained by re-immersion in the same solvent solution, minus the Coomassie Brilliant Blue.

When a more sensitive technique was required, gels were silver stained. This was performed by fixing gels for 1 hour in a solution of 10% ethanol/5% acetic acid (2 changes of solution) and then suspending the gel in a 10% solution of oxidising reagent (Bio rad, silver staining kit) for a period of 10 minutes. The gels were washed
thoroughly in double distilled water and stained with a 10% solution of Bio rad silver stain for a period of 30 minutes. Following a further wash in distilled water, the silver stain was developed with 3 changes of proprietary developer. When bands of required intensity were visualised the reaction was terminated by the addition of 5% acetic acid.

3d. Calculation of molecular weights.
To determine the molecular weight of any protein band, a series of protein standards of known molecular weight (Sigma MW SDS-200 kit), were run alongside mixtures of unknown composition. A semi-logarithmic plot of molecular weight against the relative mobility of a protein, produced a standard curve.

3e. Isoelectric focusing of proteins (IEF).
Two methods were used, the first using a polyacrylamide gel and a second using agarose.
Polyacrylamide IEF. 6 mls of stock acrylamide were added to 12 mls of 50% glycerol, 1.5 mls of Pharmalyte (pH range 3-10) and 9.9 mls of double distilled water. The resulting solution was degassed and warmed to 37°C for 10 minutes. 600ul of a 15mg/ml solution of ammonium persulphate was added and the mixture was poured into a Pharmacia casting frame containing Pharmacia "GelBond" on one internal surface. Once polymerisation was complete, unpolymerised gel was removed from the edges, and the gel
was run horizontally on a Pharmacia Flat Bed Apparatus FBE 3000.

Using a solution of phosphoric acid for the anode, and 20mM NaOH for the cathode, gels were prefocused at a constant voltage of 500V until the current had ceased falling. Samples were loaded onto the surface of the gel and run overnight at a constant voltage of 500V.

**IEF in agarose.** Agarose gels were prepared by boiling 0.3g of Agarose IEF and 3.6 g of sorbitol in 27mls of double distilled water. The mixture was cooled to approximately 75°C before adding 1.9 mls of Pharmalyte (pH 3-10). Agarose gels were cast in the same system as described for polyacrylamide. Once the gel was set, it was removed from the casting frame and allowed to harden overnight in a moist sandwich box at room temperature. Using 0.05M H_2SO_4 as the anode solution, and 1M NaOH as the cathode solution, proteins were electrophoresed at 1,500V and 15W for approximately 1.5 hours.

**3e.Fixing and staining of IEF gels.**

After completion of focusing, gels were fixed in 5% sulphosalicylic acid plus 10% trichloroacetic acid for a period of either 30 minutes (agarose), or 60 minutes (polyacrylamide). Gels were thoroughly destained in a solution of methanol:acetic acid:distilled water (3:1:6), and either immediately stained (polyacrylamide), or dried and then stained (polyacrylamide) in 0.2% Coomassie
Brilliant Blue. Protein bands were visualised by further destaining.

3f. Determining the iso-electric point.

The iso-electric point (pI) of any protein was estimated by running a series of proteins of known pI on each gel. (pI standard kit, Pharmacia) A plot of their pI versus distance from the cathode, produced a standard curve from which unknown pI’s were calculated.

3g. Modification of isoelectric focusing to select a pH at which charge differences are maximized. (128)

The composition of the polyacrylamide gel was as detailed in 2d. Following addition of ammonium persulphate, gels were cast on "GelBond" film in a Pharmacia Capillary Casting Mould with a central linear trough. The gel was focused in the first dimension at 15W constant power, unlimited current and voltage limited to 2,000V. After a period of approximately 1 hour, the electrode strips and underlying gel were removed. 50ul of sample containing approximately 100 ug of protein was loaded into the sample trough. Electrophoresis was undertaken in the second dimension at 15W, 1,000V for approximately 15 minutes.

Gels were stained and destained following the method outlined in 2e.
3h. Size exclusion chromatography by high performance liquid chromatography and fast protein liquid chromatography.

All buffers were degassed and filtered through a 0.22 micron filter on the day of use. Samples of protein were filtered.

For routine quality control of radiolabelled proteins a Zorbax GF-250 gel filtration column was used. This was run with 0.02M Na$_2$HPO$_4$, pH 7.5 as the mobile phase, at flow rates of 0.5-1ml/hour. Samples contained less than 250ug of protein and were less than 250 ul in volume.

FPLC was performed using a Pharmacia Superose column. This was run in PBS, at flow rates of 0.5-0.75 ml/min. The column capacity was limited to a 200ul sample volume.

3i. Anion exchange chromatography.

This was performed on a Pharmacia "Mono Q" column under conditions determined by prior protein titration. All buffers were degassed and filtered immediately before use.
3j. Cation exchange chromatography.
This was performed using the same procedure as for anion exchange, but using the Pharmacia "Mono S" column.

4. Estimation of protein concentration.
4a. By optical density.
The protein concentration of antibody solutions was determined by UV absorption at 280nm, assuming that a 1mg/ml solution has an absorbance of 1.4 O.D. units.

4b. By Miller's modification of Lowry's colorimetric method (127).
A standard curve was prepared using known amounts of bovine serum albumen dissolved in 100ul of PBS. The unknown protein was also prepared in 100ul of PBS. 250ul of a solution containing 5 mls of 0.5M NaOH/10% sodium carbonate and 450ul of 1% sodium tartrate was added to each tube and mixed well. A further 50ul of a solution of 5% copper sulphate was added to the tubes, mixed and left at room temperature for 10 minutes. Finally, 750ul of a solution containing a 1/10 dilution of Folin and Ciocalteau's phenol reagent was added. The tubes were heated to 50°C for 10 minutes, before their optical density was determined, using a Tungsten lamp at 650nm.
5. Biological characterisation.

5a. Isotype by Ouchterloney.

A 1% solution of agar was prepared in PBS and pipetted onto a microscope slide. After hardening at room temperature, a series of wells were punched in the agar. 2μl of the test antibody was placed in the central well, whilst 2μl of isotype specific antisera were placed in surrounding wells. Antibodies were left to diffuse into the agar in a moist sandwich box. After 8 hours, the slides were dried and rehydrated on two occasions. On the first occasion, rehydration was undertaken in 0.9% NaCl, and on the second in distilled water. Slides were stained for a period of three minutes with Coomassie Brilliant Blue, and then destained.

5b. Isotype by indirect immunofluorescence.

6μm sections of frozen neuroblastoma sections were cut at -20°C on a Bright's cryostat. 20μl of the test antibody was placed on each section under antibody saturating conditions, and incubated in a moist sandwich box for 30 minutes at room temperature. Slides were washed in PBS containing 1% FCS. 20μl of fluoresceinated, isotype specific antisera at optimal dilution, was added to the slides, reincubated for 30 minutes and washed. Neuroblastoma sections were examined for specific fluorescence, using a Zeiss Photomicroscope with epi-illumination optics.
5c. Modification of the method of indirect immunofluorescence for assessing biological activity.

To determine the biological activity of any antibody, the method described in section 4b was used. However, the starting concentration of antibody was determined by optical density, and serial dilutions of antibody tested. The biological activity was defined by the dilution at which the degree of fluorescence fell from saturation. To enable comparison between batches of the same antibody, the titre was adjusted to reflect a standard starting concentration. (Usually 1mg/ml).

5d. Modification of the immunofluorescence technique to detect antigen expression on cells within the CNS.

CSF samples were preconcentrated by centrifugation at 400xg for 5 minutes, and resuspended in 0.5mls of PBS. Aliquots of the cell suspension were placed on slides and air dried. Cells were fixed for 30 seconds in acetone at -20°C. Slides were examined with the antibody (-ies) of interest, a pan-leukocyte antibody, 2D1 as a positive control, and a negative control containing 1% FCS to estimate background immunofluorescence.

5e. Modification of the immunofluorescence technique to detect neuroblastoma infiltrates in marrow.

Bone marrow aspirates were centrifuged on Ficoll-hypaque at 400xg for 20 minutes at room temperature, and the mononuclear fraction removed. Cells were washed twice in PBS containing 1% FCS. Cells were resuspended in PBS and
the proportion of living cells calculated by Trypan Blue exclusion. Approximately $10^6$ live cells per antibody were placed in a LP3 plastic tube and incubated for 30 minutes at room temperature, with the antibody of interest. Following 2 further washes, cells were reincubated with 20ul of rabbit anti-mouse Ig fluoroscein and washed (x2) with PBS containing 1%FCS and 0.04% sodium azide. Cells were wet mounted and examined under the conditions outlined in 4b.

6. Radiolabelling.

6a. The modified Chloramine-T technique (129).
Monoclonal antibodies were radiolabelled in 0.2M PBS, pH 7.4 by addition of between 10-30 uCi of Iodine, per ug of protein and 10ul of a 0.8 mg/ml solution of Chloramine -T per 100 ul of reaction solution. The reaction was terminated at 2 minutes by addition of 10ul:100ul of a 1mg/ml solution of sodium metabisulphite and an excess of potassium iodide.

6b. The iodogen technique.
50ul of a 1mg/ml solution of iodogen was used to coat a glass reaction vessel and allowed to dry. The required ratio of protein to iodine was placed in the reaction tube and mixed gently for 10 minutes. The reaction was terminated by removing the protein from the vessel.

Following iodination with either of the above techniques radiolabelled protein was separated from unreacted iodine
by Sephadex G25 column chromatography. The 10ml column was packed with Sephadex G25, swollen and equilibrated in PBS containing 2% carrier protein. The radiolabelled protein was loaded on the column, and washed through with PBS/2% protein. The effluent was collected in sequential 0.5ml fractions and assayed for radioactivity. The first of 2 chromatographic peaks was collected and the rest discarded.

6c. Assessment of radiochemical purity by Trichloroacetic acid (TCA) precipitation.
5ul of radiolabelled protein was mixed with 95ul of PBS and 100ul of 20%TCA. The mixture was incubated on ice for 30 minutes, and then centrifuged at 10,000 x g for 30 minutes. The supernatant was separated from the precipitate, and the proportion of non-precipitable counts determined.

6d. Proportion of aggregated protein, by centrifugation.
5ul of protein solution was diluted in 95ul of PBS containing 10%FCS. The solution was centrifuged at 10,000 x g for 60 minutes, and the proportion of pelleted counts determined, compared with the supernatant.

6e. Assessment of radiochemical purity of mIBG. (157)
Differing amounts of mIBG were loaded on a silica gel thin layer chromatogram and dried by dessication. The TLC was run in a chamber containing a solution of propan-1-ol and 10% aqueous ammonia (3:1) and dried. The TLC
was autoradiographed, by incubating the TLC with a photographic plate at -70°C, overnight. The relative proportions of radioactivity in different fractions was determined by densitometry scanning of the X-Ray film.

6f. Measurement of the immunoreactive fraction, following radiolabelling.
For antibodies raised against neural antigens, immunoreactivity following radiolabelling was estimated at antigen excess. A homogenate of whole brain was prepared in PBS, aliquoted into 1 ml fractions and stored at -70oC until required.

To assay for immunoreactivity, aliquots of brain were thawed, centrifuged at 10,000 x g, and washed in PBS. Each aliquot was resuspended in a final volume of 800ul PBS. The radiolabelled antibody was diluted in 100ul of PBS/1% human serum albumin (HSA), along with 125I "irrelevant" antibody. The concentration of each antibody was adjusted so that 100ul of PBS contained, approximately 40,000 cpm of 131I and 30,000 cpm of 125I. The solution of antibody was added in triplicate to the brain homogenate, and incubated for 1 hour at room temperature. After 3 washes in PBS/1% HSA, the mixture was centrifuged, and the supernatant discarded. The radioactivity in the brain pellet was counted, and the immunoreactive fraction determined by the % of counts specifically bound to the pellet (131I-125I).
Animal maintenance and preparation for experimentation. Nude mice were supplied as specific pathogen free from the ICRF Animal Housing Unit, Clare Hall. Animals were housed in groups of five of the same sex, in a laminar flow isolation unit. Unrestricted access to water was given on days of experimentation, and thyroid blockade was achieved by adding 3 drops of Lugol's Iodine solution to this water supply.

Intravenous injection was always undertaken via a tail vein, prewarmed in heated water. Mice were killed by cervical dislocation.

Marmosets were supplied in established social groupings by the Royal College of Surgeons, Animal Unit. The larger social groups (>than 4 animals) were housed together in a large gang cage, whilst the remainder were housed in separate caging units.

Food, but not water was suspended on the morning of experimentation. 3 drops of Lugol's iodine solution was added to the water. Animals were anaesthetised for all experimental procedures, by gaseous anaesthesia with Halothane and N₂O/O₂. The proportion of Halothane varied during the study period between 2-6%, depending on the animals' tolerance. Intravenous puncture was undertaken through the femoral vein, and blood sampling either from here or from the femoral artery.
All animal experimentation was undertaken under the relevant Project and Personal licences from the Home Office.
CHAPTER 3.

The Biodistribution Of Radiolabelled UJ13A In Children With Neuroblastoma.

INTRODUCTION:
Two factors were felt to be of paramount importance in assessing the potential of monoclonal antibody UJ13A for radiation targeting. The first was the ability of the conjugate to concentrate in tumour deposits, whilst the second concerned the level of non-specific irradiation to normal tissues. To investigate these two aspects of the behaviour of $^{131}$I UJ13A, a clinical study was established. Its aims were to characterise the normal biodistribution of $^{131}$I UJ13A in children with neuroblastoma, to estimate the radiation dose delivery from $^{131}$I UJ13A to normal tissues, and to assess the sensitivity and specificity of $^{131}$I UJ13A for detecting neuroblastoma.

METHODS:
Preparation of $^{131}$I UJ13A.
Preparation of the radionuclide was undertaken in two stages. The monoclonal antibody UJ13A was prepared from murine ascites by protein A affinity chromatography (Chapter 2:1c,2b). The protein was either dialysed against 0.15M, PBS pH 7.2 and concentrated by ultrafiltration to a level of approximately 1mg/ml (Chapter 2,4a), or dialysed against 50mM ammonium bicarbonate/0.5M NaCl and lyophilised. The resulting preparation was stored at $-20^\circ$C until required.
UJ13A was radiolabelled with either iodine 123, (\(^{123}\text{I}\)) or iodine 131 (\(^{131}\text{I}\)), using a modification of the original Chloramine-T technique, to a specific activity of between 8-16 uCi/ug of protein (Chapter 2,6a). Free iodine was separated from the radiolabelled product by column chromatography using Sephadex G25 equilibrated with PBS/1\% human plasma protein fraction. An estimate of the level of free iodine was obtained by precipitation with 10\% trichloroacetic acid (Chapter 2,6c). Protein aggregation was determined by either centrifugation or high performance liquid chromatography (HPLC) (Chapter 2;6d,5h).

The immunoreactivity of the radiolabelled antibody was confirmed before and after radiolabelling using an indirect immunofluorescence assay (Chapter 2,5c). This was undertaken on neuroblastoma sections. The biological activity (B.A.) of the antibody was defined as the titre at which a reduction in immunofluorescence occurred. To enable a comparison between preparations of antibody, the titre was expressed as a dilution of a standard starting immunoglobulin concentration of 5mg/ml.

Prior to injection into patients, the protein was passed through a 22 um Millex filter.

**Patient studies.**

Children with either Evans’ stage III or stage IV neuroblastoma were entered into the study (130). Ethical committee approval and informed parental consent were obtained. To block thyroid uptake of \(^{131}\text{I}\), each patient
received 0.3mls of Lugol's iodine solution three times daily from 72 hours prior to the administration of UJ13A. This was continued for the duration of the scanning period. Sensitivity to mouse immunoglobulin was assessed by an intradermal injection of 10ug of UJ13A thirty minutes before intravenous administration of the radiolabelled conjugate.

A bolus of radioiodinated monoclonal antibody (10ug/kg) was given intravenously within 12 hours of radiolabelling. An estimate of the injected radioactivity was calculated by counting the needle and syringe on the gamma camera, both before and after administration of the isotope. Fluctuations in camera efficiency for counting $^{123}$I and $^{131}$I were monitored by repeating the measurement of syringe activity in a "Capintec" gamma counter.

**Imaging protocol.**
Imaging was performed using a Scintag-Berthold camera, fitted with a medium energy collimator at 364 Kev, and using a 20% window for $^{131}$I studies. A high resolution collimator 159 KeV, 20% window was used for $^{123}$I. Analysis of the scintigrams was achieved with the aid of an Informatek dedicated computer. Dynamic acquisition studies of isotope in abdomen and thorax were undertaken in 9 patients. Using no magnification, counts were collected in one minute frames for a period of 30 minutes.

In addition, each patient underwent sequential static scans over a period of days from injection. The times at
which these were obtained varied from 4 hours to six days after administration of isotope. At a minimum of three time points, views were obtained of the posterior chest and abdomen, anterior chest and abdomen, right and left lateral skulls with respective arms, and posterior views of both legs including the feet and pelvis. The duration of scanning for each view was 5 minutes.

**Vascular clearance of radionuclide.**

To assess the rate of clearance of $^{131}$I UJ13A from blood, serial blood samples were taken over the time period 0.5 to 48 hours. Approximately 0.5mls of blood was drawn at each time point and stored in pre-heparinised tubes. Standard 100ul aliquots were counted in an LKB ultra gamma counter. Results were corrected for isotopic decay from the time of injection.

**Data analysis.**

Estimates of organ radiation dose were obtained using the MIRD formalism (131). As the beta radiation from $^{131}$I contributes over 90% of the absorbed dose to tissue the contribution from gamma irradiation was ignored.

To calculate organ half lives, "regions of interest" were drawn around organs for analysis in the posterior view, and the total counts per minute (cpm) computed. An allowance for tissue background was made by subtracting a factor determined by the count rate in an adjoining area. Implicit in the study design was the assumption that clearance from regions of interest was monoexponential (See Chapter 9). Consequently a semi-logarithmic plot of
cpm versus time was generated from which a single effective half life of isotope (Teff) in the organ was derived.

Estimates of maximal organ accumulation of isotope were obtained by extrapolating the time activity curve to the origin. The total activity in the organ was determined by the product of the total count rate in the organ and a tissue attenuation factor for the isotope. As the estimates were undertaken from count rates in the posterior views, a variable tissue attenuation factor was used which included a depth correction for the thickness of the child in the region of the organ. The corrected tissue attenuation factor reflected the assumption that the observed counts were obtained from a tissue depth mid-way between the anterior and posterior skin surfaces. The derived activity in the organ at time 0 was expressed as a percentage of the injected dose, Ao.

The cumulated activity A, in the organ of interest was estimated from the relationship:

$$ A = Ao \times 1.44 \times Teff. \text{ (uCi.Hrs.)} \ (29) $$

The absorbed radiation dose (D) in any organ, from 1mCi of $^{131}$I UJ13A, expressed in rads/mCi, was determined from the equation:

$$ D = A \times S \times \frac{\text{Mass of adult organ}}{\text{Mass of paediatric organ}} \times 1 \frac{\text{d}}{\text{d}} \ (32) $$

Where S=the absorbed dose constant \ (132).

d=administrated dose
The dose to whole body was determined by summing the dose contribution from blood (derived from blood sampling data) and the contribution from major organs of accumulation. The % of injected activity present in the vascular compartment at any time point was derived from the estimated blood volume (Calculated from Geigy Scientific tables)(133) and the measured radioactivity per ml.

The biological half life (TBiol) was estimated from the relationship:

\[
\frac{1}{T_{\text{eff}}} = \frac{1}{T_{\text{phys}}} + \frac{1}{T_{\text{biol}}}
\]

Where TPhys is the physical half life of the radionuclide.

**Measurement of sensitivity and specificity of the imaging technique.**

All children were imaged in the context of other staging procedures for their disease. Within a two week period of imaging with UJ13A, all children underwent a $^{99}$Tc MDP bone scan, $^{99}$Tc colloid liver, spleen and bone marrow scans ("Nanocoll"), abdominal ultrasound, chest X ray, skeletal survey and in some cases computerised axial tomography (CAT scan). Interpretation of the results of UJ13A scintigraphy took place against this background.

**RESULTS:**

Full quality control was performed on 9 of the injected preparations. (Table 1) The results indicated that radiolabelling with either $^{131}$I or $^{123}$I produced a
preparation that generally contained less than 3% free iodine and was essentially free of aggregates (<3-6%). The biological activity of the injected preparation varied considerably. On neuroblastoma sections the immunoreactivity of the preparation varied between a 1/1,000 and 1/80,000 dilution of a 5mg/ml solution of UJ13A.

TABLE 1:

Quality control data on 9 patients undergoing dynamic acquisition studies.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>% I2</th>
<th>% Agg.</th>
<th>BA₁</th>
<th>BA₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>2</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>1/100</td>
<td>1/64</td>
</tr>
<tr>
<td>3</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>NE</td>
<td>1/28</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>6</td>
<td>1/100</td>
<td>1/50</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>3</td>
<td>1/80</td>
<td>1/80</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>NE</td>
<td>NE</td>
<td>1/1</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>6</td>
<td>NE</td>
<td>1/10</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>&lt;3</td>
<td>NE</td>
<td>1/10</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>6</td>
<td>NE</td>
<td>1/1</td>
</tr>
</tbody>
</table>

Where %I₂ denotes % free iodine; % Agg, % aggregated protein; BA₁, Biological activity x 10⁻³ of protein before radiolabelling; BA₂, Biological activity x 10⁻³ after radiolabelling; NE, not estimated.

A total of 19 patients were entered into the study of whom 6 were in clinical remission. Eleven patients were imaged with the ¹³¹I labelled conjugate, and the remaining 8 with ¹²³I. Observations on organ half lives and dosimetric estimates were confined to those patients receiving the ¹³¹I labelled conjugate.
Early biodistribution.

It was possible to obtain data on the early biodistribution of radiolabelled UJ13A in 9 children (Patients No. 1-4 and 6-10). Early uptake of isotope was consistently demonstrated in liver and spleen. Dynamic acquisition patterns in these organs were compared with changes in the heart. Figs 1.1a, 1.1b and 1.1c. Where the injection of the conjugate was rapid, maximal counts in the heart were obtained within the first minute. This was followed by a slow decline in measured activity. Fig 1.1a. A similar decline in counts was seen even when the administration of conjugate took several minutes. Patient 6 was atypical in the curve produced and was the only child to receive a substantial amount of free iodine in the injected preparation. With the exception of this individual, all other patients showed a progressive increase of radionuclide in the liver and spleen, in the face of falling blood pool activity. Figs. 1.1b and 1.1c.

In order to simplify analysis, data from the 8 patients administered radiolabelled monoclonal antibody containing less than 5% free iodine was pooled. Curves were generated for the mean uptake of radio-iodine in the heart, liver and spleen for set time points. Fig 1.2. Three phases of isotope accrual may be identified in the spleen; (1) a very rapid phase in which approximately 80% of the peak activity is seen in the spleen by 3 minutes; (2) a slower rate of accumulation representing 17% of peak in the following 9 minutes, and (3) a plateau phase
maintained until the end of the observation period. The pattern of liver uptake follows the splenic curve with the exception of the slightly lower proportion of peak uptake in the rapid first phase.

**Clearance of radiolabel.**

Subsequent clearance of isotope from liver and spleen was measured from 4 hours onwards by sequential scintigraphy (Fig 1.3). As with the initial acquisition of radioisotope, the rate of loss from these organs was similar. A mean biological half life of 23 hours was seen in liver (Range 12-36, n=10), compared with a biological half life of 27 hours in spleen (Range 10-42 hours, n=11). There was considerable inter-patient variation both in organ half life and estimated organ uptake at time zero; these differences resulted in variable estimates of organ radiation dosage (Table 2).
Fig 1.1a. Dynamic acquisition of iodine labelled UJ13A in the hearts of 9 patients.
A graphic representation of radioactivity present in heart during 30 x 1 minute serial images. Radioactivity is measured in counts per minute and expressed as a percentage of peak uptake.

Fig 1.1b. Dynamic acquisition of iodine labelled UJ13A in the spleen of 8 patients.
A graphic representation of radioactivity present in liver during 30 x 1 minute serial images. Radioactivity is measured in counts per minute and expressed as a percentage of peak uptake.
Fig 1.1c. Dynamic acquisition of iodine labelled UJ13A in the liver of 9 patients. A graphic representation of radioactivity present in spleen during 30 x 1 minute serial images. Radioactivity is measured in counts per minute and expressed as a percentage of peak uptake.

Fig 1.2. Composite of individual dynamic acquisition curves. Each curve represents the mean radioactivity present in the relevant organ, as measured by % peak uptake, for the patient group.
Fig 1.1c

Fig 1.2
### TABLE 2.

**Uptake and clearance of $^{131}$I UJ13A from liver and spleen.**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>$A_0$</th>
<th>$T_1/2$</th>
<th>Dose</th>
<th>$A_0$</th>
<th>$T_1/2$</th>
<th>Dose</th>
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</thead>
<tbody>
<tr>
<td>2</td>
<td>1.5</td>
<td>19.0</td>
<td>0.9</td>
<td>0.2</td>
<td>18.0</td>
<td>1.2</td>
</tr>
<tr>
<td>3</td>
<td>16.0</td>
<td>18.0</td>
<td>6.8</td>
<td>4.0</td>
<td>24.0</td>
<td>17.9</td>
</tr>
<tr>
<td>4</td>
<td>8.3</td>
<td>26.0</td>
<td>2.8</td>
<td>N.E</td>
<td>10.0</td>
<td>N.E</td>
</tr>
<tr>
<td>6</td>
<td>5.2</td>
<td>18.0</td>
<td>1.9</td>
<td>5.5</td>
<td>23.0</td>
<td>22.2</td>
</tr>
<tr>
<td>9</td>
<td>4.0</td>
<td>32.0</td>
<td>1.2</td>
<td>0.8</td>
<td>30.0</td>
<td>2.1</td>
</tr>
<tr>
<td>11</td>
<td>5.6</td>
<td>12.0</td>
<td>1.7</td>
<td>4.7</td>
<td>10.0</td>
<td>5.6</td>
</tr>
<tr>
<td>12</td>
<td>5.5</td>
<td>20.0</td>
<td>3.4</td>
<td>2.6</td>
<td>21.0</td>
<td>11.3</td>
</tr>
<tr>
<td>13</td>
<td>20.0</td>
<td>36.0</td>
<td>4.3</td>
<td>4.8</td>
<td>42.0</td>
<td>10.0</td>
</tr>
<tr>
<td>14</td>
<td>2.2</td>
<td>22.0</td>
<td>0.8</td>
<td>1.8</td>
<td>18.0</td>
<td>1.2</td>
</tr>
<tr>
<td>16</td>
<td>N.A</td>
<td>24.0</td>
<td>3.0</td>
<td>N.A</td>
<td>28.0</td>
<td>14.6</td>
</tr>
<tr>
<td>17</td>
<td>N.E</td>
<td>N.E</td>
<td>N.E</td>
<td>N.E</td>
<td>14.0</td>
<td>N.E</td>
</tr>
</tbody>
</table>

**Mean**

<table>
<thead>
<tr>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.6</td>
<td>3.1</td>
</tr>
<tr>
<td>6.3</td>
<td>1.9</td>
</tr>
</tbody>
</table>

**S.D.**

<table>
<thead>
<tr>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.7</td>
<td>2.1</td>
</tr>
<tr>
<td>1.8</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Where: Pat.No. denotes Patient number; $A_0$, estimated maximal organ uptake expressed as a percentage of the injected dose; $T_1/2$, Biological half life of the conjugate in hours; dose, estimated dose delivery in rads from 1mCi of conjugate; N.E, not estimated; N.A, not available; SD, standard deviation.

Clearance of isotope from blood followed a biphasic pattern. A semi-logarithmic plot of counts/minute/gm of blood against time, demonstrated an initial mean blood biological half life of 1.2 hours (Range 0.5-3.0, n=7). The mean biological half life of the second component was 26 hours (Range 12-52, n=8) The resultant whole body dose was estimated as a mean of 0.6 rads/mCi (Range 0.4–1.0, n=9)
TABLE 3.

Clearance of $^{131}$I UJ13A from blood and radiation dose delivery to whole body.

<table>
<thead>
<tr>
<th>Pat. No.</th>
<th>T1/2 (1)</th>
<th>T1/2 (2)</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.7</td>
<td>52</td>
<td>0.8</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>18</td>
<td>0.6</td>
</tr>
<tr>
<td>4</td>
<td>N.A.</td>
<td>N.A</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>0.5</td>
<td>32</td>
<td>0.4</td>
</tr>
<tr>
<td>11</td>
<td>3.0</td>
<td>26</td>
<td>0.5</td>
</tr>
<tr>
<td>12</td>
<td>1.0</td>
<td>19</td>
<td>0.7</td>
</tr>
<tr>
<td>13</td>
<td>1.0</td>
<td>39</td>
<td>0.6</td>
</tr>
<tr>
<td>14</td>
<td>N.E</td>
<td>12</td>
<td>1.0</td>
</tr>
<tr>
<td>15</td>
<td>0.7</td>
<td>17</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Mean 1.2 26 0.6
S.D. 0.8 13 0.2

Where Pat. No. denotes patient number; T1/2(1), The biological half life of the radiolabelled conjugate in the first clearance phase; T1/2(2), The biological half life of the radiolabelled conjugate in the second clearance phase; Dose, the estimated radiation dose to whole body (rads), from 1mCi of radiolabelled conjugate; SD, standard deviation.

Tumour uptake.

A total of 41 sites of tumour were identified by established radiological methods (Table 4). Scintigraphy with radiolabelled UJ13A resulted in the detection of 15 of these sites. Fig 1.4. Despite the theoretical advantages of $^{123}$I over $^{131}$I as an imaging isotope, no significant difference could be demonstrated between the two radionuclides. The administration of $^{123}$I UJ13A resulted in the detection of 4/12 sites (33%). This compared with 11/29 sites (38%) detected by $^{131}$I UJ13A.

The number of expected sites was possibly overestimated by inclusion of 5 tumour sites, positive by skeletal survey but negative with $^{99}$Tc MDP bone scintigraphy. Exclusion of these unconfirmed bony
deposits increased the sensitivity of UJ13A scintigraphy to 36% for $^{123}$I UJ13A and 44% for $^{131}$I UJ13A.

Imaging of thoracic tumour deposits (0/6 sites) was poor compared with skeletal deposits, (9/27, 33%) Figs 4a,b, and primary abdominal tumour, (6/7, 86%). Whilst it was not feasible to examine the antigenic profile of the thoracic sites, all patients with thoracic disease had expressed the UJ13A antigen on tumour isolated from bone marrow at diagnosis.

Whilst the sensitivity of the imaging technique was poor, specificity was high. Only 3 sites of accumulated radiolabelled UJ13A were reported that did not correspond to known sites of disease. These represented 3 bony sites, 2 femora and 1 skull deposit. The abnormal areas were not biopsied and UJ13A scans were not repeated. Consequently, it was not possible to interpret the results as false or true positives, particularly as the patients continued to receive therapy for systemic disease.
Fig 1.3. Scintigram demonstrating hepatic and splenic accumulation of iodine labelled UJ13A (\(^{123}\)I), 4 hours after administration.

Fig 1.4. Scintigrams demonstrating \(^{131}\)I UJ13A in (a) Right tibial metastasis, (b) Right femoral metastasis, 24 hours after administration of conjugate. T=tumour.
TABLE 4.

Concordance between conventional radiological staging* and immunoscintigraphy using 123I and 131I UJ13A.

<table>
<thead>
<tr>
<th>SITE OF DISEASE</th>
<th>Bones.</th>
<th>Soft tissue.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Skull</td>
<td>Long</td>
</tr>
<tr>
<td>No of sites.</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>UJ13A+</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

*Bone marrow infiltration by neuroblastoma, as determined by bone marrow aspiration and histopathology, is not recorded as a site. Eight patients had bone marrow infiltrated by tumour at the time of immunoscintigraphy. This was not detected by immunoscintigraphy.

DISCUSSION:

This study aimed to define the biodistribution of radio-iodine labelled, monoclonal antibody UJ13A, in patients with neuroblastoma. A total of 19 patients were studied, resulting in the characterisation of the distribution and pharmacokinetics of the compound.

Both liver and spleen have been identified as organs of uptake for UJ13A. In the face of falling levels of radioactivity in the vascular compartment, the biphasic shape of the dynamic acquisition curves for liver and spleen, indicates that conjugate is acquired in these organs for at least 30 minutes after injection. Estimates of uptake range between 2 - 20% for liver, and 1 - 5% for spleen. As neither organ express the UJ13A antigen, acquisition of isotope presumably reflects non specific reticuloendothelial uptake of mouse Ig (134).
Whilst the magnitude of this uptake appears highly variable between patients, there are large sources of error inherent in estimating organ uptake. These include uncertainty in defining organ boundaries, choice of background area and correction of the "S" value for estimated organ mass. In addition, the use of a single posterior scintigram and a depth correction for tissue attenuation does not take account of organ geometry. The result is a consistent overestimate of hepatic isotope accumulation, and an underestimate of splenic uptake.

Notwithstanding these reservations, several factors might be expected to result in a variable reticuloendothelial uptake of conjugate. These include the formation of immune complexes with a spontaneously occurring heterophile antibody binding mouse Ig (73), and the complexing of antibody with tumour shed antigen. It has recently become clear that UJ13A recognises a carbohydrate epitope on the neural cell adhesion molecule, NCAM. (180, 145, 120 KDa isoforms). (Personal communication from Dr. K.Patel, ICRF Oncology Laboratory, Institute of Child Health). This antigen is expressed on cellular membranes. However preliminary investigations suggest that the antigen may be present in a solubilised form in serum. The UJ13A antigen was not known at the time of this study, and so the investigation was unable to look at the influence of this factor on tumour localization and reticuloendothelial acquisition of radionuclide. The relative importance of such factors may need to be determined if liver and splenic
irradiation appears to limit dose escalation of antibody, or if they significantly reduce tumour uptake.

However experience with targeted radiotherapy in other series would suggest that the organ most likely to limit dose escalation is bone marrow (30)(63). The principle source of the dose to marrow is from blood with a small contribution from distant organs such as liver (135). The clearance of UJ13A from the vascular compartment follows the biphasic clearance kinetics observed with other antibodies (136). Interestingly, the rate of clearance is significantly faster than that reported for adults administered UJ13A (110). In a small series of 4 patients, Richardson et al. reported a mean biological half life for the second clearance component of 53 hours. It is not clear whether this observation reflects the influence of age on dehalogenation and metabolic rate, or is a facet of the factors influencing liver and splenic uptake.

The estimated mean whole body dose dose of 0.6 rads/mCi from blood is approximately equal to the bone marrow dose if one assumes no specific uptake of radiolabel. Neuroblastoma is a disease with a propensity for marrow spread so dose delivery will be influenced by the degree and pattern of marrow infiltration. An estimate of 0.6 rads/mCi to bone marrow should be considered as a conservative estimate of dose to this organ.

Radiation dose delivery to tumour was not estimated. This was due to the difficulty in assessing tumour mass
at the sites of bony disease, and the errors inherent in assuming that isotope would be uniformly distributed in a tumour volume. These two factors compound inaccuracies inherent in dosimetric methodology, and it was felt that no confidence could be placed in derived estimates of tumour dose delivery.

The disappointing feature of the study has been the low proportion of tumour sites (44%) identified with radiolabelled UJ13A. The preliminary results of Goldman are not maintained when extended to a larger series of patients. Several factors may contribute to these apparently poor results. Firstly, one's expectation of high sensitivity in immunoscintigraphy is often based on small series, on further manipulation of the radionuclide (137)(138) or scanning statistics (142). These features are partly illustrated by considering the immunoscintigraphy of colorectal cancer.

The imaging protocol in one reported series is similar to our own (58). Twenty patients with colorectal cancer, received $^{131}$I MoAb against a tumour related glycoprotein, TAG. Results were analysed against established staging investigations, and without computer aided subtraction. In this study, less than 50% of tumour sites were successfully detected by immunoscintigraphy. The low rate of detection was not attributable to damaged antibody as immunoreactivity was carefully monitored through the study. Moreover, tumour resection data taken at between 6-13 days after injection of the radionuclide, lay between 0.04 and $10.37 \times 10^{-3}$.
4% of tissue. This result is well within the reported range of tumour uptake of monoclonal antibodies, and would suggest that the imaging protocol is important in detecting low levels of isotope accumulation.

This is illustrated by the work of Goldenberg who has used 131I extensively for immunodetection of colorectal carcinoma. Goldenberg has reported on the results of immunoscintigraphy with a 131I radiolabelled polyclonal antibody in 142 patients (139). An imaging sensitivity of 85% was documented for tumour deposits of colorectal cancer, 88% for ovarian carcinoma, 71% for lung carcinoma and 64% for breast carcinoma. These results were obtained with computer aided subtraction. The author comments "Our experience again showed that we needed to perform computer-assisted subtraction of the radioantibody images in order to allow interpretation of the scans".

Polyclonal sera are often of higher affinity than their monoclonal equivalents. However an imaging sensitivity of 61% in 47 patients with gastric, oesophageal and colorectal carcinoma, was obtained by Allum et al. using an anti CEA monoclonal antibody (140).

Additional features influencing disease detection have been summarised by Bradwell as; tumour area, tumour depth, body thickness, "signal to noise" ratio, and tumour uptake ratio (141). The first three factors probably account for the inability of 131I UJ13A to detect thoracic sites of disease. These sites are generally in the mediastinum rather than in the lung.
parenchyma, so identification of the tumour sites is further compounded by vascular retention of isotope. The signal to noise ratio is amenable to improvement by the use of antibody fragments, second antibodies and improved radionuclides (142).

The uptake ratio is probably dependent on factors influencing access of antibody to antigen and the immunoreactivity of the conjugate after radiolabelling. A major criticism of the data presented in the current study is the choice of the indirect immunofluorescence assay for determining immunological activity. At the specific activities used in this study, a proportion of the immunoglobulin remains unlabelled. This results in a highly insensitive system, detecting the immunoreactivity of both labelled and unlabelled proteins. Any future studies should require a more sensitive assay system that is specific for the radiolabelled protein. Our current policy is to utilise the technique described by Lindmo et al. in which the immunoreactive fraction of radiolabelled monoclonal antibody is determined in a radiobinding assay at antigen excess. (143)

Despite the insensitivity of the assay system used, major fluctuations in the immunoreactivity of the administered protein were demonstrated. (Table 1) This may be attributable to variations in immunological activity in the purified protein or to subsequent damage during radiolabelling. The first of these factors is addressed in Chapter 5, whilst the latter requires further study of alternative labelling techniques.
SUMMARY.

The aim of this study was to characterise the biodistribution of $^{131}$I UJ13A in man, with a view to assessing its potential for therapeutic targeting. The study suggests that the antibody is likely to induce radiation toxicity through prolonged vascular carriage of isotope. Hepatic toxicity will be dependent on the highly variable uptake of isotope in this organ. Whilst further manipulation of the conjugate could be undertaken to improve the imaging ability of the monoclonal antibody, the low proportion of sites imaged in this series, seriously questions the potential of the $^{131}$I radiolabelled antibody for therapeutic targeting. Improving the therapeutic index of targeted radiotherapy requires attention to improved tumour uptake, reduced vascular carriage and hepatic uptake of isotope.
CHAPTER 4.

Experience With Administration Of High Dose $^{131}$I UJ13A To Children With Chemoresistant Or Recurrent Neuroblastoma.

INTRODUCTION:
To test the predictions of the scintigraphic study, 6 children were recruited to a pilot study of high dose, intra-venous, radiolabelled UJ13A. The purpose of the study was to investigate the feasibility and toxicity of administering $^{131}$I UJ13A as a therapeutic agent in children with neuroblastoma.

The study was initiated by Dr A. Goldman (Clinical Research Fellow, ICRF Oncology Laboratory, Institute of Child Health, London.), and continued by me. The data presented in this chapter is a retrospective analysis of the information available on the first 4 patients as well as prospective data from patients 5 and 6.

METHODS:
$^{131}$I UJ13A was prepared to a specific activity of approximately 15 uCi/ug using the modified Chloramine-T technique (Chapter 2,6a). To obtain high doses of radiolabelled conjugate, antibody was radiolabelled on the morning of administration, in batches of 5mCi. The radiolabelled product was examined for free isotope by TCA precipitation (Chapter 2,6c), aggregated protein by high speed centrifugation (Chapter 2,6d), and biological
activity by the indirect immunofluorescence assay
(Chapter 2.5c).

Clinical aspects.
Patients with chemoresistant or recurrent stage IV
neuroblastoma were eligible for this study, provided that
their tumour had been demonstrated to express the UJ13A
antigen, either at the primary site or from infiltrated
bone marrow (Chapter 2,5e). Conventional staging
procedures were used to assess the extent of disease,
including marrow aspiration from multiple sites, $^{99}$Tc MDP
bone scan, abdominal ultrasound, CT scan of relevant
sites and urinary VMA/HVA estimation. Additional
"baseline" investigations, for monitoring potential
toxicity included serum TSH, T4, plasma electrolytes and
creatinine, liver function (serum alkaline phosphatase,
bilirubin, serum alanine aminotransferase and serum
aspartate aminotransferase), haemoglobin, white cell
count and platelet count. All haematological and
biochemical parameters were measured by the routine
pathology services at the referral hospital.

Informed parental consent was obtained. Patients
were prepared for treatment with Lugol's iodine to block
the thyroid and skin tested to detect immediate
hypersensitivity (See Chapter 3). One week before
administration of the high dose radionuclide, the first 4
patients were administered a tracer amount of
radiolabelled UJ13A (10ug/kg). The pharmacokinetics of
this tracer administration were followed using the
methods outlined in Chapter 3. Repeated blood sampling was undertaken over the time period 0-192 hours and sequential static scintigraphy was performed on at least three occasions.

Patients 5 and 6 did not undergo a tracer study. Instead, the same detailed pharmacokinetic studies were performed after the therapy doses.

To study toxicity, children received a dose of $^{131}$I UJ13A escalated from 35mCi to 55mCi. When toxic levels were reached, the dose was reduced. Electrolytes, full blood counts and liver function tests were monitored at weekly intervals.

**RESULTS:**

Six patients were entered into the study. All patients had been heavily pretreated with conventional chemotherapy. This consisted of 10 or 11 courses of OPEC in patients 1, 3, 5 and 6 (See Table 1). The remaining 2 patients received additional therapy in the form of total body irradiation combined with DTIC and adriamycin. Patient 2 received these drugs because of relapse following 10 courses of OPEC. Patient 4 was changed to this protocol after 6 courses of OPEC, when clinical deterioration became apparent. In this patient, VP16 had already been substituted for VM26 because of an anaphylactic response to a second administration of VM26.

No patient had achieved a "good partial response" to conventional therapy, and each apart from patient 6, had evidence of metastatic disease. ("Good partial response"
is defined by European Neuroblastoma Study Group criteria as: clearance of all evidence of metastatic disease and greater than 90%, but less than 100% removal of the primary tumour). Patients 2, 4 and 5 were rapidly deteriorating at the time of antibody administration.

### TABLE 1.

Patients recruited to the study.

<table>
<thead>
<tr>
<th>No.</th>
<th>Patient Age (yrs)</th>
<th>Weight (kg)</th>
<th>Prior Therapy Type</th>
<th>No. of doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>16</td>
<td>OPEC</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>17</td>
<td>CVA/DTIC</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OPEC</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TBI/DTIC+ adriamycin.</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>13</td>
<td>OPEC</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>23</td>
<td>OPEC</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TBI/DTIC+ adriamycin.</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>25</td>
<td>OPEC</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>27</td>
<td>OPEC</td>
<td>8</td>
</tr>
</tbody>
</table>

CVA denotes cyclophosphamide, vincristine and adriamycin; TBI, total body irradiation; OPEC, Vincristine, cis-Platinum, VM26 and cyclophosphamide.

Toxicity (Table 2).

Acute effects.

In 5/6 patients the immediate effects from administration of $^{131}$I UJ13A were minimal. A mild pyrexia lasting 2-4 hours was observed in 3/6 patients, and 2 patients
experienced mild nausea and vomiting. Patient 4, who had an atopic history developed acute bronchospasm.

Intermediate effects.
Bone marrow suppression was the principle side effect noticed during therapy. This was observed in both patients receiving 55mCi of $^{131}$I UJ13A. The first of these patients, patient 2, had been particularly heavily pretreated (Table 1). Bone marrow samples aspirated just prior to therapy showed no evidence of disease. Tracer doses of antibody conjugate predicted a whole body dose of 0.9 rads/mCi. Six days after administration of the conjugate there was a rapid fall in blood count. Nadirs in white cell (WCC), $0.2 \times 10^9$/l and platelet count, $(1 \times 10^9$/l) were reached on day 28. Repeat marrow aspirates did not show any evidence of disease progression, but did demonstrate a hypocellular marrow. Marrow function did not recover, and the patient died with advancing disease at 12 weeks from therapy. In view of this complication, all subsequent patients had bone marrow harvested, depleted of tumour cells using the magnetic bead technique of Treleavan et al (144), and cryopreserved prior to targeting.

A whole body dose of 1.3 rads/mCi was predicted in the second patient to receive 55 mCi. The WCC and platelet count fell on day 6, and the patient required 4 platelet transfusions until the WCC and platelets recovered spontaneously, 5 weeks after administration of the radiolabel. A dose reduction to 50 mCi was
undertaken in the following 2 patients. No haematological toxicity was observed at a measured whole body dose of 87 rads in patient 5, whilst patient 6 developed a mild leukopaenia at a measured whole body dose of 65 rads. The WCC nadir was reached by day 16 at a level of 1.84 x10⁹/l.

Hepatotoxicity.
The tracer studies suggested that a significant radiation dose would be targeted to liver. Nevertheless, no acute disturbance in liver function tests was observed in 5/6 patients. In patient 5, there was a rise in liver enzymes by day 10. A rise in serum Alanine aminotransferase occurred from a baseline level of 44 u/ml to 64 u/ml (Normal range: 6-35 u/ml), whilst serum Aspartate aminotransferase increased from 49 u/ml to 274 u/ml. (Normal range: 15-40 u/ml). This coincided both with sero-conversion to Australia antigen positivity and with disease progression. Histology of the liver at seven weeks after antibody administration demonstrated a widespread infiltration by neuroblastoma and no evidence of radiation induced changes.
TABLE 2.

Complications observed in 6 patients receiving therapeutic amounts of $^{131}$I UJ13A.

<table>
<thead>
<tr>
<th>Pat.</th>
<th>Estimated dose delivery</th>
<th>Dose mCi.</th>
<th>Toxic effect.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>W.B.</td>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>1.7</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>26.0</td>
<td>55</td>
</tr>
<tr>
<td>3</td>
<td>1.3</td>
<td>16.0</td>
<td>55</td>
</tr>
<tr>
<td>4</td>
<td>0.8</td>
<td>14.9</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>1.7*</td>
<td>11.9*</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>1.5*</td>
<td>6.4*</td>
<td>50</td>
</tr>
</tbody>
</table>

Estimated dose delivery W.B: the estimated radiation dose to whole body from lmCi of radiolabelled conjugate (rads/mCi). (*) denotes that estimates are derived from therapy measurements, and() indicates that the estimate is derived from tracer scintigraphy. Liver: as for W.B but estimates derived for liver. Dose: mCi of $^{131}$I UJ13A administered as therapy. **Subsequently shown to be Hepatitis B positive.

Therapeutic effect.

Although strictly a phase I study, a note was made of any clinical response to administration of $^{131}$I UJ13A. A response was seen in one patient (Patient 3). This patient had 2 small skull deposits and bone marrow infiltrated with neuroblastoma. There was evidence of clearance of the bone marrow infiltrate as well as radiological and histological evidence of healing at the bony tumour deposits. This complete response was maintained for a period of 8 months.
The remaining patients in the series exemplified many of the problems associated with the use of antibody therapy (Table 3). Of these, patient 1 was administered 35mCi of conjugate. Isotope uptake in the primary tumour was demonstrated on scintigraphy, with a tumour half life of 74 hours. The patient was well at the time of administration despite active disease at 2 sites. No change in clinical or disease status occurred after therapy, and the patient remained well for a further 5 months. A subsequent attempt at therapy resulted in an extremely rapid clearance of conjugate with an effective half life in blood of 4 hours.

A similar effect was seen in patient 4, whose prior exposure to the antibody was confined to the tracer dose. An increased clearance of conjugate was seen on second exposure, and as a result only a low dose of radiation was achieved.

To avoid sensitisation to the murine antibody, the following two patients were not given tracer amounts of antibody. Despite immunoreactive disease present in the marrow at the time of therapy in patient 5, and UJ13A positive disease in marrow at presentation in patient 6, no uptake of isotope was demonstrated at the tumour sites.

A similar disappointing result was seen in patient 2 who had bulky abdominal disease. Despite pre- and post-therapy scans demonstrating accumulation of isotope in the tumour mass, there was no objective evidence of tumour regression.
TABLE 3.

Tumour uptake of 131 I UJ13A and clinical response to therapy.

<table>
<thead>
<tr>
<th>Pat. No.</th>
<th>Tumour site.</th>
<th>T1/2</th>
<th>Ao</th>
<th>Response.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Paraspinal mass, cervical nodes.</td>
<td>79</td>
<td>NE</td>
<td>None.</td>
</tr>
<tr>
<td>2</td>
<td>Abdominal/pelvic mass.</td>
<td>71</td>
<td>65</td>
<td>None.</td>
</tr>
<tr>
<td>3</td>
<td>2 Skull deposits Bone marrow.</td>
<td>89</td>
<td>1</td>
<td>CR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>59</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Skull deposit.</td>
<td>84</td>
<td>1</td>
<td>None.</td>
</tr>
<tr>
<td>5</td>
<td>Abdominal mass.</td>
<td>--</td>
<td>NE</td>
<td>None.</td>
</tr>
<tr>
<td>6</td>
<td>Abdominal mass.</td>
<td>--</td>
<td>NE</td>
<td>None.</td>
</tr>
</tbody>
</table>

T1/2, Biological half life of conjugate in tumour (hours); Ao, Estimated maximal tumour uptake of isotope, expressed as a percentage of injected dose; CR, complete response, i.e. clearance of all measurable disease.

DISCUSSION:

131I UJ13A conjugate at high dose, (35-55 mCi; 3-10 mg of protein), was generally well tolerated. The episode of acute bronchospasm in a patient with a history of allergic manifestations suggests that atopy may be a contraindication to treatment. As predicted, bone marrow damage was the major toxicity and occurred at levels of approximately 55mCi. We studied too few patients to comment on whether 55mCi was the threshold at which marrow toxicity consistently occurred. However, this was
the dose at which major myelosupression was observed. The relationship between administered dose and marrow toxicity is complex, reflecting observed variation in vascular clearance, reticuloendothelial uptake, tumour distribution and marrow reserve. This is illustrated by patients 2 and 3. A study of the available data would suggest that patient 2 was least likely to suffer intractable marrow failure. (Lower dose/kg, faster whole body clearance and marrow uninvolved by disease). However, it was this patient who failed to recover marrow function.

Two main conclusions were drawn from this phase I study. Firstly, considerable variation in tumour uptake was observed. However, even where there was tumour uptake on tracer scintigraphy, a clinical response was seen in only 1/4 patients. This response occurred in a patient with smaller deposits and more diffuse disease.

The apparent success of clearing disease from bony sites and bone marrow, is consistent with results from other series. (See Chapter 1, for a detailed discussion). The failure of $^{131}$I UJ13A to clear solid disease, is also consistent with clinical experience and the theoretical arguments of Vaughan and others. (See Chapter 13). Both the results of this study and the experience of others, suggest that $^{131}$I UJ13A is unlikely to provide an effective means of delivering radiotherapy without major toxicity, in patients with large, solid tumour deposits.
Secondly, a change in the pharmacokinetics of the conjugate was noted on second and third administration. (Patients 1 and 4). Although not measured, the change was attributed to the emergence of a circulating human anti-mouse immunoglobulin (Ig). It is important to validate this conclusion as a rapidly emerging immune response will limit the usefulness of UJ13A as a targeting agent. This problem along with a strategy to reduce the immunogenicity of UJ13A is explored in Chapter 6.
CHAPTER 5.
Recloning And Characterisation Of UJ13A.

INTRODUCTION:
A reduction in the quality and yield of UJ13A was noted during the course of the clinical studies. At the beginning of the clinical programme, 1ml of ascites generated approximately 5mg/ml of purified UJ13A. The biological activity of the purified protein as defined by the indirect immunofluorescence assay on neuroblastoma sections, was of the order of 1/20,000 of a 1mg/ml solution of antibody. Over a period of 1 year the yield of antibody dropped to less than 1 mg/ml with a titre averaging 1/1000.

Several batches of affinity purified UJ13A were unstable when radiolabelled. This was demonstrated in vitro by HPLC examination of the radiolabelled protein, and in vivo by examination of the radioactivity profile present in marmoset plasma.

Testing of hybridomas by the cell enriched PPLO agar assay, indicated that the cell lines were contaminated with a Mycoplasma species. (Courtesy of Ms. A. Hayles, Imperial Cancer Research Fund). Whilst this did not necessarily explain all the difficulties encountered in preparing UJ13A, a decision was made to return to an early cloning of the hybridoma, reclone and treat the hybridoma with the anti-Mycoplasma antibiotic BM-cycline. An early vial of UJ13A was re-established in culture, and re-cloned by limiting dilution (Chapter 2;
Following 3 cyclical treatments with BM-cycline (Chapter 2;1b), seed lots of Mycoplasma free UJ13A were frozen down for future use (Chapter 2;1d).

To maintain a firmer check on the quality of antibody for clinical work, UJ13A was recharacterised from these seed lots. Hybridomas were grown up in a designated "clean area", and antibody production expanded by growth as peritoneal tumours in Balb/c mice (Animal House, Institute of Child Health), and by large scale tissue culture (Imperial Cancer Research Fund, Clare Hall facility).

**Characterization of UJ13A.**

**Purification.**

UJ13A can be purified from ascites by Protein A affinity chromatography under the conditions described in Chapter 2,2b. To establish the optimal conditions for purification of UJ13A from ascites, 1ml of ascites was loaded onto a protein-A column equilibrated in phosphate buffered saline (PBS), pH 7.4. The column was sequentially eluted with 0.1M Citrate buffer at pH intervals of 6, 5, 4 and 3. At pH 6 and 5 eluted protein was collected into 0.2M phosphate buffer, pH 7.4. Below pH 5 any eluted protein was collected into 0.75M Tris HCl buffer, pH 8.5. If necessary, each fraction was rapidly pH adjusted to between 7.0-7.4 by the addition of further collecting buffer. Fractions containing protein were dialysed at 4°C against 0.15M phosphate buffered saline, pH 7.2 overnight.
The unbound fraction was tested for biological activity by indirect immunofluorescence (Chapter 2;2c), as was each fraction at the different pH intervals. Under these conditions, greater than 95% of the bound protein eluted at pH 5-6 (data not presented), and 1ml of ascites yielded 3 mg/ml of purified protein. Biological activity at a starting concentration of 1mg/ml was 1/10,000. No residual activity was detected in the ascites.

In view of this result, subsequent purifications were undertaken by eluting bound antibody from protein A, at pH 5. The method was extended to recovery of antibody from large scale tissue culture, with the addition of a pre-concentration step using ammonium sulphate. (Chapter 2, 2a).

Purity.
The purified immunoglobulin was examined by SDS polyacrylamide gel electrophoresis, in reduced and non-reduced form. (Chapter 2,3a,b and c). Under non-reducing conditions, both the antibody obtained from ascites and from tissue culture resolved into 4 bands. Reduction with dithiothreitol, produced three bands. The band with lowest mobility consistently ran with an apparent molecular weight of 52 kD and the more mobile bands with molecular weights of 32 and 26 kD (Chapter 2; 3d). (Fig 3.1).

To investigate the composition of the protein mixture, the affinity purified antibody was examined by
electrophoretic titration across a pH gradient 3-10. (Chapter 2;3g). Under the conditions of the experiment, the protein mixture consistently migrated towards the cathode (Fig 3.2). No separation into multiple subunits was noted.

In view of the net positive charge of the protein below pH 8.4, the protein mixture was examined by FPLC, using a Mono S cation exchange column. (Chapter 2;3i)

Separation of the protein mixture on the cation exchange column was performed by using 0.05M acetic acid, pH 4.8 as running buffer, and eluted with a 0-100% 0.5M sodium chloride gradient.

The protein mixture separated into 2 components (Fig 3.3). Both components were immunologically active when tested by indirect immunofluorescence on foetal brain.

A similar pattern was observed on anion exchange chromatography, using 0.05M Tris.HCl, pH 7.5 as the mobile phase, and eluted with 0.5M sodium chloride gradient. Fig 3.4. The two immunologically active fractions, separated by anion exchange chromatography, were run separately on an 8% polacrylamide gel. Each fraction resolved into similar doublets as the parent protein, although there was a suggestion of a further band in the lower subunit. Fig 3.5.

Digestion of the protein with pepsin, (see Chapter 7) produced a F(ab')2 fragment that maintained the doublet pattern on gel electrophoresis. Fig 3.6.
**Fig 3.1.** Polyacrylamide gel electrophoresis of monoclonal antibody UJ13A. Antibody run in reduced and non-reduced form on a 12% gel, and silver stained. (a) UJ13A (non-reduced), staining as 4 protein bands, (b) Molecular weight markers, (c) UJ13A (reduced), running as three bands of molecular weights 52 kDa, 32kDa and 26 kDa.

**Fig 3.2.** Electrophoretic titration curve of UJ13A. 60ug of UJ13A run across a pH gradient of 3-10, and stained with Coomassie Blue, demonstrating no separation into multiple subunits.
Fig 3.3 Cation exchange chromatography of UJ13A.

UJ13A loaded on a Mono S, cation exchange column.
Flow rate: 0.5mls/min PBS, eluted with 0-100% sodium chloride gradient.
Fig 3.4. Analysis of UJ13A by anion exchange chromatography. 50ul of UJ13A, separated on a Pharmacia "Mono Q" anion exchange column. Mobile phase:0.05M Tris.HCl, pH 7.5; eluted with a 0-100% sodium chloride gradient.

Fig 3.5 Comparison of fractions obtained by anion exchange chromatography of UJ13A, by polyacrylamide gel electrophoresis. 8% polyacrylamide gel, stained with Coomassie Blue.

(i) Fraction 1 separated by anion exchange chromatography, as seen from Fig 3.4.
(ii) Fraction 2 separated by anion exchange chromatography.
**Fig 3.6.** Polyacrylamide gel electrophoresis of a pepsin digest of UJ13A. 8% polyacrylamide gel, stained with Coomassie Blue. (M) Molecular weight markers, (a) Pepsin digest before Protein-A affinity chromatography, (b) Pepsin digest post Protein-A affinity chromatography (See Chapter 7).

**Fig 3.7.** Isotype of UJ13A as demonstrated by radial immunodiffusion (Ouchterloney). Central well contains 2ul of UJ13A, peripheral wells contain 2ul of isotype specific antisera. An immunoprecipitate between UJ13A and goat anti-mouse IgG2ab and IgG2a is demonstrated by staining with Coomassie Blue.
Fig 3.8. Isoelectric focusing of UJ13A. Focusing undertaken in agarose, across a pH gradient 3-10, and stained with Coomassie Blue. UJ13A focused in the estimated pH range 7.4-8.2.
Fig 3.8 Isoelectric focusing of UJ13A

Focused in agarose

pH 3-10
Isotype.
The isotype of the purified UJ13A was established by the method of Ouchterlony and by indirect immunofluorescence using fluoresceinated isotype specific antisera. (Chapter 2;5a,b) Both methods indicated that UJ13A was of the IgG2a subclass. Fig 3.7.

pI.
Protein A affinity purified UJ13A was analysed by isoelectric focusing in a pH gradient of 3-10. (Chapter 2;3e,f,g). Six sub units with pI’s lying between 7.4-8.2 were identified. Fig 3.8.

Equilibrium constant.
The equilibrium constant of UJ13A was defined by the method of Scatchard (145), on the cell line JR1 (146).

Monoclonal antibody UJ13A was radiolabelled with $^{125}$I using the modified Chloramine-T technique (Chapter 2;6a). The specific activity of the radiolabelled protein was estimated by assuming an 80% protein recovery after G25 column chromatography, and by measuring the radioactivity present in an aliquot of the solution.

The cell line JR1 was grown in complete RPMI 1640 medium. All cells were harvested during the exponential phase of growth.
Estimating the amount of cold antibody required to saturate the binding sites in the assay system.

To establish the level of non-specific binding of radiolabelled antibody to cells and plate, a preliminary experiment was undertaken to estimate the amount of unlabelled antibody required to just saturate the binding sites on 2.5 x 10^5 cells. Cells were harvested and centrifuged at 10,000 x g for 5 minutes. Supernatant was discarded and the cells resuspended in phosphate buffered saline. Following a second centrifugation and resuspension, the proportion of viable cells was estimated by Trypan blue exclusion (Chapter 2:le).

2.5 x 10^5 viable cells, in a volume of 100 ul were pipetted into 36 wells of a 96 well microtitre plate which had been blocked with a solution of 5% bovine serum albumin (BSA) for 4 hours at 4°C. Each of 12 serial dilutions (in triplicate), of a known concentration of UJ13A was mixed with 100,000 cpm of 125I UJ13A and added to each well. The final volume of each well was adjusted with phosphate buffered saline to 100ul. After incubating antibody and cells for 30 minutes at room temperature, the microtitre plate was centrifuged at 10,000 x g for 5 minutes. Cells were resuspended and washed with 1% BSA on a further two occasions. The amount of radioactivity bound in each well was then measured in an LKB ultra gamma counter.

The minimum amount of cold antibody required to saturate binding sites on 2.5 x 10^5 cells was estimated

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as 10 ug. This value was chosen to derive the estimate of non specific binding in the Scatchard analysis.

Scatchard analysis.
The plate assay was set up as previously described. To measure total antibody binding, $2.5 \times 10^5$ viable cells were added in triplicate to each of 10 wells. A second series of identical wells was set up to estimate the degree of non-specific binding. In the first series of wells, serial dilutions of 100,000 cpm were added, in a final volume of 100ul. To establish the level of non specific binding, the serial dilutions of antibody were repeated but with the addition of 10ug of unlabelled antibody. Antibody binding was allowed to proceed at room temperature for 30 minutes. Plates were then washed twice and counted in the manner previously outlined.

Specific antibody binding (cpm), was calculated by subtracting the radioactivity bound in wells containing 10ug of unlabelled protein from the total radioactivity bound in wells containing only radiolabelled UJ13A.

To derive the equilibrium constant, the law of mass action as used by Scatchard, and elaborated by Trucco (147) was utilised.

Using the equation:

$$\frac{r}{(A-x)} = Kn - Kr$$

(Where $r$ denotes the specific binding of antibody to a single cell at a given dilution, expressed as molecules per cell; $(A-x)$, the molar concentration of free antibody; $n$, the maximum number of antibody molecules
bound to one cell; and $K$, the equilibrium constant in litres/mole), a plot of $r/(A-x)$ versus $r$ describes a linear function, the slope of which generates $K$.

Results.

Radiolabelling of UJ13A yielded a preparation containing an estimated 0.476 ug of protein per $10^6$ cpm.

Scatchard 1.

<table>
<thead>
<tr>
<th>Counts Added $x(10^3)$</th>
<th>Total Bound $x(10^3)$</th>
<th>Specific Bound $x(10^3)$</th>
<th>$r x(10^5)$</th>
<th>Unbound $x(10^3)$</th>
<th>$(A-x) x(10^{-10})$</th>
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<tbody>
<tr>
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<td>1.13</td>
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<td>2.11</td>
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<tr>
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<td>7.65</td>
<td>6.66</td>
<td>4.92</td>
<td>29.92</td>
<td>18.37</td>
</tr>
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</table>
This experiment was repeated with a new batch of radiolabelled protein with a specific activity of $10^6$ cpm per 0.596 ug of protein.

**Scatchard 2.**

<table>
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<td>$x(10^2)$</td>
<td>$x(10^3)$</td>
<td>$x(10^{-10})$</td>
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<td>31.75</td>
<td>24.34</td>
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</tbody>
</table>

The Scatchard plots of UJ13A binding to the cell line JR1 (Fig 3.9) indicated an equilibrium constant of $4.3 \times 10^8$ M$^{-1}$ from the first experiment, and $5.0 \times 10^8$ M$^{-1}$ from the second.
**Fig 3.9.** Scatchard plot of the binding of $^{125}$I UJ13A to cell line JR1. Ordinate: $r/(A-x)$, where $r$ denotes the specific binding of antibody to a single cell at a given dilution, expressed as molecules per cell; $(A-x)$, the molar concentration of free antibody, Abscissa: $r$. The slope of the curve is generated by the method of least squares analysis.
Scatchard Analysis: Binding of 125I UJ13A to cell line JR1.

1.

2.
DISCUSSION.

The initial description of the monoclonal antibody UJ13A, confined itself to a report of its production, tissue specificity, and isotype. Other information including yield and mobility on SDS PAGE is available from laboratory records.

Treatment of the hybridoma with BM-cycline, resulted in the successful clearance of mycoplasma. Both the yield and immunological activity of the antibody improved, when there was no further evidence of mycoplasma infection. The improvement in these parameters has been sustained.

However, several important differences are now apparent between the monoclonal antibody used in our initial studies, and the recloned UJ13A. The first is the apparent change in isotype of the immunoglobulin. UJ13A was initially reported as an antibody of the IgG_1 subclass. In this study, it is clear that the antibody is of the IgG_2a subclass. This result has been confirmed repeatedly, both in our own laboratory and independently. (Personal communication: Dr D.Bigner, Duke University, North Carolina.)

There are two possible explanations for the discrepancy. Either a mistake was made in the initial assessment of isotype, (assessed by Ouchterlony), or there has been a spontaneous isotype switch in the hybridoma. The first explanation cannot be discounted, as none of the early aliquots of purified UJ13A are
available for testing. However, it should be noted that the frequency of a spontaneous isotype switch has been reported as approximately $10^{-5}$ to $10^{-6}$/cell/generation (148). The matter may be further elucidated by examining other seed lots from the initial cloning. This is underway.

This matter is of interest, because of the different biological properties of the isotypes. Antibodies of the IgG$_{2a}$ subclass are much more likely to induce antibody dependent cell mediated cytotoxicity, which may be of therapeutic value (32).

A second area in which this antibody appears to differ from the reagent used in earlier studies, is in the electrophoretic pattern produced on SDS PAGE. A distinct doublet is now apparent, with each band containing 2 further subunits. The pattern is similar whether the antibody is derived from ascites or from tissue culture. If the second major band represents a contaminant co-purifying with the antibody, then it is present in equal measure in both the mouse peritoneum, and in the tissue culture fluid. This explanation seems unlikely as the pattern was not observed in earlier purifications (Results available from early laboratory records).

The alternative explanation is that there has been an intrinsic change in the immunoglobulin species secreted by the hybridoma. Examination of the purified protein in reduced form by SDS gel electrophoresis, suggests three components, 2 major bands running in with the expected
molecular weight of light and heavy chain, (25/50 KD),
and a smaller amount of protein running in an
intermediate position (approximately 30 KD). This third
band is only apparent on silver staining, and does not
quantitatively reflect the apparent heterogeneity in the
unreduced protein.

Resolution of the protein mixture by ion exchange
chromatography, separates the purified protein into two
components, both of which are immunologically active.
This would suggest that the observed change in
electrophoretic pattern of unreduced UJ13A, may be
largely attributable to post-translational modification
of the protein e.g. glycosylation or phosphorylation. It
would appear from the SDS PAGE characteristics of the
F(ab')2 protein, that any modification must involve sites
proximal to the antibody hinge region.

The identity of the third band in the reduced protein
is uncertain. As the P3-X63-Ag8-653 fusion partner is
not an endogenous light chain producer (50), this cannot
represent contaminating light chains from the mouse
myeloma. Neither has this pattern been observed in other
monoclonal antibodies resulting from the same initial
fusion.

One explanation is that the antibody is not
monoclonal. However the antibody was repeatedly
subcloned during its initial production, and has since
been recloned at each passage.

The issue is not resolved by the data presented in
this chapter, and further studies will be required if the
analysis is to be taken further. However, apart from the issue of isotype, additional information on antibody structure/composition is unlikely to be of any direct relevance to its clinical application. Scatchard analysis, demonstrates that the antibody has a reasonably high affinity for its antigen, and in a series of studies investigating the immunoreactive fraction following radiolabelling, it has been possible to obtain 80-85% immunoreactivity. (Personal communication: R. Mosely. Brain Tumour Research Group, Frenchay, Bristol). No change has been noted in its tissue specificity, as determined by a recent international investigation of monoclonal antibodies of interest in neuroblastoma (85).

These features indicate that the current radiolabelled conjugate retains high specificity for neuroectodermal structures. The detailed characterisation of UJ13A presented in this chapter, along with pyrogenicity and viral screening, has resulted in the antibody gaining FDA approval as a bone marrow purging agent, as part of an immunomagnetic separation device.
CHAPTER 6.
The Preparation And In Vivo Distribution Of $^{131}$I Radiolabelled Fragments Of UJ13A.

INTRODUCTION:

It is clear from the results of the preliminary clinical studies with $^{131}$I UJ13A, that any further developments must centre on improving the therapeutic index of the radiotherapy. Extrapolating from data on the relative radiosensitivity of neuroblastoma, normal tissues, and the pharmacokinetics of monoclonal antibodies, Wheldon has postulated that targeted radiotherapy with $^{131}$I monoclonal antibodies remains a feasible proposition (149). Targeted radiotherapy may be beneficial in the clinical context of minimal residual disease, provided the therapist accepts significant bone marrow toxicity. Wheldon argues that the therapeutic index may be further enhanced by the appropriate scheduling of multiple doses (150) (and see Chapter 13). The suitability of UJ13A as a candidate for targeting radiotherapy to neuroblastoma under these conditions is dependent on its immunogenicity in vivo. Changing pharmacokinetics have been observed in the few patients administered multiple doses of UJ13A (Data discussed but not presented; Chapter 4). This would suggest that the antibody is rapidly immunogenic.

One potential method of reducing both immunogenicity and non-specific whole body irradiation, is to produce antibody fragments. In studies with polyclonal sheep, digoxin-specific, Ig and Fab fragments, Smith et al.
demonstrated reduced immunogenicity and faster vascular clearance of the Fab fragment in rabbits and baboons (151). These observations have been endorsed by a number of other investigators (152)(153).

To investigate any potential therapeutic advantage in the use of fragments of UJ13A, a study was established to compare the biodistribution and immunogenicity of the whole immunoglobulin (Ig), F(ab’)2 and Fab fragments.

Choice of an animal model:

A substantial amount of investigative work on the distribution of radiolabelled UJ13A had been undertaken in the Balb/c mouse (82)(83). This species was considered unsuitable for the proposed study as it would not produce a similar immune response to murine immunoglobulin as the patient population. The sub-human primate, the common marmoset was identified as suitable candidate for investigation. The distribution of the UJ13A antigen on tissues from this animal closely parallels the distribution of the UJ13A in human tissue, and is only found on tissues of neuroectodermal origin. (Personal communication, Dr DH Jones, ICRF, The Institute of Child Health, London).

To further characterize the common marmoset as a model for the biodistribution of monoclonal antibodies, a small colony of primates was established at the Institute for Child Health, London.
Determination of circulating blood volume.

Under N\textsubscript{2}O/O\textsubscript{2} and halothane anesthesia, 5 marmosets were administered a known quantity of \textsuperscript{125}I radiolabelled marmoset albumin through a femoral vein (0.15-0.25 mCi). Blood was sampled from the opposite femoral vein 3-5 minutes after administration. The circulating volume was estimated by the Fick principle, assuming that the \textsuperscript{125}I albumin was uniformly distributed in the vascular compartment. The results indicated a mean vascular volume of 9% body weight. (Table 1).

**TABLE 1.**

Blood volume and organ size in 5 marmosets.

<table>
<thead>
<tr>
<th></th>
<th></th>
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<td>516</td>
<td>242</td>
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<td>300</td>
<td>9</td>
<td>16</td>
<td>5</td>
<td>0.7</td>
<td>0.2</td>
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<td>0.4</td>
</tr>
<tr>
<td>454</td>
<td>394</td>
<td>11</td>
<td>21</td>
<td>5</td>
<td>0.9</td>
<td>0.2</td>
<td>2.0</td>
<td>0.5</td>
</tr>
<tr>
<td>128</td>
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<td>17</td>
<td>5</td>
<td>0.5</td>
<td>0.2</td>
<td>1.3</td>
<td>0.4</td>
</tr>
<tr>
<td>517</td>
<td>307</td>
<td>11</td>
<td>12</td>
<td>4</td>
<td>0.4</td>
<td>0.1</td>
<td>1.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Mean: 309 9 16 5 0.6 0.2 1.4 0.5

Where weights (Wt.) are expressed in gramms, Blood volume (Blood vol.) and organs are expressed as a percentage of body weight.

Position of liver, spleen and kidneys by scintigraphy.

To determine the position of the liver and spleen by scintigraphy, 3 marmosets were administered 0.25 mCi of \textsuperscript{99}Tc "Nanocoll" colloid intravenously under N\textsubscript{2}O/O\textsubscript{2} and halothane anesthesia. Marmosets were imaged 15 minutes
Fig. 4.1. $^{99}$Tc colloidal scan (Nanocoll) of liver (L) and spleen (S) in a marmoset.

Fig. 4.2. Clearance of $^{131}$I UJ13A from blood of marmosets. Estimated percentage of injected dose present in blood with time-pooled data, 2 animals.
after injection with a Siemens gamma camera fitted with a high resolution collimator (169KeV).

The position of the liver and spleen were clearly defined and are illustrated in Fig 4.1. A similar procedure was undertaken to indicate the position of the kidneys. Each animal was administered 0.2 mCi of $^{99}$Tc DMSA and the position of the kidneys demonstrated scintigraphically.

**Biodistribution of $^{131}$I UJ13A.**

Following thyroid blockade with Lugol's iodine, 2 animals were injected intravenously with 10ug/kg $^{131}$I UJ13A. To calculate the injected dose the syringe was counted through 10 sheets of perspex, before and after injection of isotope. Measurements were made using a medium energy collimator (410KeV) and a 20% window. Dose estimates were ascertained from the derived efficiency of the gamma camera for counting $^{131}$I in the relevant syringe volume (Chapter 10).

The 2 animals had a total of 7 blood samples withdrawn from the femoral vein over the time interval 0.25-26.80 hours. After each blood sample an equivalent volume of sterile 0.9% sodium chloride was infused into the femoral vein. The percentage of injected dose remaining in the vascular compartment at each time interval was estimated on the basis of a circulating volume of 9% body weight. To assess the distribution of the isotope at 4 hours, animals were imaged in the supine position.
The clearance of isotope from blood suggested a similar pattern of clearance to the patients, and was significantly different to the rate of clearance in mice. Fig 4.2. The scintigrams demonstrated accumulation of isotope in the hepatic region but in contrast to the pattern observed in patients, no splenic uptake was discerned. This may have represented a true interspecies difference in biodistribution of conjugate, or may have reflected a difficulty in differentiating liver from spleen in a small animal. To improve spatial resolution in the subsequent studies, it was decided to radiolabel the antibody with $^{125}$I.

STUDY OBJECTIVES:

A new colony of 12 marmosets was established with a view to evaluating the biodistribution of $^{125}$I UJ13A; whole Ig, F(ab')$_2$ and Fab fragment. The aim of the study was; 1) to compare the pharmacokinetics of the different delivery systems, and 2) assess the impact of the host response on the pharmacokinetics of multiple administrations.

METHODS:

Fragment preparation.

Preparation of antibody fragments was undertaken with a concentration of protein determined by Miller's modification of the Lowry method (Chapter 2;4b).
\(F(\text{ab'})_2\) preparation.
The optimal time for digesting UJ13A had been established by a co-investigator. (Dr DH Jones, ICRF.) Using these conditions, 59.8mgs of protein-A purified UJ13A were digested in 0.2M sodium acetate/0.2M sodium chloride buffer.acetic acid, pH 4.05, with a 50:1 Wt:Wt ratio of antibody to pepsin. The reaction mixture was incubated at 37°C for a period of 7 hours and then dialysed extensively against 50mM ammonium bicarbonate/0.5M sodium chloride, pH 7.2. To remove low molecular weight digestion products, the fragment was dialysed in tubing with a 50,000 molecular weight cut off. The resultant digest was lyophilised and stored at -20°C until required.

Fab preparation.
The Fab monomer was prepared by papain digestion of UJ13A. This was undertaken in phosphate buffered saline with the addition of 2mM EDTA and 10mM cysteine as enzyme activators plus a 1:10 (wt:wt) ratio of papain to antibody. The reaction mixture was incubated under reducing conditions at 37°C for varying periods of time. Aliquots of the digest were removed at 2, 4, 6 and 8 hours and the reaction terminated by the addition of 500mM of iodoacetamide.

A 2 hour reaction time was found to be satisfactory, with a single band evident on SDS polyacrylamide gel electrophoresis. (Fig 4.3).
Fig 4.3. Polyacrylamide gel electrophoresis of fragments of UJ13A. 8% gel, stained with Coomassie Blue. 
(M) Molecular weight markers, 
(a) Preparative pepsin digest, 
(b) Papain digest undertaken for different digestion times.
**Fig 4.3** 8% polyacrylamide gel stained with coomassie blue.

a) $\text{Fab}_2$  
   b1) 2h  
   b2) 4h  
   b3) 6h 
   b4) 8h  

papain digest
A preparative digest was performed with a reaction time of 2 hours to produce a single batch of antibody for the primate experiments. To ensure removal of contaminating Fc chains, the digested protein was diluted in distilled water at 4°C and ultrafiltrated across a YM30 filter membrane. The buffering solution was exchanged for 50mM ammonium bicarbonate in the Amicon filtration unit, and the preparation lyophilised and stored at -20°C.

SDS polyacrylamide gel electrophoresis was performed to check the purity of the resulting preparation (Chapter 2;3a). Biological activity was estimated by an indirect immunofluorescence assay on neuroblastoma sections (Chapter 2;5c).

Radiolabelling.
UJ13A and its fragments were radiolabelled with $^{123}$I supplied by Harwell. Using a modified Chloramine-T technique, protein was radiolabelled to a specific activity of 1.4-4.6uCi/ug of protein (Chapter 2;6a). The biological activity of the preparations was determined before and after radiolabelling by an indirect immunofluorescence assay. Free iodine was estimated either by TCA precipitation or by instant thin layer chromatography (ITLC) in a solution of 85% methanol. Protein aggregation was determined by centrifugation of an aliquot of the preparation in PBS with 2% foetal calf serum at 10,000 x g.(Chapter 2;6c,6d)
Animal studies.

3 animals were entered into groups to receive exposure to either whole immunoglobulin, F(\text{ab}')_2 fragment, Fab fragment or a solution of ^{123}\text{I}. Prior to the administration of the radiolabel, thyroid blockade was undertaken by adding 3 drops of Lugol's iodine solution to a liquid feed. Under Halothane, N_2O/O_2 anesthesia, all animals had 0.5 mls of blood withdrawn and stored as plasma. Experimental animals were placed supine on the gamma camera and injected intravenously with 10\mu g/kg of radiolabelled protein dissolved in a 300ul of sterile phosphate buffered saline. Control animals received 300ul of a solution of free ^{123}\text{I}. All administrations were followed by a further injection of 200ul of 0.9\% saline to flush the syringe and needle. Repeat administrations of the radiolabelled conjugate were performed on 3 further occasions 4-8 weeks apart.

Measurement of the injected dose was performed by counting the syringe and needle on the gamma counter before and after injection. The early biodistribution of the conjugate was studied using the gamma camera. The radiolabelled conjugate was injected with the animal lying supine on the camera. A dynamic acquisition series was commenced at the moment of injection in 15 x one minute frames. Information on organ half life was obtained by sequential static scintigraphy over the time period 1 hour to 48 hours. The frequency and duration of
scanning was determined by the rapidity of fall of counts to background level.

Time activity curves were generated for the vascular compartment by intermittent venous sampling over the time period 30 minutes to 24 hours. A further blood sample was obtained at approximately 4 weeks after injection. Plasma was separated off and stored at -70°C.

Preparation of Rabbit anti-Marmoset immunoglobulin and measurement of an anti-mouse immunoglobulin in marmoset sera.

Immunoglobulin was purified from the sera of marmosets by ammonium sulphate precipitation (Chapter 2:2a). The immunoglobulin was emulsified in complete Freunds adjuvant and 2mg of the protein preparation was injected subcutaneously into rabbits at multiple sites. Injections were repeated at 5 x monthly intervals using Ig emulsified in incomplete Freunds. Rabbit anti-marmoset Ig was affinity purified using an affinity column of marmoset Ig coupled to cyanogen bromide activated Sephadex 4B. After dialysis against PBS, the immunoglobulin was concentrated to 1mg/ml and radiolabelled with $^{125}$I to a specific activity of 100uCi/ug.

To assay for circulating anti-mouse immunoglobulin response, monoclonal antibody UJ13A was coated onto 96 well vinyl plates. This was undertaken by incubating each well with 1 ug of purified protein overnight at 4°C. Plates were washed twice in PBS/1%BSA before incubation
with PBS/1%BSA for 1 hour at 37°C. Serial dilutions of control and test plasma were added to the wells and incubated at 37°C for a further 1 hour. The plates were washed twice with PBS/1%BSA before adding 100,000 cpm of $^{125}$I radiolabelled rabbit anti-marmoset Ig. Following a further 1 hour incubation, plates were washed twice in fresh buffer. The proportion of counts bound was assessed by counting individual wells in a LKB ultra-gamma counter.

**Data analysis.**

**Dynamic acquisition curves.**

To construct a dynamic acquisition curve, a 5 pixel "region of interest" (ROI) was constructed over the estimated centre of the organ. Counts per minute/unit area (cpm/ua) were recorded from sequential scintigrams. To enable a comparison of organ uptake during successive administrations of radionuclide, counts were normalised for injected dose. This was undertaken by expressing the cpm/ua as a ratio of the injected dose. To minimise any error from daily fluctuations in camera efficiency, injected dose was expressed by the whole body count two minutes after injection.

**Vascular clearance of isotope.**

Time-activity curves were generated by converting the cpm/g of blood at different time points into the percentage of injected dose remaining in the vascular
compartment. The total activity in blood was determined by assuming a vascular compartment of 9% body weight. Data for 3 animals were corrected for isotope decay, pooled and represented graphically, on a semi-logarithmic plot.

Organ half lives.
The 5 pixel ROI was utilised to estimate the cpm/ua for sequential scintigrams. A further 5 pixel area over thigh was selected to represent background activity and the cpm in this area was subtracted from the organ cpm. The resulting estimate of organ uptake was graphed as a function of time on a semi-logarithmic scale.

RESULTS:
The pepsin digest of UJ13A resulted in a doublet of approximately 105 kDa and the papain digest as a single band of approximately 50 kDa on SDS polyacrylamide gel electrophoresis. (Fig 4.3)

The whole immunoglobulin and fragments were shown to be immunoreactive by indirect immunofluorescence assay on neuroblastoma tissue sections. Both the F(\(ab')_2\) and Fab fragment were assessed as having lost approximately 50% of their biological activity by this technique. A further reduction in biological activity was observed following radiolabelling (Table 2).
TABLE 2.

**Quality control of $^{123}$-I/U13A in sequential administrations of U13A.**

<table>
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<th>Method</th>
<th>%Agg</th>
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<th>BA*</th>
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<td>1/10</td>
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**Fab$_2$ fragment**

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<th>Method</th>
<th>%Agg</th>
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<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>TCA</td>
<td>NE</td>
<td>1/10</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>TCA</td>
<td>6</td>
<td>1/10</td>
<td>1/10</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>ITLC</td>
<td>31</td>
<td>1/10</td>
<td>1/10</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
<td>ITLC</td>
<td>2</td>
<td>1/10</td>
<td>1/10</td>
</tr>
</tbody>
</table>

**Fab Fragment**

<table>
<thead>
<tr>
<th>No</th>
<th>%I</th>
<th>Method</th>
<th>%Agg</th>
<th>BA</th>
<th>BA*</th>
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<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>TCA</td>
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<td>1/10</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>TCA</td>
<td>5</td>
<td>1/10</td>
<td>1/10</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>ITLC</td>
<td>8</td>
<td>1/10</td>
<td>1/10</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>ITLC</td>
<td>6</td>
<td>1/10</td>
<td>NE</td>
</tr>
</tbody>
</table>

No: number of animal exposure; %I: percentage of free iodine in labelled protein; Method: method used to determine free iodine (see text); %Agg: percentage of aggregated protein in the radiolabelled protein; BA: biological activity of unlabelled protein; BA*: biological activity of radiolabelled protein; NA: not immunoreactive; NE: not estimated.

Other parameters reflecting the quality of the injected product also varied. These included the level of protein aggregation and free iodine and were directly related to the nature of the $^{123}$I supplied during the course of the experiment.
Vascular clearance of UJ13A.

On first administration of $^{123}$I UJ13A (Ig), isotope was cleared relatively slowly from the vascular compartment Fig 4.4a. The clearance was biexponential, with a fast component having an effective half life of 4.8 hours and a second slower component with an effective half life of 19.6 hours. Isotope in the blood remained attached to immunoglobulin as in excess of 98% of the counts were precipitable with 10% TCA.

This was clearly different from the pharmacokinetics of isotope alone. Although a biphasic clearance curve for free iodine was suspected, the first component was so rapid that it could not be accurately measured. Only 20% of the injected dose remained in blood thirty minutes after injection. The mean half life of the second component was 6 hours. No significant change in the pharmacokinetics were seen with repeat exposure. Initial vascular loss was persistently estimated at approximately 20% of the injected isotope at each exposure. (Range 19-24%), and the mean effective half life of the second component was 5.5 hours, 5.2 hours and 5.7 hours on second, third and fourth exposure.

These results contrasted with those for repeated administrations of whole immunoglobulin. After each injection the clearance of radionuclide became faster. This was particularly marked for the first half life, where a sequential change from 4.8 hours to 0.3, 0.2 and 0.1 hours was seen on second, third and fourth
administration. A similar trend was demonstrated for the second component, where changes from 19.6 hours to 17.3, 14.4 and 11.0 hours were documented on successive exposures. Figs. 4.4b,4c,4d. Variability became more marked with each injection of 123I UJ13A, as indicated by the dispersion of counts around the mean.

The rate of clearance of the Fab fragment from blood lay between those observed for the whole immunoglobulin and the free isotope. Suprisingly the kinetics of the F(ab')2 fragment more closely mirrored the distribution of the free isotope.

For the Fab fragment an initial loss of 50% of the injected activity occured from the vascular compartment. The initial clearance phase described an effective half life of 0.26 hours. The pattern of early clearance was similar on successive exposures with subsequent half lives of 0.27, 0.25 and 0.27 hours. (Figs. 4.5a,5b,5c and 5d). Some variation in the estimate of the late clearance component was seen, but this did not follow any consistent trend.

Administration of the F(ab')2 fragment resulted in a faster early clearance of isotope from the vascular compartment. A rapid initial drop in vascular activity to approximately 20% of injected activity was measured. This was a consistent observation in successive administrations (Fig 4.6a,6b and 6c). Despite the initial kinetic similarity to free isotope, the second clearance component was between 2 and 2.8 x as long as documented for free isotope. In addition a clear
Fig 4.4. Clearance of $^{123}$I UJ13A from blood of marmosets. Sequential plots of estimated % of injected dose against time (Hours), for 1st (4a), 2nd (4b), 3rd (4c) and 4th (4d) administrations of $^{123}$I UJ13A.

Pooled date for 3 animals.
Fig 4.4c

Fig 4.4d
Fig 4.5. Clearance of $^{123}\text{I}$ UJ13A Fab fragment from blood of marmosets.
Sequential plots of estimated % of injected dose against time (Hours), for 1st (5a), 2nd (5b), 3rd (5c) and 4th (5d) administrations of $^{123}\text{I}$ Fab.

Pooled data for 3 animals.
Fig 4.6. Clearance of $^{123}$I UJ13A F(ab')$_2$ fragment from blood of marmosets.
Sequential plots of estimated % of injected dose against time (Hours), for 1st (6a), 2nd (6b) and 3rd (6c) administration of $^{123}$I F(ab')$_2$.

Pooled data for 3 animals.
**Fig 4.6a**

1st Administration

**Fig 4.6b**

2nd Administration
Fig 4.6c
difference in biodistribution was demonstrated by scintigraphy.

**Gamma camera scintigraphy.**

The qualitative differences in organ accrual of isotope is illustrated in the 2 hour scintigrams. Figs. 4.7a, 7b, 7c and 7d. In animals administered free isotope, diffuse, homogenous activity was measured over the head, thorax and abdomen. No organ could be identified in the first 15 minutes of scanning, with tissue distribution of isotope reflecting tissue mass rather than specific organ uptake. By two hours a region of activity was visible in the left hypochondrium, and a smaller amount around the mouth. Fig 4.7a. In later scans the activity in the left hypochondrium had dispersed, and isotope was diffusely distributed throughout the abdomen.

Analysis of the 2 hour scans after injection of $^{123}$I UJ13A indicated that the main tissue accumulating IgG was liver, whilst liver and kidney both accumulated the F(ab')$_2$ and Fab fragment. Fig 4.7c and 7d. The relationship between organ uptake of isotope and successive exposures to UJ13A was explored by examination of the dynamic acquisition series which were successfully obtained in 7/12 administrations of $^{123}$I UJ13A. These indicated a rapid early accrual of isotope in liver over the first 5 minutes. The rate of uptake was observed to slow over the following 8 minutes, before a decline in activity was observed. Fig 4.8a.
Fig 4.7. Biodistribution of UJ13A and its fragments as demonstrated by scintigraphy.
Scintigram of a marmoset taken 2 hours after 1st administration of radionuclide.

Fig 4.7a Following injection of free isotope, demonstrating accumulation in the left hypochondrium (S).

Fig 4.7b Following injection of $^{123}$I UJ13A (IgG), demonstrating accumulation in liver (L) and blood pool (H).

Fig 4.7c Following injection of $^{123}$I UJ13A (Fab), demonstrating accumulation in liver (L) and kidneys (K).

Fig 4.7d Following injection of $^{123}$I UJ13A (F(ab')$_2$) demonstrating accumulation in liver (L) and kidneys (K).
With successive exposures, dynamic acquisition studies indicated a progressive increase in the amount of radiolabel sequestered in the liver. Increasing hepatic accumulation of radiolabel was independent of fluctuations in the level of aggregated protein.

A similar analysis of hepatic and renal sequestration of isotope was made for the F(ab')\(_2\) and Fab fragment. The unchanging vascular distribution of isotope was reflected in similarity of the dynamic acquisition curves obtained over liver and kidney. Information was obtained on 8/12 exposures to the Fab fragment. After the initial rapid distribution of isotope to the liver and kidney, a plateau in the rate of isotope accrual was demonstrated. No quantitative or qualitative change in the pattern of organ uptake was noted during successive exposures. Fig 4.8b.

The pattern of radiolabelled F(ab')\(_2\) accumulating in liver and kidney was inconsistent over the experimental period. Uptake of \(^{123}\text{I}\) F(ab')\(_2\) is illustrated with regard to a single animal (3 serial exposures). Fig 4.8c.

The first administration of biologically inactive protein resulted in rapid renal excretion of the antibody with minimal hepatic accumulation of isotope. Administration of the biologically active protein suggested an increase in hepatic uptake on the third exposure.
Fig 4.8. Dynamic acquisition curves for UJ13A and its fragments in liver and kidneys of experimental animals. Change of counts per minute/unit area with time, sampled from a 5 pixel region of interest over organ. Time 0-15 minutes after injection.

Fig 4.8a Curves for 3 different animals given $^{123}$I UJ13A (IgG), during 1st, 2nd and 4th administration.

Fig 4.8b Curves for 3 different animals given $^{123}$I Fab fragment, during 1st, 2nd, 3rd and 4th administration.

Fig 4.8c Curves for 1 animal given $^{123}$I F(ab’)$_2$ fragment, during 1st, 2nd and 3rd administration.

N.A. = Not available.
Fig 4.8a

Fig 4.8b
**Fig 4.9.** HPLC trace of unlabelled and radiolabelled F(ab')\(_2\) fragment. Solid line; protein trace as detected by changing optical density (280nm), broken line; radioactivity trace as detected by cpm/fraction.

Running conditions: Column Zorbax G250 gel filtration column. Solvent, 0.1M sodium acetate.0.5M sodium chloride, pH 6. Flow rate; 0.5mls/min.
Whole body and organ clearance of radionuclide.

The subsequent loss of isotope from organs and whole body was determined by static scintigraphy. Clearance of Ig described a monoexponential curve over the observation period. The rate of clearance followed the trend of the vascular loss and became increasingly rapid at each administration. The same phenomenon was observed for hepatic clearance of radiolabelled immunoglobulin. (Table 3).

TABLE 3.

Effective half lives of blood and organs following successive administration of $^{123}$I UJ13A.

<table>
<thead>
<tr>
<th></th>
<th>Blood T1/2(1)</th>
<th>Blood T1/2(2)</th>
<th>Liver T1/2</th>
<th>Whole body T1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.8</td>
<td>19.6</td>
<td>10.6</td>
<td>14.1</td>
</tr>
<tr>
<td>2</td>
<td>0.3</td>
<td>17.3</td>
<td>8.8</td>
<td>10.0</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>14.4</td>
<td>6.9</td>
<td>8.1</td>
</tr>
<tr>
<td>4</td>
<td>0.1</td>
<td>11.0</td>
<td>4.3</td>
<td>7.6</td>
</tr>
</tbody>
</table>

Half lives are expressed in hours. Values represent a mean of 3 observations.

Observations on the clearance of the Fab fragment were confined to the whole body clearance, where mean effective half lives of 6.0, 7.5, 5.5 and 5.0 hours were recorded for injections 1, 2, 3 and 4 respectively. Analysis of data for the hepatic half life was not practicable as the conjugate cleared very rapidly from this organ. By 6 hours after injection count rates were similar to that measured in background.
Unlike the Fab fragment, hepatic count rates remained persistently elevated over background during the scanning period. Measurements from the administration of the F(ab')2 fragment suggested a trend to faster clearance of the radiolabel from whole body and liver at successive exposures, although the interpretation of the results are once again complicated by the fluctuation in injected preparation.

**Interpretation of results with respect to the quality of the injected product and immune response.**

Interpretation of the results is complicated by the fluctuation in the quality of the injected radionuclide. The magnitude of the variation was larger than expected from experiences with ¹³¹I (Chapter 3). Variation in level of free iodine, aggregation and biological activity correlated with an apparently unpredictable labelling efficiency. (Table 4). During the course of the experiment it was noted that the supply of ¹²³I fluctuated in the concentration of sodium hydroxide contaminating the isotope. Examination of the radiolabelling results with respect to this parameter, suggested that the provision of ¹²³I in conjunction with high levels of sodium hydroxide was detrimental to the labelling procedure. This was manifest as a reduction in labelling efficiency and an increase in protein aggregation.
TABLE 4.

Variation in efficiency of labelling and protein aggregation with sodium hydroxide concentration of $^{123}$Iodine.

<table>
<thead>
<tr>
<th></th>
<th>HIGH</th>
<th>LOW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean.</td>
<td>Range.</td>
</tr>
<tr>
<td>mCi/mg NaOH.</td>
<td>15.5</td>
<td>11-18</td>
</tr>
<tr>
<td>Efficiency.</td>
<td>8.8</td>
<td>0-25</td>
</tr>
<tr>
<td>Aggregates.</td>
<td>15.3</td>
<td>3-36</td>
</tr>
</tbody>
</table>

Efficiency refers to the percentage incorporation of added iodine, into the monoclonal antibody. Aggregates refers to the percentage of aggregated protein present in the radiolabelled product.

The fall in labelling efficiency with the use of high sodium hydroxide content $^{123}$I, did not appear to reflect a pH related phenomenon. The $^{123}$I was supplied in dried form and reconstituted for radiolabelling in 0.1-0.8M phosphate buffered saline, pH 7.4. The final pH of the $^{123}$I solution was determined to be in the range 7.2-7.4 before addition to protein.

Whilst these results accounted for the variation in labelling efficiency and protein aggregation, they did not appear to account for fluctuations in the estimated levels of free iodine. This problem appeared to be principally confined to the radiolabelling of the $F(\text{ab}')_2$ fragment. To determine the nature of the problem, an aliquot of the radiolabelled $F(\text{ab}')_2$ protein was examined by high performance liquid chromatography and compared.
with the undigested parent protein UJ13A, and the
unlabelled F(ab')\textsubscript{2} fragment. (Performed courtesy of
Dr. S. Mather, ICRF, Lincoln's Inn Field, London.)

**Figs. 4.9**

The sequential runs of these proteins indicated that
a large proportion of the radioactivity was associated
with a small molecular weight fraction. The unlabelled
F(ab')\textsubscript{2} resolved into 5 major peaks. The position of the
first two with respect to the parent UJ13A indicated that
these peaks represented a small amount of undigested
immunoglobulin. The third peak, quantitatively the main
component of the F(ab')\textsubscript{2} digest, was interpreted as
equivalent to the major band demonstrated on SDS PAGE at
molecular weight 105 kDa. Two further fractions were
identified, at lower molecular weight.

Following radiolabelling, two additional fractions
were documented. Of these the major fraction was
estimated to have a molecular weight of 30 kDa, whilst
the last and smallest fraction was interpreted as free
isotope. These results were confirmed in a further
radiolabelling in which sequential 0.5 ml fractions were
collected after loading the protein onto a Sephadex G-25
column **Figs. 4.10a, 10b and 10c.** Column chromatography
with Sephadex G-50 and G-75 improved the recovery of the
F(ab')\textsubscript{2} fraction and suggested a method for improving the
purity of the fragment. (Chapter 7).
**Fig 4.10.** Radioactivity trace of $^{123}\text{I} \text{F(ab')}_2$ following gel filtration.

Fig 4.10a Profile following gel filtration on Sephadex G25. Column equilibrated in PBS/2% FCS.

Fig 4.10b Profile following gel filtration on Sephadex G50. Column equilibrated in PBS/2% FCS.

Fig 4.10c Profile following gel filtration on Sephadex G75. Column equilibrated in PBS/2% FCS.
Fig 4.10
Emergence of the anti-mouse immunoglobulin.

In view of the problems encountered with the radiolabelling of the F(\text{ab'}\text{)}_2 fragment, it was decided to restrict the analysis of circulating anti-mouse Ig response to the plasma of the control group and those animals receiving whole IgG and the Fab fragment. Sufficient plasma was available only on a limited number of antibody exposures. This was because initial difficulties were encountered in setting up the assay system, using up precious amounts of stored serum. Sequential titres were available on a single animal administered whole immunoglobulin, and in 3 animals in both the control and the group administered Fab fragment.
TABLE 5. Percentage specific binding, refers to the proportion of $^{125}\text{I}$ Rabbit anti-marmoset Ig to serial dilutions of marmoset serum. This is expressed as the counts bound in wells containing marmoset serum and UJ13A, as a percentage of added counts. (Mean of 3 observations).

The data records the level of specific binding for sequential administrations (1-4) of free isotope, Fab fragment and IgG, at different dilutions of marmoset serum. The data is represented graphically in Fig 4.11.
Results.

**TABLE 5.**
Specific binding of 125I Rabbit anti-marmoset Ig to circulating anti-UJ13A Ig in the serum of marmosets exposed to free 123I.

**Percentage specific binding.**

<table>
<thead>
<tr>
<th>Dil</th>
<th>539M</th>
<th>135M</th>
<th>543F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>1.</td>
<td>2.</td>
<td>3.</td>
</tr>
<tr>
<td>1/5</td>
<td>1.1</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>1/10</td>
<td>1.1</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>1/20</td>
<td>1.0</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>1/40</td>
<td>0.7</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>1/80</td>
<td>0.5</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>1/160</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>1/320</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Specific binding of 125I Rabbit anti-marmoset Ig to circulating anti-UJ13A in the serum of marmosets exposed to 123I Fab fragment.

**Percentage Specific Binding.**

<table>
<thead>
<tr>
<th>Dil</th>
<th>539F</th>
<th>540M</th>
<th>603M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>1.</td>
<td>2.</td>
<td>3.</td>
</tr>
<tr>
<td>1/5</td>
<td>1.1</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>1/10</td>
<td>1.0</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>1/20</td>
<td>0.9</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>1/40</td>
<td>0.6</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>1/80</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>1/160</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>1/320</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
</tbody>
</table>

Specific binding of 125 I Rabbit anti-marmoset Ig to circulating anti-UJ13A in the serum of marmoset exposed to 123 I whole immunoglobulin.

**Percentage Specific Binding.**

<table>
<thead>
<tr>
<th>Dil</th>
<th>540F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
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</tr>
<tr>
<td>1/5</td>
<td>1.2</td>
</tr>
<tr>
<td>1/10</td>
<td>1.9</td>
</tr>
<tr>
<td>1/20</td>
<td>2.8</td>
</tr>
<tr>
<td>1/40</td>
<td>1.3</td>
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<tr>
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<td>1.3</td>
</tr>
<tr>
<td>1/160</td>
<td>0.4</td>
</tr>
<tr>
<td>1/320</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Specific binding of 125I Rabbit anti-marmoset Ig to circulating anti UJ13A.

**FIRST EXPOSURE.**

- 539M
- 135M
- 543M
- 539F
- 540M
- 603M
- 540F

**SECOND EXPOSURE**

- 539M
- 135M
- 543F
- 539F
- 540M
- 603M
Specific binding of rabbit anti marmoset Ig to circulating anti UJ13A.

**THIRD EXPOSURE.**

![Graph showing specific binding of rabbit anti marmoset Ig to circulating anti UJ13A for the third exposure.](image)

- 539M
- 135M
- 543F
- 539F
- 540M
- 540F

**FOURTH EXPOSURE.**

![Graph showing specific binding of rabbit anti marmoset Ig to circulating anti UJ13A for the fourth exposure.](image)

- 539M
- 135F
- 543F
- 539F
- 540M
- 540F

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Within the limits of the available sera, the results indicate a serial rise in anti-mouse Ig titre in the animal receiving the whole immunoglobulin. Fig 4.11. No such response was demonstrated in the iodine control group or in 2/3 animals in the Fab group. Animal 603 was recruited late in the study to replace an animal that died from infective causes. Consequently only two administrations of 123 I Fab were possible during the time course of the experiment. However, there is an indication of a rising anti-UJ13A Ig titre in this animal.

**DISCUSSION.**
This study set out to investigate whether fragments of UJ13A offered any advantages over whole immunoglobulin as targeting agents. It is clear from the data that repeated administration of whole immunoglobulin results in rapidly changing pharmacokinetics. On the basis of the serum available from animal 540F, this would appear to correlate with a rise in anti-mouse titre. Unfortunately, it was not possible to measure serial titres in the other animals administered whole immunoglobulin, due to initial difficulties in establishing the assay system. The small volumes of serum obtained from animals, were quickly utilised in a less sensitive assay system based on measuring serum complex formation with UJ13A and Clq, and in an unsuccessful attempt at the assay system finally used in the study. The preliminary attempts at using the method
described in the thesis were frustrated by an external supply of non-immunoreactive Rabbit anti-marmoset Ig. Production of our own polyclonal sera resulted in a successful radio-immunoassay.

However, for animals in the IgG group, changes in kinetics were very similar suggesting a common underlying cause. The rapid early loss of isotope from the vascular compartment, and commensurate increase in hepatic sequestration are entirely consistent with a rising circulating anti-mouse Ig in all animals.

The production of such immune complexes does not favour successful tumour targeting. A significant proportion of the injected dose, is complexed and not available for radiation therapy. Immune complexes are cleared through the reticulo-endothelial system, increasing radiation dose to dose limiting organs such as bone marrow.

The results from the animals receiving the $^{123}$I Fab fragment indicate that the strength of the immunological response is diminished by the use of this conjugate. However, animal 603 suggests that the response may vary between individual animals. This is consistent with the observations made in clinical studies, in which the anti-mouse Ig response has been characterised as principally anti-Fc, but with emergence of an anti-idiotypic response in some patients (75).

The low incidence of anti-murine Ig in the Fab group is accompanied by a stable biodistribution of isotope following serial exposures. If these observations can be
reproduced in the human population, the data would suggest an exploitable advantage of the Fab fragment over whole immunoglobulin. Several additional factors favouring the use of the Fab fragment as a targeting agent are suggested by this study. Firstly, a reduction in whole body radiation dose is likely because of the rapid excretion of the radionuclide. The rate of fall of $^{123}$I UJ13A administered as the Fab fragment is 1-2.4 x that of whole IgG.

A second feature of interest is the apparent increase in the volume of distribution of the Fab fragment. Despite the early distribution of isotope to the kidneys, the rapid clearance of isotope from whole body does not appear to be due to renal filtration of conjugate. This can be deduced from comparing the vascular and whole body clearance patterns. Vascular loss of isotope occurs in at least two phases with a rapid initial fall to approximately 45% of the injected dose. Clearance of isotope from the whole body is monoexponential over the same period indicating loss to a second compartment. This is consistent with the work of several authors who have studied the biodistribution of fragments in a variety of species (152)(153)(154). Covell et al. constructed a compartmental model of the biodistribution of monoclonal antibody and its fragments in mice. Based on tissue resection data they deduced that the Fab fragment has a larger distribution volume than whole IgG, that it distributes more rapidly, and that the fragment produces higher interstitial:plasma ratios of conjugate.
However, the number of cycles of the Fab fragment through interstitial space before metabolism and excretion are considerably smaller than that observed for IgG. (Ratio IgG:Fab 7:1) This may be an important kinetic factor in reducing the efficacy of the Fab fragment for targeting.

A further limitation to the potential use of the Fab fragment is the likely reduction in association constant produced on preparation of an univalent fragment. The importance of this parameter is greater with low affinity antibodies and requires characterising for the UJ13A Fab fragment.

In previous studies the kinetics of the F(ab’)2 fragment have fallen between those described for the whole immunoglobulin and its Fab monomer. This has not been borne out by the observations on the vascular loss of the radiolabelled F(ab’)2 fragment of UJ13A. This apparent paradox is explained by examining the change in composition of the F(ab’)2 fragment on radiolabelling. Radiolabelling has resulted in the preparation of fragment contaminated by a small molecular weight species, which is presumably cleared in a rapid manner. It is not apparent from the comparative HPLC traces of radiolabelled and unlabelled F(ab’)2 whether this represents the preferential radiolabelling of a small molecular weight contaminant, or if the radiolabelling procedure has resulted in degradation of the F(ab’)2 protein. This is of more than academic importance. The advantages of the Fab monomer in reducing the anti-mouse Ig response may be extrapolated to the F(ab’)2 fragment.
A similar shift in biodistribution may be expected with a potential advantage of using a bivalent species. These advantages are only likely to be realised if the problems with the $F(ab')_2$ preparation result from a contaminant in the purified protein.

Several strategies are possible. The initial $F(ab')_2$ preparation requires better initial purification. This was not initially attempted by size fractionation using matrices such as Sephadex column chromatography, as UJ13A is reported to specifically bind this compound. (Personal communication, Dr JT Kemshead, ICRF Oncology Unit, Institute of Child Health, London.) Alternative methods such as ion exchange chromatography may prove useful.

Degradation of the protein due to radiolabelling conditions, may be circumvented by exploring alternative radiolabelling strategies. The Chloramine-T technique subjects the protein to relatively harsh oxidation and reduction. Other techniques such as the Bolter-Hunt method may be less injurious. Continued degradation, despite changing radiolabelling conditions, may be improved by column chromatography of the radiolabelled product. Production of a purer $F(ab')_2$ may be produced by column chromatography of the radiolabelled product. It would appear from experiments with Sephadex G25, 50 and 75, that this approach is feasible. No binding of IgG was noted to Sephadex under the conditions of this experiment, and probably reflects the "blocking" effect of 2% carrier protein.
Further studies will proceed, to ascertain optimal conditions for preparation of a $\text{F}(\text{ab}{'})_2$ fragment of UJ13A. These are reported in Chapter 7.
CHAPTER 7.

Further Attempts At The Production Of A Stable F(ab')2 Fragment.

INTRODUCTION:

Two problems were identified in the preparation and subsequent use of the F(ab')2 fragment. (Chapter 6). These were the problems of purity and stability. High performance liquid chromatography of an aliquot of the protein demonstrated a small amount of whole immunoglobulin contaminating the F(ab')₂ digest, plus an additional amount of a small molecular weight protein of approximately 30 KDa. (Chapter 6, Fig 4.9). This result was based on an analysis of protein that had been lyophilised and stored at -20°C for a period of 8 months.

Several explanations are available for the presence of the small molecular weight contaminant. Either dialysis in tubing with a molecular weight cut off of 50,000 did not remove all of the low molecular weight digestion products, or degradation of the protein occurred whilst in storage or on reconstitution.

The second problem was the emergence of the small molecular weight fraction as an important contaminant on radiolabelling. The radioactivity trace obtained after HPLC of the radiolabelled protein, demonstrated a large amount of radioactivity associated with the small molecular weight fraction. This could have arisen from preferential radiolabelling of the small molecular weight
fraction, or from further degradation of the protein, under the radiolabelling conditions.

Using the recloned UJ13A it was decided to repeat the digests with pepsin and to assess whether the problems encountered in Chapter 6 were due to inadequate purification or instability in the fragment.

Repeat attempts at digesting UJ13A under the conditions used in the initial experiments persistently produced a low yield of fragment, with a high yield of the low molecular weight species. (Fig 5.1) In view of this a repeat pH, time course experiment was undertaken to find the optimal conditions for digestion of the recloned UJ13A.

**Establishing the optimal time and pH for digestion of UJ13A.**

UJ13A was prepared for this experiment from the supernatant of large scale tissue culture. The protein concentration of the protein-A purified antibody was determined by Miller's modification of the Lowry technique, as 1.6 mg/ml. Four 5mg aliquots of monoclonal antibody UJ13A were dialysed against 0.2M sodium acetate/0.2M sodium chloride.acetic acid buffer at pH intervals 3.5, 4.0, 4.5 and 5.0. Digestion of the protein was commenced with insolubilised pepsin which had been cross linked to beaded agarose. The ratio of enzyme to UJ13A was maintained at a level of 64 i.u. of enzyme activity/mg of monoclonal antibody.
The reaction mixture at each pH interval was incubated at 37°C for time periods of 2, 4, 6 and 24 hours. To terminate the reaction, the reaction vessel was cooled rapidly to 4°C, and the pH adjusted to above pH 6 by adding 0.5M Tris.HCl buffer, pH 8.8. The reaction vessel was centrifuged at 10,000 x g for 5 minutes to separate out the insolubilised pepsin. Each aliquot of protein was then dialysed overnight at 4°C against phosphate buffered saline pH 7.4.

The dialysed protein was recentrifuged at 10,000 x g for a further 5 minutes to remove any residual pepsin. An aliquot of each reaction time and pH interval was analysed by SDS polyacrylamide gel electrophoresis.

Comparison of the different digestion conditions indicated that a digestion time of 4 hours, at pH 4 was satisfactory. (Fig 5.2). Under these conditions the major products of enzyme digestion were two protein bands of molecular weight 112,000 Da and 103,000 Da. Small amounts of undigested UJ13A and protein at an estimated molecular weight of 65,000 were visible.

All further digests were undertaken at pH 4, and digested for 4 hours. In a larger scale pepsin digest, 10 mgs of UJ13A yielded 5.4 mgs of digested protein, representing a 46% loss of protein during digestion.
Fig 5.1. Polyacrylamide gel electrophoresis of pepsin digest of recloned UJ13A. 10% gel, stained with Coomassie Blue. UJ13A digested under the conditions outlined in Chapter 6. (Channels b, c and d). Channel a: Molecular weight marker at 29kDa.

Fig 5.2. Polyacrylamide gel electrophoresis of pepsin digest of recloned UJ13A. 10% gel stained with Coomassie Blue. UJ13A digested for different time periods at different pH's.

pH intervals: 3.5, 4.0, 4.5 and 5.0.
Time intervals: 2 hours, 4 hours, 6 hours and 24 hours.

Molecular weight markers not shown.
Removal of undigested immunoglobulin and estimation of the proportion of small molecular weight contaminants.

A further 17.6 mg of UJ13A was digested. The protein preparation was stored frozen in phosphate buffered saline until required.

The removal of undigested immunoglobulin was attempted by affinity chromatography with protein-A sepharose.

2.43 mg of the digest was loaded onto the column at pH 7.4, using phosphate buffered saline as the binding buffer. Bound protein was eluted with 0.1M citrate buffer, pH 3. Successive fractions of unbound and bound protein were collected. Protein concentration was determined by optical density (Chapter 2.4a).

The protein eluted in two peaks, with the unbound fraction accounting for 60% of the total protein loaded on the column. A comparison of the protein mixture applied to the column and the protein in the unbound fraction was made by SDS polyacrylamide gel electrophoresis. (Fig 5.3). The appearances of the two protein fractions suggested that protein-A column chromatography removed the undigested immunoglobulin, but had no impact on the contaminant at 65,000.

Because of the gel composition and the technical difficulty in fixing peptides, this technique was insensitive to the presence of small molecular weight fragments. Aliquots of both protein mixtures were analysed further by size exclusion chromatography in a fast protein liquid chromatography (FPLC) system. The
Fig 5.3. Polyacrylamide gel electrophoresis of pepsin digest, pre and post Protein-A column chromatography. 8% gel, stained with Coomassie Blue. (M) Molecular weight markers. (a) 4 hour digest at pH 4, pre Protein-A column chromatography. (b) An aliquot of (a) post Protein-A column chromatography.
Fig 5.3

M  a  b

116-

67-
Fig 5.4 Size exclusion chromatography of pepsin digest (a) Pre-, (b) Post-, protein-A affinity chromatography. Superose column, run at 0.75 ml/min, in PBS.
initial protein digest eluted from the Superose column as a single major peak with two shoulders (Fig 5.4a). Comparing the chromatographic profile with the polyacrylamide gel electrophoresis, the two shoulders are interpreted as whole immunoglobulin and its Fab fragment at approximately 65,000. The loss of the IgG fraction, following protein-A column chromatography, is reflected in the loss of one of the shoulders (Fig 5.4b). The small molecular weight contaminants account for approximately 20% of both the original digest and the protein A purified preparation.

Stability of the digest.
The stability of this digest was ascertained, by investigating any changes in composition with time. A further 10 mgs of UJ13A were digested, and dialysed against PBS. 5.4 mgs of protein (54% of the original protein) was recovered from the dialysis tubing, at a concentration of 0.47 mg/ml. The protein was divided into three batches, and concentrated to 0.94 mg/ml, 1.88 mg/ml and 4.7 mg/ml, by placing the solution in dialysis tubing and surrounding with dry Ficoll.

An aliquot from each solution was drawn at time 0, and examined by FPLC, size exclusion chromatography. The remainder of the concentrated protein was stored at 4°C, and aliquots were drawn and examined at 1 hour, 4 hours and 24 hours. Over the time course of the experiment, there was no appreciable change in the chromatographic profile of the digest at any
concentration. The results are illustrated by the sequential chromatograms of the digest, concentrated to 4.7 mg/ml. (Fig 5.5).

Removal of the low molecular weight contaminant.

To remove, the low molecular weight contaminant, 15 mgs of UJ13A were redigested. After correcting the ph to >6 and centrifuging off the insolubilised pepsin, the digest was filtered in a 20ml capacity Amicon filtration unit, fitted with an XM50 filter. (50,000Da cut off). The buffering solvent was exchanged for PBS, three times during ultrafiltration.

Residual protein in the ultrafiltration chamber was Protein A affinity purified, concentrated to 3 mg/ml by further ultrafiltration, and frozen at -20°C for further analysis. The yield of protein after digestion, purification and reconcentration was 3 mgs. i.e 20% of the original protein. By SDS PAGE, the protein mixture, separated into two fractions, a doublet at approximately 105 KDa, and a second at 55 KDa. However, it was evident on size exclusion column chromatography, that a small amount of low molecular weight digest products remain (Fig 5.10). The protein separated once again into two major, biologically active fractions, and a smaller amount (<10%) of low molecular weight, biologically inactive protein. Examining sequential fractions by SDS PAGE, demonstrated a mixture of F(ab’)2 and Fab in the two main peaks, with F(ab’)2 predominating in the earliest fractions, and Fab in the later fractions. The
**Fig 5.5.** Size exclusion (Superose) column chromatography of pepsin digest.
Protein traces of pepsin digest at time 0 (a), and after 24 (b) hours at 4°C.
"Superose" column run at 0.75ml/min in 0.15M Phosphate buffered saline, pH 7.2. 50ul of solution at 4.7 mg/ml loaded on the column.
Fig. 10 Examination of the pepsin digest by size exclusion column chromatography, after protein-A affinity chromatography and ultrafiltration.

The pepsin digest of UJ13A was run on a Superose column, at 0.5 ml/min in PBS. Protein from peaks a, b and c were analysed by polyacrylamide gel electrophoresis. 8% gel, silver stained.

Red/white boxes represent fractions taken from along the Superose run.
low molecular weight contaminants, identified by column chromatography did not appear on SDS PAGE.

**Removal of the 65KDa contaminant.**
Several methods are available for further purification of the F(\(ab'\))\(_2\) fragment. These include size exclusion chromatography, affinity purification and ion exchange chromatography. A purified form of the UJ13A antigen, NCAM, was not available for affinity chromatography.

Size exclusion chromatography on Sephadex G150 had been tried by co-worker Dr DH Jones, during his initial work on the F(\(ab'\))\(_2\) preparation. Using a 1 metre column, he found that there was a high level of non specific binding to the column, further reducing the yield of fragment. Consequently, it was decided to attempt purification of the digest by ion exchange chromatography.

**Protein titration curve.** (Chapter 2;3g)

100 ug of the F(\(ab'\))\(_2\) digest was run by isoelectric focusing, across a ph gradient 3-10. (Fig 5.6). The mixture resolved into a maximum of 6 discernable bands at pH 8.15. The protein mixture migrated towards the cathode throughout the ph gradient. Consequently, a cation exchange column was chosen for separation using 0.05 M HEPES, pH 8.1 as the running buffer and 0.05 HEPES, 0.5M NaCl, pH 8.1 as the eluant.

**Cation exchange chromatography.** (Chapter 2;3j)
Fig 5.6. Protein titration curve of pepsin digest. Pepsin digest electrophoretically focused across a pH gradient 3-10.

Fig 5.7. Cation exchange column chromatography of pepsin digest. 50ul of protein digest loaded onto a "Mono S" cation column. Running buffer: 0.05M HEPES, pH 8.1, and eluted with a 0-100% sodium chloride gradient.
Fig 5.6

Fig 5.7
Fig 5.8. Polyacrylamide gel electrophoresis of fractions following cation exchange column chromatography and G150 gel filtration.
8% gel, silver stained.

Fractions 15, 16, 18, 19, 23 and 24, correspond to the fractions collected during cation exchange column chromatography, (Fig 5.7), and b and c refer to the fractions obtained during gel filtration (Fig 5.9).
**Fig 5.9** Separation of the pepsin digest by size exclusion chromatography.
Sequential protein fractions obtained on a 30 x 2 cm column containing Sephadex G150.
Fig 5.11 Polyacrylamide gel electrophoresis of sequential fractions of pepsin digest, after separation by Sephacyl S200 column chromatography. Each lane represents protein from sequential 1 ml fractions. Column dimensions 1m x 5cm. Flow rate, 1.5 mls/min.
Cation exchange chromatography, resolved the mixture into three main fractions, two of which were biologically active by indirect immunoflorescence on foetal brain. (Fig 5.7) Each fraction was examined by SDS PAGE, on a silver stained 8% gel. The two immunologically active fractions, b and c, ran as two major doublets with molecular weights of 105 and 65 KDa. No protein bands were detected in fraction a. This was assumed to correlate with the low molecular weight fraction demonstrated on size exclusion chromatography.

Gel filtration.
In view of the failure to separate F(ab')2 from Fab on cation exchange chromatography, it was decided to look again at the problem of non specific binding of protein to Sephadex.

G150 was swelled in an excess of PBS, pH 7.4 at 80°C for 5 hours. A 30 cm x 2 cm column was poured at room temperature, and allowed to equilibrate in PBS, flow rate 20 mls/hour. 500 ul of 1.3 mg/ml F(ab')2/Fab mixture was loaded on the column and washed through with PBS, at a flow rate of 10 mls/hour. Sequential 1ml fractions were collected and their optical density determined. (Fig 5.9).

Two protein peaks were determined by optical density. Each peak accounted for approximately 0.3mgs and 0.4mgs of protein. An aliquot of pooled fractions 47-53, and 67-72 were analysed by SDS PAGE. The silver stained gel demonstrated the familiar F(ab')2 doublet/Fab pattern in
the first peak, and no discernable protein in the second peak.

The column dimensions were increased to 100cm x 5cm, and packed with Sephacryl-S200, Superfine (Pharmacia). 3.8mgs of digest were loaded on the column at a flow rate of 1.5 mls/minute. Sequential 1 ml fractions were collected, and an aliquot of each run on an 8% polyacrylamide gel (Fig 5.11). The gel demonstrated that the F(ab')₂ could be separated from the Fab fraction, by collecting the early protein fractions.

DISCUSSION.
A small number of experiments have been undertaken to improve the preparation of UJ13A F(ab')₂ fragment. Affinity chromatography and ultrafiltration are available methods for reducing undigested IgG and small molecular weight contaminants, but they substantially increase protein loss. A single step method for fractionating the F(ab')₂ from all contaminants is desirable. Ion exchange chromatography was a likely method but proved to be unsuccessful at separating F(ab')₂ from Fab under the conditions described. The conditions were chosen after the methods of Haff et al. who validated the use of electrophoretic titration curves to predict optimal chromatographic conditions for FPLC of proteins. The protein titration curve indicated that optimal separation of the protein constituents in the digest would be achieved at a pH of approximately 8.1. Unfortunately,
this was not demonstrated under the conditions used.
Haff postulates that most of the discrepancies between
electrophoretic and chromatographic behaviour of proteins
may be explained by an inhomogenous charge distribution
on the protein. The relevance of this observation to the
chromatographic behavior of UJ13A requires further study.
Investigation of the charge distribution on the protein
by physical measurements, such as the dielectric
increment may be necessary.

The electrophoretic behavior of the F(ab’)_2 digest
was also unusual in suggesting a highly alkaline pI. The
protein mixture is never stationary across the pH
gradient, inferring that the pI is greater than 9. This
result has been confirmed by isoelectric focusing of the
digest in agarose. Over a pH gradient 3-10, the digest
was found to focus poorly at the extreme of the pH range.
(Measured as approximately pH 9.3).

Despite the apparently unusual electrophoretic
/chromatographic behavior of the digest, the difficulties
encountered in separating the Fab fragment from F(ab’)_2
is not dissimilar from those reported by Kurkela et al.
(155). They were unable to successfully purify pepsin
digested antibody by ion exchange chromatography, due to
overlapping pI values of the respective fragments. The
contribution of this factor to the failure of ion
exchange chromatography to purify the digest, may be
examined by determining the pI of our papain digest.
This has not yet been undertaken.
Kurkela successfully purified their digest by gel filtration using a Sephacryl S200 column. The yield of fragments was 24 +/- 11% of the theoretical maximum. The success of gel filtration as a single step method for purifying the fragment, is endorsed by the work of Parham who reports a yield of 10mg of pure F(\text{ab'})_2, from 100mg of an IgG_{2a} antibody after Sephadex G200 column chromatography (156).

Despite the pessimistic results of initial experiments undertaken at this laboratory, repeat gel filtration with Sephadex G150 did not result in any appreciable protein loss on the column. Although both Fab and F(\text{ab'})_2 were detected in the first protein peak, analysis was undertaken with a small column, and on a portion of the pooled antibody. Increasing the dimensions of the column produced an adequate separation of F(\text{ab'})_2, from the Fab monomer. This result is entirely consistent with the work of Parham, who reports successful separation of F(\text{ab'})_2 from Fab on columns of 100-150 cm length in preparative amounts. Consequently, this method remains the most promising method of purifying the F(\text{ab'})_2 digest. More experiments are required to finalise this, and quantify the final yield. This work is continuing, along with further assessment of the stability and affinity of the resulting fragment.
The Biodistribution Of meta-Iodobenzylguanidine (mIBG) In Animal Models.

Establishing animal models:
Meta-Iodobenzylguanidene is an alternative radiopharmaceutical for radiation targeting. (See Chapter 1). As with monoclonal antibodies, the potential of the approach hinges on the differential dose delivery between tumour and normal tissues. This chapter concerns itself with the biodistribution of mIBG in animal models.

The first portion of the study characterises the in vivo distribution of mIBG in two animal systems. The nude mouse was chosen as the first model, because of its capacity to host a human neuroblastoma xenograft. $^{131}$I mIBG has also been evaluated is the common marmoset, so that a specific comparison of radiation dose delivery could be made between immunoglobulin and mIBG.

METHODS.
Mice:
mIBG was radiolabelled with $^{125}$I to a specific activity of 2.5 uCi/ug. (Chapter 9).

Following thyroid blockade with Lugol's iodine, 58 nude mice were injected through a tail vein with 0.1mls of mIBG ($2.14\times10^6$ cpm). Animals were killed by cervical dislocation at time intervals 2, 5, 15, 30 minutes and 1, 2, 4, 6, and 24 hours after injection. A sample of blood was obtained at death by severing the carotid artery, and
aspirating into a 1ml syringe. Liver, kidney, spleen, heart and lung were dissected out and placed in pre­weighed tubes. Tubes were re-weighed and counted for radioactivity in a LKB ultra-gamma counter.

The percentage of isotope in any organ was graphed as a function of time. (Figs 6.1,2). The blood volume of the nude mouse had been previously estimated by the Fick principle as 1 ml, and the percentage of injected dose in blood was calculated on this basis. (Personal communication Dr D.H. Jones, Imperial Cancer Research Fund, ICH).

During the course of the experiment the 6 animals forming the 24 hour group were kept in a metabolic cage. A 24 hour urine collection was performed, and an aliquot of the urine analysed by HPLC for excretory products of mIBG. (Courtesy of Dr S.Mather, Imperial Cancer Research Fund, Lincoln's Inn Field, London).

Marmosets:
The purpose of this experiment was two fold. Firstly, to quantitate organ uptake of mIBG in the primate, and secondly, to estimate the gamma-camera efficiency for counting $^{131}$I in "organs of interest".

This was undertaken in a group of 12 animals who had been previously used to investigate the biodistribution of intrathecally administered monoclonal antibodies. The group had not been used in the preceding month and no residual radioactivity was measured by gamma scintigraphy above background levels in the animal group.
Thyroid blockade was undertaken by the addition of Lugol's iodine to the water supply for 48 hours before administration of mIBG. Under Halothane/nitrous oxide anesthesia, each animal was injected with 20 ug/kg of $^{131}$I mIBG (obtained from Amersham International: S.A.2.5uCi/ug), then scanned with the Siemens gamma camera fitted with a medium energy collimator at 410keV. Scintigraphy was performed at time points varying between 20 minutes and 26 hours from injection.

Immediately following scintigraphy, animals were killed by inhalation of excessive halothane and nitrous oxide. Organs were removed by tissue dissection and blood removed by cardiac puncture. Tissue was weighed and counted in an LKB ultragamma counter.

A standard "region of interest" was created over tissues visualised on the gamma camera, and the counts per unit area at the time point immediately before death were correlated with counts/gm of tissue in the relevant organs. In this way tissue attenuation for $^{131}$I was established for each organ. Additionally, time-activity curves were generated from this resection data.

During the first hour after administration of the isotope, aliquots of urine were collected from animals. This was achieved by lining the base of the animals' recovery box with plastic 96 well microtitre plates. Urine was aspirated from the wells and was analysed for the presence of mIBG and metabolites, by comparing excreted radioactivity with the administered radionuclide on thin layer chromatography (TLC). (Chapter 2:6e)
Fig 6.1. and 6.2. Clearance of $^{125}$I mIBG from tissues of mice. Graphs show the mean percentage of injected dose/g. of tissue with time. The range of results in a minimum of 6 animals are also shown.
Fig 6.1

Fig 6.2
A similar exercise was undertaken for isotope in plasma. A single aliquot of plasma was examined by TLC and compared with the administered mIBG at 20 minutes after injection.

**RESULTS:**

Following intravenous injection in the mouse, there was rapid loss of isotope from blood. The mean percentage of injected dose remaining in the blood at 30 minutes from injection was 1.55%/g. (Range: 1.28-1.99, N=7). Table 1, Fig 6.1. The isotope was rapidly distributed to the various tissues. All tissues with the exception of liver and spleen demonstrated an immediate fall in activity with time. (Figs 6.1,2). In contrast, a rise in activity was measured in the liver in the first 30 minutes after injection, before decreasing at a rate of 1.8%/g/hr. This rapid rate of excretion was not sustained. By 24 hours, the rate had slowed, although there were insufficient time points to define the half life of this phase. A similar pattern was demonstrated in spleen, although the initial phases of isotope acquisition and loss were of shorter duration. Early splenic uptake lasted for a period of approximately 15 minutes, before isotope levels began to drop.

Analysis of injected mIBG and its excretory products in urine by HPLC, is demonstrated in Fig 6.3. The radiolabelled mIBG eluted in two fractions. The initial fraction comprised less than 5% of the total radioactivity and was interpreted as free
Fig 6.3  HPLC of administered mIBG (A) and excretory products in urine of mice (B).

Column Vidaq C18; Solvent 0.1% Trifluoric acetic acid
Gradient 0-50% acetonitrile.
isotope/iodide/iodate. The larger main peak was interpreted as mIBG. Examination of an aliquot of the 24 hour urine collection demonstrates a change in the elution profile of the radioactivity. Approximately 50% of the isotope excreted in the 24 hour urine collection eluted in the same fraction as the main peak demonstrated in the injected product. The remaining radioactivity eluted in three earlier fractions. The position of the first suggested that this was free isotope, the remaining two were assumed to represent metabolites of the mIBG.

**TABLE 1.**

The percentage injected dose per gram of tissue at different time points, after intravenous injection of $^{125}$I mIBG to Nude mice.

<table>
<thead>
<tr>
<th>TIME</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Heart</th>
<th>Lung</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>4.52</td>
<td>19.82</td>
<td>3.07</td>
<td>22.90</td>
<td>28.14</td>
<td>7.11</td>
</tr>
<tr>
<td>0.08</td>
<td>5.32</td>
<td>12.78</td>
<td>4.38</td>
<td>21.69</td>
<td>22.58</td>
<td>3.51</td>
</tr>
<tr>
<td>0.25</td>
<td>8.22</td>
<td>5.33</td>
<td>5.98</td>
<td>17.45</td>
<td>15.90</td>
<td>1.98</td>
</tr>
<tr>
<td>0.50</td>
<td>9.56</td>
<td>4.65</td>
<td>4.09</td>
<td>12.48</td>
<td>11.15</td>
<td>1.55</td>
</tr>
<tr>
<td>1.00</td>
<td>9.10</td>
<td>3.03</td>
<td>4.35</td>
<td>6.54</td>
<td>8.33</td>
<td>1.27</td>
</tr>
<tr>
<td>2.00</td>
<td>7.83</td>
<td>2.26</td>
<td>3.67</td>
<td>4.90</td>
<td>6.66</td>
<td>0.95</td>
</tr>
<tr>
<td>4.00</td>
<td>4.13</td>
<td>1.75</td>
<td>2.34</td>
<td>3.60</td>
<td>4.15</td>
<td>0.59</td>
</tr>
<tr>
<td>6.00</td>
<td>2.42</td>
<td>1.06</td>
<td>1.95</td>
<td>1.57</td>
<td>2.02</td>
<td>0.39</td>
</tr>
<tr>
<td>24.00</td>
<td>0.4</td>
<td>0.19</td>
<td>0.44</td>
<td>0.39</td>
<td>0.36</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Time is expressed in decimal fractions of hours. Each value for percentage of injected dose per gram of tissue, represents the mean value of the animals sacrificed at that time point (Minimum of 6 animals).

Marmosets.

Following injection of $^{131}$I mIBG, isotope rapidly cleared from the vascular compartment. Multiple blood samples taken from all of the animals, indicated that
Fig. 6.4. Clearance of $^{131}$I mIBG from blood of marmosets. Graph shows the relationship between % of injected dose in blood with time. (Pooled data from 12 animals).

Fig. 6.5. Comparison of radioactivity profile in urine of marmosets with administered mIBG. Urine and mIBG analysed simultaneously, by silica gel thin layer chromatography. TLC run in 3:1 ratio of propan-1 ol and 10% ammonium hydroxide. Radioactivity visualised by autoradiography.
Fig 6.4

Fig 6.5

URINE

0 - 20 mins
20 - 60 mins
0 - 20 mins
20 - 60 mins

MIBG
approximately 2% of the injected dose remained in the vascular compartment at 15 minutes (Range 1.5-2.5%, N=6). A further small decline in radio-activity was measured over the following 4 hours, before levels of isotope stabilised at 1.1% of the injected dose. (Table 2 and Fig 6.4).

During the period of rapid vascular loss of radionuclide, isotope accumulated in the kidneys. This was evident on both the 2 and 20 minute scintigrams. The level of radioactivity then fell rapidly, as by 2 hours the level of isotope in kidneys was at background levels. Measurements of isotope in resected kidneys indicated that the magnitude of the initial uptake was around 5% of the injected dose/kidney at 20 minutes after injection. (Range 4.0-5.8%, N=3) Table 2. Subsequent clearance of isotope was rapid with only 0.3% of the injected dose remaining in each kidney by 4 hours. (Range 0.2-0.3%, N=3).

The fall in whole body activity during this time period reflected the renal uptake of isotope. This indicated that the isotope was undergoing renal excretion rather than renal metabolism. This was confirmed by examination of urine in the time period 0-60 minutes. Excreted isotope was principally unchanged mIBG. Fig 6.5. In addition a small amount of larger molecular weight bands were identified in the urine. These were not necessarily metabolites of mIBG but appeared to reflect the excretion of compounds contaminating the injected product.
Data from resected organs, suggested a biphasic clearance pattern in all of the tissues studied. Figs 6.6. The first clearance phase was rapid in kidney, myocardium and liver, suggesting that this early phase was unlikely to contribute significantly to the final organ radiation dose. The major dose contribution appeared to come from retention of isotope in tissues after 4 hours. The rate of isotope clearance from liver was deduced to be monoeponential over the time period 4-24 hours, with an extrapolated maximal tissue uptake of 6.4% of the injected dose at time 0 and a Teff of 7.5 hours. A similar rate of clearance was noted for kidney, (Teff, 7 hours). Myocardial retention was longer, with an estimated Teff of 12.6 hours. Extrapolating the second clearance component for myocardium to the ordinate, resulted in an estimated tissue accumulation of isotope of 0.46% of the injected dose.

The only organ which persistently retained isotope over background levels, as measured by scintigraphy, was liver. The relationship between counts per minute/unit area and counts per minute in the resected tissue are shown graphically in Fig 6.7. The relationship appeared monoeponential over the range of activity in this
Fig 6.6. Clearance of $^{131}$I mIBG from the tissues of marmosets. Graph shows the change in % of injected dose for resected organs with time. (Pooled data 12 animals).

Fig 6.7. Relationship between counts per pixel, taken from a standard "region of interest" over liver, and the counts per gramm of tissue.
experiment.

**TABLE 2.**

**Percentage of injected dose of mIBG in tissues of Marmosets.**

<table>
<thead>
<tr>
<th>TIME</th>
<th>Blood</th>
<th>Heart</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Lung</th>
<th>Adrenal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>2.2</td>
<td>1.1</td>
<td>13.0</td>
<td>0.1</td>
<td>4.0</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>0.3</td>
<td>1.5</td>
<td>0.7</td>
<td>12.6</td>
<td>0.2</td>
<td>5.4</td>
<td>0.6</td>
<td>---</td>
</tr>
<tr>
<td>0.3</td>
<td>2.2</td>
<td>1.2</td>
<td>7.4</td>
<td>0.2</td>
<td>5.8</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>3.8</td>
<td>1.0</td>
<td>0.4</td>
<td>4.5</td>
<td>0.1</td>
<td>0.3</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
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</table>

Time is expressed in hours from administration of mIBG. Values represent the percentage of injected dose in resected organs of individual animals.

**DISCUSSION.**

The relevance of these two animals as model systems for further investigation of mIBG depend on how closely the biodistribution parallels the human distribution of mIBG. Much of the work on the pharmacokinetics of mIBG, and radiation dose extrapolations are based on kinetic observations in rats and dogs (157). These observations supplemented by information on two Rhesus monkeys form the base of human radiation dose estimates at the Radiopharmaceutical Internal Dose Information Centre, Oakridge Tennessee (158). From the two Rhesus monkeys
studied, the implication is that primates may have a higher hepatic accumulation of the compound than lower species.

The distribution of $^{131}$I mIBG has been reported in the human in a limited number of studies. Nakajo reported that mIBG invariably accumulated in liver, spleen and bladder in patients administered the compound for scintigraphy (159). Accumulation of isotope in the lung bases, colon and left ventricle was also seen but were a less frequent phenomenon.

Tissue uptake of mIBG has been quantitated in a study of 5 normal adults by Kline et al. (160). This study reported a rapid biphasic loss of isotope from the vascular compartment. The initial loss of isotope from the vascular compartment are reported as % kg dose/gm of blood. Assuming a mean body weight for the group of 60 kg, this would translate to approximately 6% of the injected dose present in the vascular compartment at 15 minutes from injection. Urinary samples were obtained at 3 hours from injection, at which time approximately 35% of the injected dose had been excreted. The pattern of mIBG clearance from heart was reported by Kline as monoexponential. Maximal uptake of isotope was estimated by scintigraphy as 0.63% of the injected dose (Range 0.45-0.78%).

The biodistribution of mIBG in children with neuroblastoma is reported both in Chapter 9, and in a larger study involving a collaboration between the Royal Marsden Hospital, Sutton, and the Hospital for Sick
Children, London (161). Similar rapid renal excretion of isotope is documented in the dynamic acquisition series resulting in an initial renal loss of between 11 and 26% of injected dose. No renal persistence of isotope is seen on later images.

Vascular loss and hepatic uptake of isotope was quantitated as a mean of 4.4% of the injected dose in blood at 60 minutes after injection. Initial hepatic uptake of isotope was 6.3%. These figures compare favourably with the initial distribution data in the marmoset. The pattern of isotope distribution is qualitatively similar, and the figures for initial hepatic uptake (6.4% marmosets v 6.3% patients), early vascular loss (98% marmosets v. 95.6% patients), myocardial uptake (0.46% marmosets v. 0.63% adult volunteers). However, the observations in marmosets differ in describing a biexponential fall in activity in the myocardium. A rapid initial loss of isotope of short duration is seen, followed by a longer clearance phase. Although this is not reported in the scintigraphic work of Kline, the observation is consistent with data on the behavior of the sympathetic ganglion blocker guanethidine.

The efflux of radiolabelled guanethidine from isolated slices of rat heart is also biphasic, with an initial rapid loss followed by a slower second phase (163). The data is taken to indicate loss from two kinetically distinct storage compartments. This conclusion is substantiated by the influence of
amphetamine on reserpine blocked heart slices. Reserpine blocks amine storage in neurosecretory granules. In spite of this, amphetamine is able to displace radiolabelled guanethidine from myocardium, suggesting that the drug may be bound to other cytoplasmic components. Our data suggests that a similar two compartment storage occurs in the marmoset myocardium. It is possible that loss of mIBG is similar in the human myocardium, but is not of sufficient magnitude to be detected by scintigraphic studies.

The nude mouse shows a similar tissue distribution of mIBG as the marmoset. However, the results in this experiment were not expressed as % of injected dose per organ and so it is difficult to make a quantitative comparison. Qualitatively, with the exception of the splenic accrual of isotope, it would appear that this animal will model the biodistribution of mIBG sufficiently closely to make some extrapolations from neuroblastoma xenograft experiments.
A Comparison Of The Biodistribution And Radiation Dose Delivery Of mIBG And UJ13A In Patients And Animal Models.

INTRODUCTION:
To make a direct comparison of the biodistribution and radiation dose delivery of UJ13A and mIBG, it was decided to administer both compounds serially in the same animal. This was undertaken in the marmoset, as the animal had been demonstrated to model the biodistribution of both compounds. (Chapters 6 and 8).

In view of the paucity of detailed clinical pharmacokinetic data for mIBG, a parallel investigation was undertaken in children with neuroblastoma.

Comparison of the biodistribution of mIBG and UJ13A in the marmoset.

METHODS:
UJ13A was prepared and radiolabelled with $^{131}$I as specified in Chapter 2;6a. $^{131}$I mIBG was obtained from Amersham International at a specific activity of 2.5 uCi/ug. Routine quality control was undertaken. The level of protein aggregation and free isotope in the radiolabelled UJ13A was assessed by high performance liquid chromatography, Chapter 2;5h, and the composition of mIBG by thin layer chromatography. (Chapter 2;6e).

Six marmosets, none of which had undergone any previous experimental procedure were used in the study. After thyroid blockade, and under Halothane/N₂O/O₂ anaesthesia, each animal was sequentially administered
$^{131}$I mIBG and $^{131}$I UJ13A over a period of three weeks. Doses were calculated so that each animal received approximately 10ug/kg of the radiolabelled product. Actual dose administered was estimated by counting the full and empty syringe on the camera face, before and after administration. The measured counts were converted to injected dose by comparison to a series of $^{131}$I standards counted on the gamma camera on the day of injection. In addition to quantifying administered dose, the standards acted as a check for fluctuations in camera efficiency over the time course of the experiment.

Using the camera settings outlined in Chapter 6, whole body scintigrams were performed at 1 minute, 20 minutes, 1,2 4, 6, 24 and 28 hours. Time-activity curves for whole body and liver were constructed using the methods outlined in Chapter 6.

Using the data generated for tissue attenuation of $^{131}$I in Chapter 8, cpm/ua were converted to cpm/g of liver. When the animals were culled, and the weight of the liver known, this data was finally converted to percentage of injected dose in the organ.

Aliquots of blood and urine were collected from animals receiving $^{131}$mIBG under the conditions described in Chapter 8. Blood was assayed for total radioactivity/gm, and in 2 animals, plasma was separated and examined by thin layer chromatography (Chapter 2;6e) for radioactivity. A similar exercise was undertaken on aliquots of urine at time intervals 0-20 minutes, 21-60 minutes and at 24 hours.
RESULTS.
The design of the experiment required the use of two batches of mIBG and two radiolabellings of UJ13A. The quality of the injected product varied with administrations. For the two separate batches of mIBG, densitometry scanning of the autoradiograph estimated 59% and 80% of radioactivity associated with mIBG respectively (Rf value; 0.5 and 0.53). The second largest isotope peak, ran with a Rf value of 0.7-0.87 and constituted 8 and 23% of the radioactivity (Fig 7.1). The two separate radiolabellings of UJ13A provided a product with no appreciable free iodine. The percentage of radioactivity associated with aggregated protein varied by 0 and 9%.

The biodistribution of the radiolabel followed the pattern detailed in Chapters 3, 6 and 8. After administration of mIBG, there was an initial rapid loss of isotope from the whole body. The early loss was sustained for a period of 1 hour and accounted for a mean of 17% of the injected dose (Range 12-21% N=6). During this time interval, the kidneys were clearly visible on successive scintigrams. After 1 hour the apparent renal uptake of isotope diminished and corresponded with a decrease in the rate of excretion of isotope from the whole body. The mean effective half life of the second whole body clearance component was estimated as 22 hours (Range:19-26 hours N=6). Table 1.
Fig 7.1 Densitometry scan of autoradiograph of injected mIBG-marmosets
TABLE 1.

Effective half life and maximal organ uptake of radiouclide:whole body.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Ao</th>
<th>Teff</th>
<th>Ao</th>
<th>Teff</th>
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</thead>
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</tr>
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<td>22</td>
</tr>
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<td>23</td>
</tr>
<tr>
<td>Mean</td>
<td>100</td>
<td>66</td>
<td>83</td>
<td>22</td>
</tr>
</tbody>
</table>

Where Ao is the maximal radioactivity present in whole body at time 0 as determined by extrapolating the second clearance phase to the ordinate and expressed as a percentage of injected dose. Teff is the effective half life in hours.

The correlation of renal imaging and the fall in whole body radioactivity suggested renal excretion of isotope. It was not possible to obtain a complete urine collection within the first hour to investigate the correlation. However, it was possible to examine aliquots of urine to study the pattern of urinary isotope excretion. Examination of urine collected between 0-20 minutes and 21-60 minutes revealed that the isotope was principally excreted as mIBG. Radioactivity with an Rf value equivalent to radiolabelled mIBG, accounted for a mean of 83% of radioactivity excreted in urine during the first 20 minutes, (Range 74-97% N=3). The pattern was maintained in the time period 21-60 minutes (Mean 83%, Range 74-92% N=4). A small amount of isotope was associated with fractions running behind the main mIBG band (Rf values; 0.48 and 0.52). These fractions were
Fig 7.2  Autoradiograph of mIBG and plasma of marmoset after silica gel TLC
present in all of the animals and time intervals studied, and corresponded to a similar band in the supplied mIBG. During this time period, radioactivity in blood fell to between 0.9 and 1.6% of the injected dose. Examination of an aliquot of plasma at 20 minutes indicated that greater than 90% of the radionuclide was present in the mIBG fraction. Fig 7.2.

No early period of renal excretion could be identified for $^{131}$I UJ13A. Clearance of conjugate from the whole body followed monoexponential kinetics. The rate of clearance was both variable between animals and prolonged. (Mean Teff:66 hours, Range 52-81 hours N=5). Extrapolating the main clearance component to the origin, and then calculating the accumulated activity in whole body per 1mCi of administered conjugate, the results indicate a mean of 3.6 x greater whole body dose from UJ13A than from mIBG. (Range 2.6-5.3, N=5), Fig 7.3.

Both mIBG and UJ13A were initially taken up by liver. This was evident on scintigrams as a steady rise in hepatic activity over the first hour. Following this initial accrual of activity, $^{131}$I UJ13A cleared at a rate described by a single exponential function. The clearance of mIBG followed a different excretory pattern, with an initial rapid loss, followed by a slower second phase. Fig 7.4.

By extrapolating the prolonged excretory phase to the origin, a value was obtained for estimated maximal organ uptake (Ao). This was assessed as 7% of the injected dose of administered mIBG, for the six animals undergoing
**Fig. 7.3.** Clearance of low specific activity (SA) mIBG from the liver of marmosets. Graph demonstrates the change in estimated % of injected dose with time for 5 animals.

**Fig. 7.4.** Comparison of the estimated cumulated activity from 1 mCi of radiolabelled UJ13A and 1mCi of mIBG in the whole body of marmosets.

**Fig. 7.5.** Comparison of the estimated cumulated activity from 1 mCi of radiolabelled UJ13A and 1mCi of mIBG in liver of marmosets.
Fig 7.3

Fig 7.4

Fig 7.5
scintigraphy (Range: 5-9% N=6). The effective half life, (Teff) of this excretory phase was defined as lying between 8 and 18 hours (Mean Teff: 11 hours, N=6). These figures compared with an estimated mean effective half life of 28 hours (Range 20-37: N=5), and an estimated Ao of between 10 and 25% of the injected dose. Table 2.
The high uptake in animals 58 and 59, occurred in the animals administered the preparation containing aggregates.

**Table 2.**

**Effective half life and maximal organ uptake of radionuclide:liver.**

<table>
<thead>
<tr>
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<th>Teff</th>
<th>Ao</th>
<th>Teff</th>
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<td>7</td>
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</table>

Where Ao refers to the extrapolated maximal liver uptake of radionuclide, expressed as a percentage of injected dose; Teff the effective half life in hours, and * denotes a preparation containing significant aggregated protein.

Comparing the estimated dose delivery of $^{131}$I mIBG to $^{131}$I UJ13A, it would appear that the monoclonal antibody delivers between 4.2 and 8.0 x the radiation dose to liver than mIBG, for equivalent amounts of administered isotope. **Fig 7.5.**
Comparison of the biodistribution of mIBG and UJ13A in patients.

METHODS:

A study of similar design was undertaken in 10 children with neuroblastoma. Each patient had Evans Stage III/IV neuroblastoma and had been treated with between 6 and 10 courses of OPEC. (See Chapter 3). Six patients in complete remission on this protocol were consolidated with high dose melphalan and autologous marrow transplantation. In the 10 days preceding the administration of mIBG or UJ13A, the remission status of each patient was evaluated. All patients underwent bone marrow aspiration, a $^{99}$Tc MDP bone scan, $^{99}$Tc colloid ("Nanocoll") scan of bone marrow and liver and spleen. Two days prior to scintigraphy, the thyroid was blocked with 0.3mls of Lugol’s Iodine.

To limit the study period to 10 days, two isotopes of iodine were used to trace the radiolabelled conjugate. $^{123}$Iodine has a half life of 13.2 hours compared with 8.04 days for $^{131}$Iodine. The first 6 patients received $^{123}$I mIBG followed by $^{131}$I UJ13A, 5 days later. The following 4 patients had the labels reversed, receiving $^{123}$I UJ13A on day one of the study, and $^{131}$I mIBG on day 5. UJ13A was radiolabelled using the modified Chloramine-T technique and radiochemical purity and immunological activity were determined in the usual way (Chapter 2; 6a,6c,6d,6e).
At the time that this study was undertaken, $^{123}$I mIBG was not readily available from radiopharmaceutical companies. Consequently mIBG was prepared and radiolabelled by Dr DH Jones, Imperial Cancer Research Fund. This was undertaken by the addition of 10 mCi of $^{123}$I to between 1 and 2 mg of mIBG. 100 ul of glacial acetic acid was added to this mixture, followed by 3-5 mgs of ammonium sulphate. The bottle was sealed and heated for 45 minutes at 140-160°C in a glycerol bath. 1.5 mls of 0.005M acetate buffer/pH 4.1 was added, and free iodine removed from the preparation by passage through a Cellnex D ion exchange column. Radiolabelled mIBG was sterilized by passage through a 22 um Millex filter and collected in an evacuated vial. The radiochemical purity was determined by silica gel, thin layer chromatography (Chapter 2;6e).

All patients were injected with approximately 10 ug/kg of either conjugate whilst lying supine on the camera face. The injected dose was calculated from counting the syringe on the gamma camera, before and after administration of the radiolabel. To check the efficiency of the camera, the full syringe was also counted in a "Capintec" gamma counter.

All patients underwent a dynamic acquisition series as detailed in Chapter 3. Serial static images were also obtained following the methods described in Chapter 3 at 4, 24 and 48 hours for the $^{123}$I labelled conjugate, and 4, 24 and 72 hours for the $^{131}$I labelled compound.
Vascular sampling was undertaken at 0.5, 1, 2, 4, 8, 16, 32 and 48 hours from injection of both compounds.

Time activity curves and estimates of tissue radiation dose were performed using the methods detailed in Chapter 3.

**RESULTS:**

The detailed biodistribution data for UJ13A is contained in Chapter 3. The patients contributing data to both Chapters are indicated by *. Data on the biodistribution of UJ13A has been selected out for comparison with mIBG and is presented in Tables 3 and 4.

mIBG routinely ran as two bands on the thin layer chromatogram. The major band, which ran with an Rf value of 0.50-0.52, contained 90-97 % of the injected counts.

The biodistribution of mIBG in patients.

Following a bolus injection of radiolabelled mIBG, activity cleared from the blood following biexponential clearance kinetics. The first clearance component was very rapid with between 1.5 and 10% of the injected dose remaining in blood at 60 minutes. (mean activity 4.4% N=8). Subsequent loss was monoexponential over the assay period of 48 hours. The biological half life of this clearance component was extremely variable between patients and lay between 9 and 130 hours. Table 3, Fig 7.6.

The early biodistribution of the compound was followed using the dynamic acquisition series. Within
Fig. 7.6. Clearance of mIBG from blood of patients.
The graph demonstrates the change in % of injected dose with time for individual patients. (Minimum of 7 observations for each patient).
Fig 7.6
the first 30 minutes the radiolabel was observed to be excreted by the kidney, and isotope was demonstrated to rapidly accumulate in the bladder. **Figs 7.7a and b.** This phase of renal filtration was of short duration and after 15-20 minutes no further increase in bladder activity was seen. In patient c and f, an estimate of maximal uptake per kidney was undertaken during the dynamic phase. This was calculated as 4.4 and 3.6% of the injected dose respectively. The calculated renal accumulation and excretion of isotope would not account for the rapid vascular loss.

Additional loss occurs due to selective organ uptake. Accumulation of counts within the liver occurred rapidly over the first 5 minutes. **Fig 7.7c.** During the following 25 minute observation period the activity remained constant. In patients c and f maximal uptake was estimated as 7.5 and 9.0% respectively. Counts measured over the heart fell at a linear rate over 30 minutes. **Fig 7.7d.** Maximal radioactivity was seen in the first minute. This fell to 75% of the peak uptake by 30 minutes after injection.

Scintigrams performed after the dynamic acquisition series consistently demonstrated high concentrations of radionuclide in salivary tissue, myocardium and liver. **Fig 7.8.** Images at 4 hours also showed mIBG uptake in the basal third of the lungs. This uptake was transient and no radioactivity above background level was observed at 24 hours. The normal left adrenal was visualised on only one occasion, and the uptake of isotope in bowel was
Fig.7.7.-7.10. Dynamic acquisition of mIBG in various organs of patients. Results are expressed as a percentage of peak activity.

Fig.7.7 Increase in radioactivity with time-kidney.
Fig.7.8 Increase in radioactivity with time-bladder.
Fig.7.9 Increase in radioactivity with time-heart.
Fig.7.10 Increase in radioactivity with time-liver.
Fig 7.9

Fig 7.10
variable between patients. No uptake was demonstrated in the spleen, bone or bone marrow that was uninvolved by the disease process.

The hepatic uptake of isotope was determined from extrapolating the time activity curve to the origin. This data provided a range of hepatic accumulation of isotope that lay between 0.5 and 10% of the injected dose. (Mean 4.9% N=8) The rate of hepatic excretion of isotope varied, with biological half lives between 8 and 66 hours (Mean 26.5 hours N=8).

Comparison with UJ13A.

The rates of clearance of mIBG and UJ13A are compared for blood in Table 3, and for liver in Table 4.

TABLE 3.

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<th>UJ13A</th>
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</tr>
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<td>NE</td>
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<td>j</td>
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</table>

Mean: 4.4 37 69 44

Where percentage of injected dose, 1 hour, indicates the estimated percentage of injected dose remaining in the vascular compartment, 1 hour after injection. T(Biol) represents the biological half life in hours.
TABLE 4.

Clearance and estimated radiation dosage to liver, of 1mCi of administered radionuclide.

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<th>Ao</th>
<th>T(Biol)</th>
<th>Dose</th>
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</tbody>
</table>

Ao is the percentage of injected dose present in liver at time 0; T(Biol), the biological half life of the radionuclide in liver in hours; dose, the estimated radiation dose to liver from 1mCi of injected conjugate.
Abnormal sites of accumulation of mIBG.

A total of 18 abnormal sites of isotope accrual were seen in the 10 patients studied. 13 of these sites were confirmed as areas of tumour, by alternative conventional staging methods. (Table 5).

Abnormal sites of accumulation of mIBG.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td>OPECx9 Melphalan</td>
<td>Remission (9/12)</td>
<td>Mediastinum</td>
<td>CAT -ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CXR -ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BS -ve</td>
</tr>
<tr>
<td>b.</td>
<td>OPECx10 Melphalan</td>
<td>Remission (3/12)</td>
<td>Mediastinum</td>
<td>CXR +ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VMA -ve</td>
</tr>
<tr>
<td>c.</td>
<td>OPECx10</td>
<td>Persistent disease.</td>
<td>None</td>
<td>BM +ve</td>
</tr>
<tr>
<td>d.</td>
<td>OPECx10 Melphalan</td>
<td>Remission (23/12)</td>
<td>L. Adrenal</td>
<td>US -ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VMA -ve</td>
</tr>
<tr>
<td>e.</td>
<td>OPECx10</td>
<td>Persistent disease.</td>
<td>BM</td>
<td>BM +ve</td>
</tr>
<tr>
<td>f.</td>
<td>OPECx10</td>
<td>Relapse.</td>
<td>Mediastinum</td>
<td>CXR +ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lymph nodes</td>
<td>Palpable</td>
</tr>
<tr>
<td>g.</td>
<td>OPECx9 Melphalan</td>
<td>Remission (6/12)</td>
<td>L. Adrenal</td>
<td>US -ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VMA -ve</td>
</tr>
<tr>
<td>h.</td>
<td>OPECx9</td>
<td>Remission</td>
<td>R. Tibia*</td>
<td>BS -ve</td>
</tr>
<tr>
<td>i.</td>
<td>OPECx10</td>
<td>Relapse</td>
<td>L. pubic ramus</td>
<td>BS +ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Abdomen</td>
<td>US +ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mediastinum</td>
<td>CXR -ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L. knee</td>
<td>BS +ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L. humerus</td>
<td>BS +ve</td>
</tr>
<tr>
<td>j.</td>
<td>OPECx10</td>
<td>Relapse</td>
<td>Abdomen</td>
<td>US +ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R. knee</td>
<td>BS +ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R. jaw</td>
<td>BS +ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bone marrow</td>
<td>Asp +ve</td>
</tr>
</tbody>
</table>

*Subsequently reassessed as normal.

CAT; CAT scan, CXR; chest XRay, BS; Bone scan, US; ultrasound.
Fig. 7.11. Scintigram taken at 4 hours after administration of 123I mIBG, demonstrating normal distribution of mIBG.

S: Salivary tissue.
H: Heart (myocardium)+Tumour.
L: Liver.
Bo: Bowel.
B: Bladder.

Fig. 7.12. Scintigram taken 24 hours after mIBG administration, demonstrating mIBG persistence in mediastinal mass (T) (Probably ganglioneuroma—see text).
Fig. 7.13. Scintigrams demonstrating abnormal patterns of mIBG distribution.

Fig 7.13a Uptake in abdominal recurrence of neuroblastoma (T1) and in marrow infiltrate of neuroblastoma (T2).

Fig 7.13b Uptake in a tibial (bony) metastasis of neuroblastoma; in contrast to:

Fig 7.13c Symmetrical uptake of mIBG in the femoral and tibial marrow cavity, secondary to diffuse bone marrow infiltration by neuroblastoma.
Of the remaining 5 sites, one site was considered as normal when reassessed after gaining experience in interpreting mIBG scintigrams. A further abnormal area of isotope accumulation was seen in the mediastinum of a patient who had relapsed at a variety of additional sites. The extra site was felt to be consistent with the clinical pattern. Three sites of abnormal isotope accumulation were demonstrated in which there was no supporting evidence of disease. The sites were all in the area of the primary tumour. No clinical evidence of disease was apparent in two of the patients until 22 and 27 months from imaging with mIBG. Both these patients were alive with disease at 3 years from initial mIBG scintigraphy. The remaining patient with an abnormal area in the mediastinum (Patient a), remains in clinical remission at 43+ months from scintigraphy. In addition, patient b, who had an intense uptake of mIBG in two mediastinal lymph nodes (Fig 7.12) remains with no active disease at 43+ months from the mIBG scan.

DISCUSSION.
The purpose of this study was to characterise the distribution of mIBG and to compare the radiation dosimetry from two potential delivery systems in neuroblastoma. It can be seen from the data generated in this study that the major difference in the pharmacokinetics of these two radiolabelled conjugates is the rapid vascular loss of mIBG. This does not appear to
be a species specific phenomena and results from organ uptake, renal loss of isotope and distribution to a second space. The size of the initial loss would suggest that the second space is the total body water. In the marmosets a steady state in the vascular compartment is rapidly achieved. This presumably reflects a state of equilibrium between the extravascular compartment and blood, with a rate of loss of mIBG to blood equivalent to the rate of loss from the whole body.

Only one patient with neuroblastoma appeared to achieve a steady state (Patient a). This patient was not obviously different from the other patients in the study. She had similar initial distribution kinetics, and normal renal function. However, for the majority of patients, mIBG cleared rapidly from blood suggesting that the rate of renal excretion generally exceeds the rate of transfer of isotope from the extravascular compartment.

The monoclonal antibody has a much slower distribution phase than mIBG. The size of the initial loss is much smaller and is largely accounted for by reticuloendothelial loss rather than any transfer across the capillary network. A detailed examination of the relative merits of the two delivery systems requires compartmental modeling, however it can still be appreciated from this study that mIBG offers advantages over UJ13A. These include the potential to target $^{131}$I rapidly across vascular barriers at lower whole body radiation dose. The data from the marmosets suggests a 3.8 fold advantage in whole body dose of mIBG over UJ13A.
This exaggerates the advantage, as a significant proportion of the administered dose is rapidly renally excreted. Excreted isotope is not available for radiation targeting and would appear to account for a mean of 17% of the injected dose. The importance of this renal loss is supported by urine collections in patients undertaken at the Royal Marsden Hospital (161). In this study, during the first three hours, a mean of 13% of the injected dose was excreted through the kidneys. (Range 11-26, N=7).

Data from the marmoset experimental group indicates that clearance of mIBG from liver is biphasic. However, the duration of the initial phase is short, and does not contribute significantly to the final radiation dose estimates. Consequently, satisfactory dose estimates may be made by assuming that clearance of isotope from liver follows monoexponential clearance kinetics.

The marmoset data indicated a clear advantage of mIBG over UJ13A in terms of the hepatic radiation dose. The radiation dose to liver was partially inflated by the administration of aggregated material. This could be allowed for by assuming that all of the aggregated material was rapidly cleared by the liver, and deducting 9% of the injected dose from the estimated activity at time 0. Manipulating the data in this way still results in a mean estimated radiation dose to liver from UJ13A in excess of mIBG. (Dose ratio UJ13A:mIBG, 3.6:1).

Although there was a trend to increased hepatic dosage from UJ13A in the patients, the size of the
difference was not as large as might have been anticipated from the marmoset data. The differences are explained by a lower proportion of isotope accumulating in liver following the administration of UJ13A, and hepatic persistence of mIBG. Several factors may account for the disparity in results. The reduction in percentage of UJ13A in the liver may or may not be a valid observation. The greater variation in body thickness in the patient group, and the reliance on posterior views, may have resulted in a consistent underestimate of isotope accumulation. However, a similar variation is built into the estimates of mIBG uptake, suggesting that these factors do not totally account for the final difference in hepatic radiation dose delivery.

An alternative explanation may result from the pronounced splenic uptake of conjugate observed in the patients. This mechanism may have reduced the initial proportion of injected dose sequestered by liver. Delayed clearance of mIBG may have also increased estimates of hepatic radiation dosage. The estimated hepatic half life of mIBG made in this patient group, has been supported observation in a larger series of patients (161). In this report which included patients from the Royal Marsden Hospital, no correlation could be established between renal function and hepatic clearance. There was no clear evidence of impaired hepatic function in the study group. All of the patients have undergone significant chemotherapy and it may be that a variety of
subtle mechanisms for metabolism of mIBG have been compromised. Alternatively, the results may simply reflect inter-species differences in rate of mIBG metabolism.

The importance of the radiation dose to liver may increase if mIBG is administered at doses calculated to ablate marrow. Careful tissue dosimetry will be required under these conditions, with attention to kidneys, bladder and pelvic organs as well as liver.

Although the study did not set out to assess the suitability of mIBG as a scintigraphic agent, it became apparent that mIBG is a highly sensitive method for detecting neuroblastoma. 13/14 sites detected by conventional techniques were imaged with this radiopharmaceutical. In addition to detecting sites of solid disease, mIBG detected 2/3 marrow sites. Failure to detect marrow disease in the third patient was a feature of low dose administration rather than mIBG negative disease, as a subsequent administration of high dose mIBG (80mCi $^{131}$I mIBG) for therapy resulted in a strong signal from the marrow.

Finally, it is interesting to comment on the accumulation of mIBG at sites in which there was no measurable disease on conventional assessment. A clinical decision was made at the beginning of the study not to change routine management on the basis of mIBG scintigraphy. This was not a difficult decision as the patients had already received the best available conventional therapy. It is possible in two of the
patients that have relapsed, that the mIBG scan was picking up early residual disease. However, it should be noted that in one of these patients, clinical relapse was associated with mIBG negative disease on repeat scintigraphy. In two other patients the clinical relevance of the positive mIBG scan is in doubt. Both have no evidence of active disease at greater than 3 years from scintigraphy. In one this presumably represents maturation of tissue to ganglioneuroma and in the second either a similar process in a small tissue volume, or a true false positive. It is clearly necessary to evaluate this further if patients are to avoid more aggressive therapy on the basis of positive mIBG scintigraphy.
A Comparison Of The Pharmacokinetics Of High And Low Specific Activity mIBG In Vivo.

Results in Chapter 9 suggest that mIBG has several advantages over UJ13A as a vector for $^{131}$I. These characteristics were demonstrated using a low specific activity (S.A), commercial preparation of mIBG normally supplied for scintigraphy (2.5 mCi/mg). In practice, therapy is undertaken with a high SA preparation of greater than 30 mCi/mg, which is reported to be relatively unstable. Users are advised to keep the radiopharmaceutical frozen until use.

It was decided to investigate in vivo, whether the reported instability of high specific activity mIBG resulted in different pharmacokinetics from the low SA conjugate. The issue is important, as a similarity in pharmacokinetics would enable physicians to utilise information from scintigraphic studies for treatment planning.

**METHODS:**

$^{131}$I mIBG (SA 30 mCi/mg) was received from Amersham International. The radiopharmaceutical was maintained at $-20^\circ$C until just before the experiment, then defrosted in a water bath at $37^\circ$C and diluted to the desired activity in a sterile solution of 0.9% sodium chloride. Marmosets were injected with the radionuclide within 40 minutes from defrosting. Where possible, an aliquot of the
injected preparation was examined by thin layer chromatography at the time of injection, and at 1 hour after administration. The efficiency of the gamma camera for counting ¹³¹I was determined over a range of activities during each experimental procedure.

The six animals reported in Chapter 8 were used for this study. They were prepared and injected with high specific activity mIBG, under the conditions described in Chapter 9. Sequential scintigraphy and subsequent data analysis was performed as described for the low S.A compound.

RESULTS.
A total of 5 batches of high S.A mIBG were used during the course of this experiment. The radionuclide was examined on two occasions for radiochemical purity. On each occasion the major band demonstrated on thin layer chromatography, (Rf value, 0.47 and 0.48) was associated with approximately 65% of the total activity (64 and 66%). A substantial proportion of the residual isotope was associated with two additional fractions. One ran in front of the main band with an Rf value of 0.75 and 0.87 and constituted 8 and 14% of the radioactivity; the second component ran with an Rf value of 0.39 and constituted 15 and 8% of the total activity respectively.

One hour after administration of mIBG, the proportion of isotope (as determined by densitometry scanning of the autoradiograph) associated with the major band, had decreased from 66% to 57%. The loss of activity in this
fraction was associated with an increase in all the measured fractions.

Administration of isotope.
Animals were studied in groups of three. The first and second administrations of high S.A mIBG came from different batches of isotope. The third administration was obtained from a single batch. Variation in the amount of administered isotope was monitored by counting the full and empty syringe and needle on the camera face, and comparing this to a series of $^{131}$I standards. Because a variable proportion of the administered dose was left in the syringe, each animal received a different dose of mIBG, as shown in Table 1. During the course of the experiment the efficiency of the gamma camera did not fluctuate substantially over the experimental dose range.

**Fig 8.1.**

**TABLE 1.**

Variation in administered dose of $^{131}$I mIBG, for successive studies.

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>1st Dose (uCi.)</th>
<th>2nd Dose (uCi.)</th>
<th>3rd Dose (uCi.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>46</td>
<td>52</td>
<td>--</td>
</tr>
<tr>
<td>47</td>
<td>47</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>58</td>
<td>27</td>
<td>24</td>
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</tr>
<tr>
<td>59</td>
<td>53</td>
<td>14</td>
<td>34</td>
</tr>
<tr>
<td>62</td>
<td>53</td>
<td>61</td>
<td>31</td>
</tr>
<tr>
<td>63</td>
<td>41</td>
<td>26</td>
<td>37</td>
</tr>
</tbody>
</table>
Fig. 8.1. Fluctuation in camera efficiency over the time span of the experiment. Graph of known quantity of $^{131}$I (uCi) against counts per minute recorded on the gamma camera.

Fig. 8.2. Estimated cumulated activity (whole body), from 1 mCi of $^{131}$I mIBG, for serial administrations (1,2,3). Data for 6 marmosets (first two exposures), and 5 marmosets (third exposure). The rise in cumulated activity with serial exposure is statistically significant. (p=0.003).
Whole body clearance of mIBG.

The pattern of isotope clearance was similar to that described for low specific activity mIBG. An initial rapid clearance phase was observed, followed by a second slower phase. The initial loss of isotope accounted for a mean of 88.5% (Range 80-94%) of the injected dose, during the first administration of the high specific activity compound. The subsequent rate of mIBG clearance from the whole body was monoexponential with a mean effective half life of 21 hours (Range 17-27 hours).

Table 2.

The mean estimated cumulative activity in whole body was 26863 uCi.hrs after the first dose of high SA mIBG. (Range 21888-32695 uCi.hrs). With repeated administrations of the high SA mIBG there was a trend towards increasing whole body dose (Fig 8.2 and Table 2). The mean accumulated activity rose from 26863 uCi.hrs (Range 21888-32659 uCi.hrs) through 35635 uCi.hrs (Range 23616-40140 uCi.hrs) to 39305 uCi.hrs at the third administration (Range 30800-48720). The initial rise was principally explained by a decrease in the rate of excretion during the period 1-24 hours, whilst the second increase resulted from a reduction in the rate of isotope excretion during the first 60 minutes.
TABLE 2.

Changes in excretion patterns of $^{131}$I from whole body of marmosets during repeated administration of high SA mIBG

<table>
<thead>
<tr>
<th>Animal</th>
<th>1st admin.</th>
<th>2nd admin.</th>
<th>3rd admin.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ao</td>
<td>Teff</td>
<td>Ao</td>
</tr>
<tr>
<td>44</td>
<td>92</td>
<td>24</td>
<td>92</td>
</tr>
<tr>
<td>47</td>
<td>87</td>
<td>23</td>
<td>86</td>
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<td>94</td>
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<td>62</td>
<td>94</td>
<td>17</td>
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</tr>
<tr>
<td>63</td>
<td>84</td>
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<td>94</td>
</tr>
<tr>
<td>Mean:</td>
<td>88</td>
<td>21</td>
<td>87</td>
</tr>
</tbody>
</table>

Ao indicates the percentage of injected dose present in whole body at time 0 as determined by extrapolating the second effective half life to the ordinate. Teff is the effective half life in hours.

TABLE 3.

Changes in estimated cumulated activity (whole body) during successive administrations of high SA mIBG.

<table>
<thead>
<tr>
<th>Animal</th>
<th>1st admin.</th>
<th>2nd admin.</th>
<th>3rd admin.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>uCi.hrs</td>
<td>uCi.hrs</td>
<td>uCi.hrs</td>
</tr>
<tr>
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<td>31795</td>
<td>33649</td>
<td>--</td>
</tr>
<tr>
<td>47</td>
<td>28814</td>
<td>39628</td>
<td>48720</td>
</tr>
<tr>
<td>58</td>
<td>23011</td>
<td>38880</td>
<td>37320</td>
</tr>
<tr>
<td>59</td>
<td>21888</td>
<td>40140</td>
<td>30800</td>
</tr>
<tr>
<td>62</td>
<td>23011</td>
<td>23616</td>
<td>40176</td>
</tr>
<tr>
<td>63</td>
<td>32659</td>
<td>37900</td>
<td>39510</td>
</tr>
<tr>
<td>Mean:</td>
<td>26863</td>
<td>35635</td>
<td>39305</td>
</tr>
</tbody>
</table>

Hepatic clearance of mIBG.

The pattern of isotope accrual in liver, and its subsequent clearance, were also similar to that described for the low S.A conjugate. The pattern of hepatic uptake for the first administration and normalised for injected
dose, is detailed in Fig 8.3. Once again a rapid period of hepatic accumulation and loss was documented, spanning the 4-5 hours after injection. Subsequent loss on a small number of observations appeared monoexponential. This major excretory component did not change substantially with successive administrations. The mean effective half life was measured as 10 hours (Range 6-15 hours), 9 hours (Range 7-16 hours) and 9 hours (Range 8-11 hours) for serial administrations. The estimated cumulated activity after one administration of conjugate varied between 897 and 1472 uCi. hrs. (Mean activity: 1160 uCi.hrs.) With sequential administration of isotope, there was a slight trend for the group to increased retention of radionuclide (Fig 8.4, Table 4). However, when the results were examined sequentially for individual animals, no consistent pattern was identified.

**TABLE 4.**

Changing kinetics in the livers of marmosets during repeated administrations of high SA mIBG.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
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<td>15</td>
<td>5</td>
<td>11</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
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<td>58</td>
<td>13</td>
<td>6</td>
<td>9</td>
<td>8</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>59</td>
<td>8</td>
<td>8</td>
<td>17</td>
<td>7</td>
<td>9</td>
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<tr>
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<td>10</td>
<td>9</td>
<td>9</td>
<td>10</td>
<td>9</td>
</tr>
</tbody>
</table>

Where Ao indicates the extrapolated percentage of injected dose present in liver at time 0, and Teff the effective half life in hours.
**Fig 8.3.** Clearance of high SA mIBG from liver of marmosets. Clearance curves for 6 different animals.

**Fig 8.4.** Change in estimated cumulated activity in liver of marmosets during serial administration of high SA mIBG.
TABLE 5.

Changes in estimated cummulated activity (liver) during successive administrations of high SA mIBG.

<table>
<thead>
<tr>
<th>Animal</th>
<th>1st admin. uCi.hrs</th>
<th>2nd admin. uCi.hrs</th>
<th>3rd admin. uCi.hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>1472</td>
<td>792</td>
<td>----</td>
</tr>
<tr>
<td>47</td>
<td>1306</td>
<td>864</td>
<td>1373</td>
</tr>
<tr>
<td>58</td>
<td>1170</td>
<td>1036</td>
<td>1198</td>
</tr>
<tr>
<td>59</td>
<td>969</td>
<td>1713</td>
<td>1325</td>
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<td>62</td>
<td>897</td>
<td>1244</td>
<td>1321</td>
</tr>
<tr>
<td>63</td>
<td>1150</td>
<td>1108</td>
<td>1108</td>
</tr>
<tr>
<td>Mean:</td>
<td>1160</td>
<td>1126</td>
<td>1265</td>
</tr>
</tbody>
</table>

Comparison with low SA mIBG.

To compare dose delivery from low S.A conjugate and from high S.A conjugate, the ratio of cumulative activity after first administration of high S.A conjugate to the cumulative activity from low S.A conjugate was constructed. Table 6. (See Chapter 9 for data on low S.A mIBG).
TABLE 6.

Ratio of estimated cumulative activity from 1 mCi of high SA mIBG to estimated cumulative activity from low SA mIBG. (Liver and whole body - marmosets)

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Ratio (liver)</th>
<th>Ratio (W.B.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>44*</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>47*</td>
<td>1.4</td>
<td>0.9</td>
</tr>
<tr>
<td>58</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>59</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>62*</td>
<td>1.8</td>
<td>0.8</td>
</tr>
<tr>
<td>63</td>
<td>0.7</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*Denotes that these animals always received mIBG from the same batch.

As cumulative activity is directly proportional to radiation dose delivery (Chapter 3), estimates of radiation dose delivery to whole body derived from the low S.A conjugate are within +30 to -20% of the estimated dose delivery from high S.A mIBG. More variation was observed in the ratios of accumulated activity in liver, (Range 0.7-1.8). These results indicate that the estimated radiation dose to liver from 1 mCi of low S.A conjugate, is within -30 to +80% of the estimated dose delivery from high S.A conjugate. When account is taken of the batch of mIBG used, this variation does not appear to be random. The first three animals to be studied accounted for all the ratios of low:high S.A mIBG over 1, whilst the second grouping had ratios under 1.
DISCUSSION:
Two observations in this study were considered to be of importance. Firstly, the reported instability of high SA mIBG does not seem to substantially effect the handling of the conjugate in vivo. The biodistribution of the conjugate was observed to be quantitatively similar to the low SA conjugate. This is reflected in the ratios of cumulated activity for whole body and liver.

This similarity in pharmacokinetics is sustained despite a 17 fold difference in benzylguanidene dose, between the low SA and high SA administration. The results support the observations of Kline et al (160), who demonstrated that no change in vascular or myocardial kinetics occurred despite a 10,000 fold difference in S.A.

These results suggest that standard tracer studies will be useful in predicting subsequent behaviour of the high S.A mIBG used in therapy. Under the conditions of this study, it would seem possible to predict the whole body radiation dosage within +/- 30%. Less confidence may be placed in estimates of hepatic dosage, where results were more varied.

In practice, the results may be more difficult to achieve. It has been possible to administer the high S.A mIBG within a short time period after defrosting. Delays would result in radiolysis of the conjugate. This is illustrated in the two measurements made at 1 hour apart
on a single batch of mIBG, where 10% of the activity has already been degraded.

The accuracy of the kinetic estimates is improved by the frequency and timing of the scanning procedure. Over the dose range used within the study it was possible to repeat the scintigrams at similar time points to those used during administration of low S.A conjugate. The dose range used also ensured that the camera efficiency for gamma counting was similar for the high SA conjugate. The disparity in isotope dose between tracer and therapeutic administration of conjugate, and the restrictions placed on patient movement after therapy, introduce major variables in the measurements of dose delivery from the high SA conjugate. Provided careful monitoring of the radiochemical purity of mIBG is undertaken, the kinetics of the tracer dose may be an adequate predictor of tissue toxicity.

This assumption should be qualified by the data on repeat administrations. The apparent rise in whole body dose with second and third administrations was a surprising finding. Unfortunately, little information is available on the metabolic fate of mIBG. It is known to be structurally similar to the drug guanethidene and like guanethidene, is transported into cells by a type 1 and type 2 mechanism (162)(164). The characteristics of the type 1 mechanism are similar to guanethidene (high affinity, low capacity, ouabain sensitive and energy dependent), whilst the type 2 mechanism has the characteristics of a diffusion process, (low affinity,
high capacity, energy and sodium independent). Whilst the type 2 mechanism is likely to predominate at high concentration of mIBG, the type 1 mechanism is more likely to influence cellular transport of mIBG after the initial distribution phase. Like guanethidine, high speed centrifugation of the dogs adrenal medulla has shown that mIBG is associated with chromaffin granules. These similarities in transport and storage suggest that mIBG might share other metabolic characteristics of guanethidine, including storage outside neurosecretory granules within neuronal cytoplasm, hepatic metabolism and renal excretion (163)(165).

Increasing whole body retention of the radionuclide, with serial exposures suggests that there has been tissue persistence of isotope or impairment of the renal excretory mechanism. The slight increase in hepatic retention of isotope is probably insufficient to account for the increase in whole body retention. The renal excretion is believed to reflect a simple filtration phenomena and it is unlikely that this had been impaired by radiation induced glomerular damage at the level of isotope administered. (approximately 150 uCi/kg.) An alternative explanation is facilitation of neuronal storage with repeat exposures to the compound. No evidence for this phenomena was found within the guanethidine literature.

It would seem impossible to account for the observation within the limits of the information available. The observation may be spurious, although an
analysis of the data by a two way analysis of variance, indicates that the trend is highly significant (p=0.003). Consequently, it would seem appropriate to repeat this experiment collecting information on the rate of vascular and renal loss. This would test the reproducibility of the data and give insight into the mechanism of whole body retention.
INTRODUCTION.
The clinical studies reported in the earlier portion of this thesis concern themselves with the intravenous administration of the monoclonal antibody UJ13A for therapy of neuroblastoma. Several problems have been identified which indicate that modification of the monoclonal is required before it is likely to be a useful clinical tool. The major problem appears to be one of antibody access across vascular barriers. This is supported by the successful targeting of antibodies to human tumour xenografts, and reports of radiation targeting at sites of increased vascular permeability. The hypothesis that the vascular barrier is the major limiting factor to radiation targeting with monoclonal antibodies may be tested in circumstances where dose delivery is independent of vascular delivery. As discussed in the Chapter 1, intracavity administration of monoclonal antibodies offers a framework in which to evaluate the approach.

A monoclonal antibody, UJ181.4 produced in our laboratory appeared suitable for investigation. This hybridoma was cloned from the same fusion as UJ13A and produces an antibody of IgG₁ isotype. The antibody recognises an antigen expressed on primitive neuroectodermal tumours and foetal brain. No UJ181.4
antigen expression is detected by indirect immunofluorescence on mature human brain (166). The availability of this antibody stimulated an investigation of monoclonal antibodies as targeting agents in the intrathecal compartment.

A large number of personnel with different skills have been involved in the study. My role has been one of developing the clinical design of the study, acting as co-ordinator, collecting, interpreting and evaluating clinical data. In addition, I have been involved in the preparation, characterisation and quality control of monoclonal antibodies administered to patients. Other members of the group have been responsible for radiolabelling monoclonal antibodies, administering radionuclide and sample collection. Supplementary studies have been undertaken on the stability of the radionuclide in CSF and blood, and on the radiobinding of monoclonals to the central nervous system (CNS).

STUDY DESIGN:
The injection of monoclonal antibodies into the thecal space was regarded as a new therapeutic procedure. Consequently, we set out to investigate the biodistribution of monoclonal antibodies after administration, as well as monitoring toxicity and therapeutic effect. The results of the pharmacokinetic study are presented along with primate data in Chapter 12. The results of the Phase I study are presented in this chapter.
METHODS:

Patient selection.

Patients were considered for inclusion in the study if they had failed an "adequate" trial of conventional therapy and had evidence of leptomeningeal dissemination of tumour. (See Table 2).

The immunophenotype of each patient's tumour was established using a panel of monoclonal antibodies. This was undertaken either by examination of a CSF cytospin or on a specimen of previously biopsied tumour. (Chapter 2;5a,5d). A single antibody was selected for radiation targeting based on its reactivity with the patient's tumour and lack of reactivity with normal central nervous system (CNS) components.

Each patient was assessed with serum and cerebrospinal fluid (CSF) biochemistry, CSF cellular count and morphology, full blood count, cranial CAT scanning with contrast, and myelography. Patients were not excluded from treatment solely on the basis of their poor clinical condition, but were excluded if they had evidence of a solid parenchymal metastasis. Those with evidence of a spinal block were referred for external beam radiotherapy to the affected spinal segment.

Preparation of the radiolabelled conjugate.

Patients 1-8 received antibodies radiolabelled by the modified Chloramine-T technique to a specific activity of between 5 and 15 mCi/mg of protein (Chapter 2;6a). The
remaining patients were administered antibody radiolabelled by the Iodogen method to a specific activity of between 5 and 10mCi/mg of protein (Chapter 2;6b). Free iodine was separated from the radiolabelled protein by column chromatography using Sephadex G25 equilibrated in phosphate buffered saline/2% human plasma protein fraction. Antibody was passed through a 22 micron filter (Millex) and collected into a sterile evacuated vial.

Quality control.
Aliquots of radiolabelled protein were examined for radiochemical purity by precipitation with 10% trichloroacetic acid. Microaggregates were assessed either by high speed centrifugation at 10,000 x g for 30 minutes, or by S300 column chromatography (Chapter 2;6c,6d).

In patients 1-5, the biological activity of the radiolabelled conjugate was assessed by an indirect immunofluorescence assay (Chapter 2;5c). Later radiolabellings were assessed in a radiobinding assay which estimated the immunoreactive fraction under conditions of antigen excess (Chapter 2;6f).
Patient preparation and administration of conjugate.
Thyroid blockade was undertaken in all patients. This was performed either with 0.3 mls Lugol's iodine t.d.s and liothyronine 8 mcg b.d, or by liothronine 80 mcg daily supplemented with 10 drops of supersaturated potassium iodide q.d.s and 200 mg of potassium perchlorate q.d.s. In anticipation of a meningitic reaction associated with the introduction of protein into the CSF, all patients were given low dose dexamethasone, 1 mg t.d.s. This was tailed off over a period of three weeks from therapy.

Radiolabelled protein was administered through a 22 um Milllex filter, either by direct lumbar administration (3/15 patients), via an intraventricular Ommaya resevoir (10/15) or a combination of intraventricular and lumbar injection (2/15). On each occasion a sample of CSF was withdrawn of equivalent volume to the solution of radionuclide. Cannulae and reservoirs were flushed with approximately 2 mls of sterile 0.9% saline.

Response to therapy.
Patients were evaluated for response, if they had neither received chemotherapy for 4 weeks prior to treatment with monoclonal antibody, or radiotherapy to all evaluable sites within the preceding 6 weeks. These conditions were waived if the patients had clear evidence of disease progression in the intervening period. Response was
assessed at 3+ months, by clinical criteria and by imaging and cytological evidence of tumour reduction.

RESULTS.
Fifteen patients with a heterogenous group of tumours were enrolled in the study (Table 2). The diversity in tumour type necessitated the use of a variety of monoclonal antibodies (Table 1). The selected antibodies were UJ181.4, an antibody of IgG1 isotype, detecting an oncofoetal antigen expressed on neuroectodermal tumours \(166\), 81C.6 (IgG2a) detecting the extracellular matrix protein, Tenascin \(167\), Mel.14, (IgG2a) detecting the a melanoma associated glycoprotein \(170\), F8-11-13 (IgG1) with B-cell specificity \(168\), and HMFG1 (IgG1) recognising a large mucin like molecule, expressed in a variety of carcinomas \(169\).

Antibody preparation.
Radiolabelling produced a preparation that was relatively free from microaggregates (Mean value: 1%, Range 0-2%, N=12), and containing a small percentage of free iodine (Mean value: 4.5%, Range 1-13%, N=12). In the first 5 patients, the antibody always retained biological activity, as measured by the indirect immunofluorescence assay. In 2 of the remaining 10 radiolabellings, the immunoreactivity of the protein was lost. (Patients 6 and 12). Table 1.
### Table 1.

**Characteristics of administered radiolabelled antibody.**

<table>
<thead>
<tr>
<th>Pat. No.</th>
<th>MoAb</th>
<th>% Free I(_2)</th>
<th>% Agg.</th>
<th>Immunoreactive Fraction (%)***</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UJ 181.4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>F8.11.13</td>
<td>6</td>
<td>2</td>
<td>NA*</td>
</tr>
<tr>
<td>3</td>
<td>UJ 181.4</td>
<td>10</td>
<td>5</td>
<td>NA*</td>
</tr>
<tr>
<td>4</td>
<td>UJ 181.4</td>
<td>5</td>
<td>2</td>
<td>NA*</td>
</tr>
<tr>
<td>5</td>
<td>Mel.14</td>
<td>4</td>
<td>1</td>
<td>NA*</td>
</tr>
<tr>
<td>6</td>
<td>UJ 181.4</td>
<td>13</td>
<td>0**</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>UJ 181.4</td>
<td>4</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>8</td>
<td>Mel.14</td>
<td>3</td>
<td>1</td>
<td>35</td>
</tr>
<tr>
<td>9</td>
<td>HMFG1</td>
<td>2</td>
<td>1</td>
<td>85</td>
</tr>
<tr>
<td>10</td>
<td>HMFG1</td>
<td>1</td>
<td>1</td>
<td>84</td>
</tr>
<tr>
<td>11</td>
<td>81C6</td>
<td>1</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>12</td>
<td>UJ181.4</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>81C6</td>
<td>1</td>
<td>0</td>
<td>90</td>
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<tr>
<td>14</td>
<td>HMFG1</td>
<td>2</td>
<td>0</td>
<td>84</td>
</tr>
<tr>
<td>15</td>
<td>HMFG1</td>
<td>2</td>
<td>0</td>
<td>85</td>
</tr>
</tbody>
</table>

Where Pat no indicates the patient in the study; MoAb monoclonal antibody administered; % Free I\(_2\), percentage free iodine measured in radiolabelled antibody; % Agg, % microaggregated protein in injected preparation, Immunoreactive Fraction %, % of immunoreactive radiolabelled monoclonal antibody; NA, not assessed; NA*, not assessed for % immunoreactive fraction, but biologically active, by indirect immunofluorescence assay; ** Multiple aggregates and fragments on S300 column chromatography.

***Performed by Dr R. Mosely, Bristol Brain Tumour Research Group.

**Sites of isotope accumulation.**

All patients were scanned as soon and as frequently as their clinical condition allowed. The first scintigram was obtained when whole body radioactivity had diminished to a level of 20 mCi. Usually, this resulted in the first scintigram at 5-7 days after therapy.

The biodistribution of radionuclide varied with both the pattern of tumour distribution and the antibody used.
All injections on radiolabelled antibody \( \text{antibody} \) followed by some accumulation of isotope in the liver and spleen. This was most marked for the antibodies Mel.14 and 81C.6. The antibody Mel.14 was also associated with pronounced skeletal accumulation of isotope (Fig 9.1).

The distribution of isotope within the neuraxis correlated well with the anticipated clinical and radiological distribution of tumour (Table 1 and Figs. 9 1-8). The effective half life of isotope at sites of focal accumulation was not measured for all patients, but results were available for patient 1, 5, 6 and 7. The effective tumour half life was estimated as 253 hours (optic nerve), patient 1; 127 hours (cerebral deposit), patient 5; 72 hours (posterior fossa), patient 6; and 307 hours (posterior fossa), 197 hours (thoracic spine), patient 7. Eight unexpected areas of isotope accumulation were noted. These were not consistent between patients, and comprised: posterior fossa, in two patients referred with relapsed spinal deposits of medulloblastoma; left cerebral cortex in a third patient with medulloblastoma; 4 additional spinal sites, (Patients 1, 11, 14 and 15); and abdominal foci in a patient with metastatic tumour from ovarian carcinoma.
Fig.9.1-9.8. Distribution of radiolabelled monoclonal antibodies, after intrathecal administration. (T) denotes known site of tumour.

Fig.9.1. Scintigrams at 5, 7 and 12 days after administration of 55mCi of $^{131}$I Mel.14. in patient with melanoma. Demonstrating accumulation in spleen, skeleton and bladder in addition to tumour in the occipital pole, and mid-thoracic spine.

Fig.9.2. Scintigrams at 7, 10, 12 and 14 days after administration of 40 mCi of $^{131}$I UJ181.4 in patient with medulloblastoma. Isotope noted to accumulate in liver in addition to tumour sites in posterior fossa and thoracic spine.

Fig.9.3. Scintigrams at 6 and 8 days after administration of 40 mCi of $^{131}$I UJ181.4 in patient with relapsed medulloblastoma. Accumulation of isotope noted in thyroid, posterior fossa (site of primary tumour) and in thoracic and lumbar spine. (Sites of tumour)

Fig.9.4. Scintigrams at 7 and 9 days after administration of 55 mCi of $^{131}$I HMFG2 in patient with neoplastic meningitis secondary to ovarian carcinoma. Uptake noted in thyroid, bowel and tumour (lumbar deposits).
Fig. 9.5. Scintigrams of head 7-15 days after administration after 40mCi of $^{131}$I F8-11-13. Accumulation of isotope in thyroid and in tumour involving facial nerve.

Fig. 9.6. Scintigrams of head 7-14 days after administration of 24mCi of $^{131}$I UJ181.4. Accumulation of isotope in thyroid and tumour infiltrating optic nerve.

Fig. 9.7. Scintigrams of head 10-20 days after administration of 60mCi of $^{131}$I Mel.14, in patient with relapsed melanome. Accumulation of isotope noted in the occiput and in basal cisterns.

Fig. 9.8. Scintigrams of head 5-21 days after administration of 40 mCi of $^{131}$I UJ181.4. Accumulation of isotope noted in shunt (Sh) and posterior fossa tumour (medulloblastoma).
Fig 9.5

Fig 9.6
**Fig 9.7**

**Fig 9.8**
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pineoblastoma</td>
<td>High CSF cell count-153/ml.</td>
<td>Left optic nerve.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Infiltration left optic nerve.</td>
<td>Marked, irregular accumulation of isotope in lumbar expansion.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Multiple spinal deposits.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Lymphoma</td>
<td>High CSF cell count-372/ml. 3rd, 4th, 6th cranial nerve palsies. 7th LMN cranial nerve palsy.</td>
<td>Persistent focus right side of face.</td>
</tr>
<tr>
<td>3</td>
<td>Spinal Teratoma</td>
<td>Malignant cells on cytospin. Spinal block L3-4.</td>
<td>No focal accumulation.</td>
</tr>
<tr>
<td>4</td>
<td>Medulloblastoma</td>
<td>Multiple spinal deposits.</td>
<td>Lumbar, sacral and mid-thoracic spine. Posterior fossa.</td>
</tr>
<tr>
<td>6</td>
<td>Medulloblastoma.</td>
<td>Cytospin + Cystic mass posterior fossa.</td>
<td>Posterior fossa*, right&gt;left.</td>
</tr>
<tr>
<td>8</td>
<td>Melanoma</td>
<td>Cytospin + Non enhancing lesion, right occiput.</td>
<td>Occipital pole right hemisphere.</td>
</tr>
<tr>
<td>Code</td>
<td>Diagnosis</td>
<td>Methodology</td>
<td>Findings</td>
</tr>
<tr>
<td>------</td>
<td>---------------------------</td>
<td>------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>9</td>
<td>Ca Ovary</td>
<td>Cytospin +</td>
<td>Superior surface</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Left 6th cranial nerve.</td>
<td>cerebral cortex.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lumbar-sacral nerve roots.</td>
<td>Sacral spine.</td>
</tr>
<tr>
<td>10</td>
<td>Ca Bladder</td>
<td>High CSF cell count &gt;1000/ml.</td>
<td>Not scanned.</td>
</tr>
<tr>
<td>11</td>
<td>Gliomatosis</td>
<td>Cytospin + Intaventricular</td>
<td>Ventricular persistence.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>extension of tumour</td>
<td>Basal cisterns.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thoracic spine.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sacral spine.</td>
</tr>
<tr>
<td>12</td>
<td>Medullo-blastoma.</td>
<td>No evaluable disease.</td>
<td>Posterior fossa*.</td>
</tr>
<tr>
<td>13</td>
<td>Gliomatosis</td>
<td>----------------------------</td>
<td>Ventricular persistence.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intra-abdominal**</td>
</tr>
<tr>
<td>14</td>
<td>Ca Breast</td>
<td>Cytospin + Multiple cranial</td>
<td>Single scintigram 21 days. 3 foci head.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nerve palsies.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sacral nerve roots.</td>
<td>Lumbar spine.</td>
</tr>
<tr>
<td>15</td>
<td>Adeno-carcinoma. ? primary.</td>
<td>Cytospin + Multiple deposits</td>
<td>Diffuse cortical uptake.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sacral recess thecal sac.</td>
<td>Single focus lumbar spine.</td>
</tr>
</tbody>
</table>

*Biologically inactive antibody.

**Leakage through a patent VP shunt.

**Toxicity**.

**Aseptic meningitis.**

Acute aseptic meningitis was the major complication after administration of the radionuclide. This occurred after 7/15 antibody administrations, and was characterised by a triad of headache, nuchal rigidity and nausea/vomiting. Symptoms typically began at 4 to 6 hours after administration of conjugate and persisted for a period of 8 to 12 hours. In 2 patients, symptoms were protracted and persisted for 48 hours. On 3 occasions the symptom
complex was associated with a mild pyrexia of between 37.5°C and 38.5°C. Where lumbar puncture was performed in the first 24 hours after administration of conjugate, results were consistent with an aseptic meningitis.

**Bone marrow suppression.**

Three patients developed reversible bone marrow suppression at an administered dose of approximately 60mCi of $^{131}$I MoAb. In two patients this occurred in combination with the antibody HMFG1 (Pat. 9 and 14) and in the remaining patient with the monoclonal antibody Mel.14 (Pat 8). The nadir in white cell count occurred at week 5 in patient 8 (WCC: $0.8 \times 10^9/1$, 11% Neutrophils), week 5, patient 9 (WCC: $3.1 \times 10^9/1$), and week 4 patient 14 (WCC $2.1 \times 10^9/1$, 85% Neutrophils). A commensurate fall in other marrow elements was noted with platelet counts reaching $23 \times 10^3/ml$, $86 \times 10^3/ml$ and $76 \times 10^3/ml$ respectively. A return to a normal peripheral blood count was noted by week 9 in all patients.

**Neurological disturbance.**

Two forms of neurological disturbance were noted in the study. The first was the occurrence of transient paraesthesiae during lumbar injection of isotope. Tingling over sacral dermatomes was reported by patient 4, during the administration of conjugate. This symptom resolved within a few minutes of completing the injection.
Of greater concern was the occurrence of seizures in patients 10 and 14. Patient 10 presented with a history of progressive dementia and two grand mal seizures. In the week prior to therapy the patient was noted to have signs of raised intracranial pressure and to be deteriorating clinically. Injection of 58 mCi of radiolabelled conjugate produced a mild headache that lasted for 4-6 hours. 48 hours after administration of the conjugate, the patient was found drowsy and unresponsive. This state reversed in 20 minutes and was attributed to an unwitnessed seizure. The patient died suddenly in bed during the night, possibly as a result of a further unwitnessed seizure. Post-mortem revealed extensive involvement of the cerebral leptomeninges by tumour. In addition marked, acute oedematous and reactive changes were noted in the ventricular and subpial white matter. Milder oedematous changes were noted in white matter away from the CSF surfaces. It was felt that the degree of oedema was disproportionate to the degree of tumour infiltration, and was possibly related to an acute radiation effect.

The second patient (No.14) fitted after 60 mCi of $^{131}$I HMFG1. Following an initial improvement in her clinical state, patient 14 suddenly developed status epilepticus, 10 days after antibody administration. The patient required treatment with a continuous Heminevrin infusion before making a clinical recovery. No clear precipitating cause was identified for the sudden deterioration.
Chronic toxicity.
Patients 1 and 2 underwent post mortem, 2 and 1 years from administration of radiolabel. Multiple sections through the brain, spinal cord and leptomeninges were taken and examined for histological evidence of radiation damage. There were no histological abnormalities attributable to radiation damage in either the parenchyma or vasculature of brain or spinal cord. In patient 2, some fibrotic changes were identified in the cerebral leptomeninges. The changes were patchy and consisted of a loose meshwork of collagen deep to the layer of arachnoid cells. This meshwork contained a mixture of fibroblasts, macrophages and chronic inflammatory cells.

Response to therapy.
Of the 15 patients enrolled in the study, 11 were considered evaluable for clinical reponse. The four exclusions were Patient 6 (Biologically inactive conjugate), Patient 7 (Received conventional radiotherapy to all evaluable sites within 6 weeks of antibody therapy), Patient 10 (Toxic death), and Patient 12 (Received conventional radiotherapy to evaluable site and biologically inactive conjugate).

Of the 11 patients evaluable for evidence of response, 10 had abnormal clinical signs at the time of treatment. Six of these demonstrated a marked improvement in clinical condition, for up to 3 months from treatment (Table 3). The improvement in
**Fig 9.9.** CAT scan of head (with contrast), showing appearances before (1), and after (2) administration of 40mCi of $^{131}$I Mel.14.
(a) Enhancing tumour rim in first scan, and tumour present in the quadrigeminal plate (b). These appearances have improved at the time of the second CAT scan.

**Fig 9.10 and 11.** CSF cell counts with time in patients 1 and 2.
TABLE 3
Clinical Summary of all Patients Entered into the Phase I Study of Intrathecal \textsuperscript{131}I MoAb.

<table>
<thead>
<tr>
<th>PATIENT</th>
<th>DISEASE</th>
<th>PRIOR RX.</th>
<th>CLINICAL STATUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Medulloblastoma</td>
<td>Surgery. Cranial DXT, 5500 rads, 1500 rads, T8-L1</td>
<td>Recurrent disease.</td>
</tr>
<tr>
<td>5</td>
<td>Melanoma</td>
<td>Surgery.</td>
<td>Residual disease.</td>
</tr>
<tr>
<td>6</td>
<td>Medulloblastoma</td>
<td>Surgery. Neuraxis DXT. Total tumour dose - 10,500 rads. VCR, CCNU.</td>
<td>Recurrent disease.</td>
</tr>
<tr>
<td>THERAPY</td>
<td>CLINICAL SIGNS</td>
<td>CLINICAL RESPONSE</td>
<td>MEASURED TUMOUR RESPONSE</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------</td>
<td>------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>24 mCi UJ181.4</td>
<td>Confused</td>
<td>CR</td>
<td>CR-CSF</td>
</tr>
<tr>
<td></td>
<td>Cachectic</td>
<td>CR</td>
<td>parameters*</td>
</tr>
<tr>
<td></td>
<td>Nuchal rigidity</td>
<td>CR</td>
<td>Repeat myelography</td>
</tr>
<tr>
<td></td>
<td>Optic atrophy</td>
<td>NR</td>
<td>not performed.</td>
</tr>
<tr>
<td></td>
<td>Paraparesis</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>40 mCi F8.11.13</td>
<td>Confused</td>
<td>CR</td>
<td>CR-CSF</td>
</tr>
<tr>
<td></td>
<td>Nuchal rigidity</td>
<td>CR</td>
<td>parameters*</td>
</tr>
<tr>
<td></td>
<td>Multiple UMN</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cranial N palsies</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LMN VII palsy</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>11 mCi UJ181.4</td>
<td>Paraparesis</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>40 mCi UJ181.4</td>
<td>Paraparesis</td>
<td>CR</td>
<td>CR-myelography.</td>
</tr>
<tr>
<td>45 mCi Mel.14</td>
<td>Raised ICP</td>
<td>CR</td>
<td>CR-CAT imaged</td>
</tr>
<tr>
<td></td>
<td>Epilepsy</td>
<td>NR</td>
<td>lesions head*</td>
</tr>
<tr>
<td></td>
<td>Ataxia</td>
<td>I</td>
<td>Repeat myelography</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>not performed.</td>
</tr>
<tr>
<td>30 mCi UJ181.4</td>
<td>Raised ICP</td>
<td></td>
<td>NOT ASSESSED</td>
</tr>
<tr>
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<td>Ataxia</td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
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</tr>
<tr>
<td></td>
<td>Paraparesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sensory level</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PATIENT</td>
<td>DISEASE</td>
<td>PRIOR RX.</td>
<td>CLINICAL STATUS</td>
</tr>
<tr>
<td>---------</td>
<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>10</td>
<td>Bladder Ca.</td>
<td></td>
<td>New disease.</td>
</tr>
<tr>
<td>12</td>
<td>Medulloblastoma</td>
<td>Surgery. Neuraxis DXT, TD 6,000 rads. Post fossa, 5,500 rads MOPP. I/T Cytosine, Mtx., hydrocortisone, Carboplatinum, Cyclo, Mtx., VCR.</td>
<td>Recurrent disease.</td>
</tr>
<tr>
<td>13</td>
<td>Gliomatosits</td>
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<td></td>
</tr>
<tr>
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<td>Ca. Breast</td>
<td></td>
<td></td>
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<td>CLINICAL SIGNS</td>
<td>CLINICAL RESPONSE</td>
<td>MEASURED TUMOUR RESPONSE</td>
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<tr>
<td>----------</td>
<td>------------------</td>
<td>------------------</td>
<td>--------------------------</td>
</tr>
<tr>
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<td>--</td>
<td>CR on CSF parameters.</td>
</tr>
<tr>
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<td>Paraparesis</td>
<td>CR</td>
<td>CR on CSF parameters.</td>
</tr>
<tr>
<td></td>
<td>Incontinent</td>
<td>CR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6th N palsy</td>
<td>I</td>
<td>Repeat myelography not performed.</td>
</tr>
<tr>
<td>58 mCi HMFG1</td>
<td>Dementia</td>
<td>--</td>
<td>TOXIC DEATH</td>
</tr>
<tr>
<td>60 mCi 81.C6</td>
<td>ICP</td>
<td>NR</td>
<td>Progressive disease.</td>
</tr>
<tr>
<td></td>
<td>Paralysis</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>downward gaze.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L homonymous hemianopia</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>48 mCi UJ181.4</td>
<td>NED</td>
<td>--</td>
<td>NOT ASSESSED</td>
</tr>
<tr>
<td>60 mCi 81.C6</td>
<td>ICP</td>
<td>NR</td>
<td>Progressive disease.</td>
</tr>
<tr>
<td></td>
<td>L Ptosis</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>56 mCi HMFG1</td>
<td>Multiple cranial nerve palsies.</td>
<td>I</td>
<td>CR-CSF parameters*.</td>
</tr>
<tr>
<td></td>
<td>Ataxia</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sensation, ---</td>
<td></td>
<td></td>
</tr>
<tr>
<td>58 mCi HMFG1</td>
<td>Paraparesis</td>
<td>NR</td>
<td>Progressive disease.</td>
</tr>
<tr>
<td></td>
<td>sacral sensation.</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VII N palsy</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ICP = Intracranial pressure.
CSF = Cerebrospinal fluid.
CR = Complete resolution of abnormal findings.
I  = Improvement in clinical status.
NR = No change or deterioration in abnormal finds.
neurological status was accompanied by an objective measurement of tumour response in these, and in a seventh asymptomatic patient.

The median survival of the total group was 8 months (Mean: 9 months, Range: 0-31) and 7.5 months (Mean: 9.6 months, Range 0-31), for the group receiving biologically active antibody and no supplementary therapy. These results have been achieved with the addition of limited spinal irradiation to a thoracic reoccurrence of tumour in patient 4, 7 months from antibody therapy, and further neuraxis irradiation 5 months after antibody treatment in patients 5 and 12. No other patients have received additional therapy.

5 patients remain alive at: 31+ months (Patient 4; No estimatable disease [NED]), 18 months+ (Patient 7; Alive with extracranial medulloblastoma), 14+ months (Patient 8; NED), 12+ months (Patient 12; NED) and 8 months+ (Patient 14; NED).
DISCUSSION.

Radioisotopic therapy of leptomeningeal tumour by colloidal gold enjoyed a vogue during the mid to late 1960's (171)(172). The major problem associated with the approach was the level of non-specific irradiation to areas uninvolved by tumour. A substantial number of patients treated in this way went on to develop delayed radionecrosis of the cord (124).

This issue is crucial to further development of radiolabelled monoclonal antibodies as treatment for leptomeningeal tumour. In choosing appropriate monoclonal antibodies, we selected antibodies that were known to react with a patient's tumour, but were not immunoreactive with normal CNS components. Administration of the radiolabelled antibody, produced a varied distribution of isotope within the neuraxis that correlated with the anticipated clinical and radiological pattern of disease. The sensitivity of the radioimmunolocalisation has not been reported, due to the difficulties in quantifying and correlating myelographic findings with scintigraphy. Firstly, myelography was frequently reported as "multiple spinal deposits", and secondly the imaging characteristics of $^{131}$I are such that it is frequently difficult to separate small foci in close proximity. The problems observed during trials of colloidal gold indicate that specificity of isotope localisation is crucial. Isotope was noted on 8

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occasions to be present at sites not radiologically involved by disease using standard criteria. The sites were not consistent between patients, and tended to reflect the known clinical pattern of disease e.g. posterior fossa in medulloblastoma patients, abdominal foci in patient with carcinomatous meningitis secondary to an ovarian primary.

This data is supportive of the specificity of I/T radioimmunolocalisation, but does not prove it. Non specific localisation is entirely possible due to disturbed vascular flow within tumours, and trapping within cysts (124)(175). These features are more likely in larger tumour deposits, and may explain the observed accumulation of isotope in the cystic tumour of patient 6 who was administered a conjugate with 2% immunoreactive fraction. Alternatively, the small proportion of immunoreactive conjugate may have been sufficient for tumour detection, but not for effective therapy.

Some evidence for the specificity of the technique, is provided by an experiment conducted by co-worker, Dr A. Davies (Bristol Brain Tumour Research Group). Patient 1, underwent a dual label tracer study prior to therapy. He was simultaneously administered $^{131}$I UJ181.4 and $^{125}$I HMFG2 into the thecal compartment. On three successive days CSF was sampled, and centrifuged to separate the malignant cell pellet from CSF. The ratio of specific (UJ181.4) to non specific antibody (HMFG2) reached 10:1 in the cell pellet, but remained at unity in the CSF. Toxicity might be expected to correlate with the
operational specificity of the radiolabelled conjugate. In practice, toxicity was related to both the introduction of protein into the CSF as well as due to the biodistribution of radionuclide. Aseptic meningitis has been documented following $^{131}$I HSA cisternography (176). This was reported as a feature of specific preparations of albumen, and was reduced by modifying the formulation (177). This suggests that aseptic meningitis may not be an invariable consequence of intrathecal MoAb therapy, and might be reduced by pharmacological manipulation of the preparation.

Other toxicities were related to the dose of administered radionuclide. Three of seven patients given the highest dose of $^{131}$I had evidence of bone marrow suppression. Two of them probably had a high bone marrow dose, due to the biological characteristics of the monoclonal antibody. Patient 8 had marked skeletal accumulation of isotope, due to Mel.14 expression in bone, whilst Patient 9 was noted to generate large molecular weight complexes in serum. These were likely to be either immune complexes resulting from tumour shed antigen or from pre-existing anti-mouse immunoglobulin, resulting in increased reticuloendothelial uptake.

Neural toxicity was noted at 60 mCi of HMFG1. Of the two patients developing seizures, one died and came to post mortem. It was not possible to be certain about the cause of death. The patient had extensive leptomeningeal infiltration by tumour, and the V-P shunt that had been inserted to control raised intracranial pressure had been
clipped off, to facilitate circulation of conjugate. Post mortem examination of the brain demonstrated wide spread cerebral oedema, which may have partially resulted from an acute radiation effect. It is likely that a sudden rise in intracranial pressure due to radiation induced cerebral oedema, was sufficient to cause in death in an already compromised patient.

Only 2 patients have survived more than 1 year and came to post mortem. We were interested to see if there was any histological evidence of toxicity. In patient 1, abnormal histological changes in the CNS were soley attributable to disease progression. Patient 2, who received 40 mCi of $^{131}$I F8.11.13, had widespread fibrosis of the leptomeninges. This may have been attributable to a radiation effect, either from targeted radiotherapy alone, or in combination with external beam therapy and I/T methotrexate. No parenchymal damage was observed. Further data on long term toxicity, requires both time, and patients who have not been extensively pretreated.

The combination of marrow toxicity and neural toxicity at 55-60 mCi of isotope, suggests that Phase II studies should be undertaken at lower doses. This is endorsed by the encouraging number of clinical responses to $^{131}$I MoAb, at or below this dose. 7/11 patients demonstrated major, sustained clinical responses to antibody treatment. The study was not sufficiently rigorous in repeating myelography to define an objective tumour response. However, a clinical response was
invariably associated with a return to normal CSF
cytology, protein and glucose.

The importance of the results should be assessed in
the context of alternative forms of treatment. The
patients studied represent many of commoner neoplastic
meningitides, and range from the highly radiosensitive,
such as medulloblastoma and pineoblastoma (178)(179), to
the tumours of variable radioresposiveness, such as
melanoma (180)(181) and carcinoma.

It is hardly suprising that the patients with the
most radiosensitive tumors, fared best. Similar
responses have been reported for medulloblastoma,
pineoblastoma and NHL with external beam radiotherapy,
+/- combination chemotherapy (182)(183)(184). Mean
survival following a spinal relapse in medulloblastoma is
of the order of 12-13 months. 47% of patients with
primary lymphoma of the brain are also alive after CNS
irradiation at 1 year, although the patient included in
this study, had progressive disease during treatment.

A more depressing prognosis is expected for patients
with carcinoma or melanoma infiltrating the leptomeninges
(185). In a prospective trial of single agent versus
combination chemotherapy in meningeal carcinomatosis,
median survival of the total group was 8 weeks. The
addition of radiotherapy improved the response rate,
although the median survival of responders was still poor
at 18 weeks. Other studies report a similarly poor
prognosis (121)(123).
The responses observed in this patient group, (4/6; melanomas and carcinomas) and their survival to between 7 and 18 months is encouraging. We plan to continue the evaluation of targeted radiotherapy in the areas of relapsed medulloblastoma, and carcinomatous meningitis. Two Phase I/II studies are underway in these tumors, with more attention to quality control i.e. better monitoring of the purity and immunoreactivity of the radiolabelled antibody, and to tighter evaluation of tumour response.
CHAPTER 12.

The Pharmacokinetics Of Intrathecal Antibody Administration.

Pharmacokinetics of intrathecally administered monoclonal antibodies in patients.
The outcome of our Phase I study of intrathecally administered radiolabelled monoclonal antibodies, has encouraged the group to persist with the investigation of radiolabelled antibodies. During the course of that study, we had several concerns. Firstly, we were uncertain that the accumulation of isotope in tumour was a specific phenomenon; secondly, although acute toxicity was tolerable below 60 mCi we were anxious about delayed toxicity to the spinal cord; and thirdly, we did not know how much of tumour dose delivery was a result of suspended radioactivity in the CSF. These issues remain under investigation. However, we have undertaken a limited number of experiments to clarify some points.

The first of these, was a clinical study which ran concurrently with the Phase I study. The first 7 patients referred for treatment with I/T ¹³¹I monoclonal antibody, were recruited to a preliminary investigation, in which the pharmacokinetics of intrathecally administered monoclonal antibodies were assessed. The aim of this study was two fold: 1) to define the pharmacokinetics of intrathecally administered monoclonal antibodies, and 2) to seek evidence of specificity in tumour targeting.
METHODS.

An initial tracer study was undertaken 5-10 days before treatment. Following the methods outlined in Chapter 11, thyroid blockade was started 24 hours before administration of tracer antibodies. Patients were assessed for hypersensitivity to mouse protein by intradermal injection of 10ug of mouse immunoglobulin in 100ul of PBS. Thirty minutes later, each patient was injected intrathecally via the lumbar route with a mixture of tracer amounts of "specific" (tumour binding) antibody (11-60 ug), and "non-specific" antibody (22-55ug). As with the Phase I study, "specific" antibody was chosen for its immunoreactivity on tumour but not on human brain. The "non-specific" antibody was matched for isotype with the specific antibody, and was neither immunoreactive with tumour or brain.

"Specific" antibody was radiolabelled with $^{131}$I and "non-specific" antibody with $^{125}$I by the modified chloramine-T technique to a specific activity of between 5-10 uCi/ug (Chapter 2;6a). The amount of free iodine contained in the radiolabelled preparation was estimated by TCA precipitation, and aggregation either by centrifugation at 10,000 x g for 30 minutes, or by Sephacryl S300 column chromatography.

To follow the pharmacokinetics of administered antibodies, blood was sampled at frequent intervals over a period of 5 minutes to 5 days. In patients with indwelling CSF resevoirs, CSF was withdrawn at 30
minutes, 4, 12, 24 hours, and daily thereafter. Additional information on the clearance of the $^{131}$I was provided by external counting with a collimated scintillation probe (containing a 25mm x 25 mm sodium iodide, thallium activated crystal-Nuclear Enterprises, Edinburgh). Measurements were made over standard reference points of the occiput, C4, D5, D12 and S1 at approximately 2 and 8 hours, and twice daily to 7 days.

Sequential scintigraphy was performed on an International General Electric 400T Gamma camera, with a medium or high energy collimator and linked to a DEC PDP computer fitted with Gamma 11 software. Serial scintigraphy was undertaken as long as significant radioactivity remained in the neuraxis. The first scan was obtained at 10 minutes after injection, then at 4 hours and daily thereafter.

Estimates of radioactivity in tissue samples were made by counting aliquots, against a standard activity in a scintillation well counter. The total radioactivity remaining in blood or CSF, expressed as a percentage of injected dose, was estimated from standard normograms for CSF and blood volume. Time-activity curves were generated by scintigraphy for selected areas of the neuraxis, using standard "region of interest" analysis. Counts per minute/unit area, were corrected for background activity by background subtraction. For spinal segments, an equivalent area immediately lateral to the spinal segment was used as background, whilst for head, an area was taken from thigh.
RESULTS:
Six of the patients studied, had progressive disease at the time of study, and are patients 1, 2 and 4-7 detailed in Chapter 11. A sixth patient was referred because of a suspected re-occurrence of an ependymoma (symptoms of compression on sacral nerve roots, and marginal abnormalities on a CT scan of spinal cord). Following the tracer study, the patient's symptoms spontaneously recovered, and the patient did not proceed to treatment.

Radiolabelled antibody.
Six different antibodies were used in the study (Table 1). Where the "specific" antibody was an IgG₁, HMFG2 was generally used as the "non-specific" antibody. This policy was deliberately changed in the last patient. Radiolabelling produced a preparation that varied in quality. 2/4 radiolabellings of UJ181.4 produced protein that was significantly aggregated (9% and >10% aggregated protein in the injected dose). Other antibodies labelled satisfactorily, with minimal levels of free isotope or aggregates. (% Aggregation: Range <2-4, and % free iodine, <2-6)
<table>
<thead>
<tr>
<th>Pat. No.</th>
<th>MoAb</th>
<th>Isotype</th>
<th>% Agg.</th>
<th>% Free I2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UJ181.4</td>
<td>IgG1</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>HMFG2</td>
<td>IgG1</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>2</td>
<td>F8.11.13</td>
<td>IgG1</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>HMFG2</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>4</td>
<td>UJ181.4</td>
<td></td>
<td>9</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>HMFG2</td>
<td>2</td>
<td></td>
<td>&lt;2</td>
</tr>
<tr>
<td>5</td>
<td>Me1.14</td>
<td>IgG2a</td>
<td>&lt;2</td>
<td>&lt;2</td>
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<tr>
<td></td>
<td>4C.6</td>
<td>IgG2b</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>6</td>
<td>UJ181.4</td>
<td></td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>HMFG2</td>
<td>&lt;2</td>
<td></td>
<td>&lt;2</td>
</tr>
<tr>
<td>7</td>
<td>UJ181.4*</td>
<td>&gt;10</td>
<td>&lt;2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HMFG2</td>
<td>&lt;2</td>
<td></td>
<td>&lt;2</td>
</tr>
<tr>
<td>16</td>
<td>FD32</td>
<td>IgG1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>UJ181.4</td>
<td>&lt;2</td>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>

* 20% immunoreactive fraction by radiobinding assay. All other preparations immunologically active by indirect immunofluorescence assay; NE, not estimated; %Agg, % aggregated protein in administered product; % Free I2, % of free isotope.
Biodistribution of radiolabelled protein.

Following intrathecal administration of the radiolabelled antibodies there was a rapid distribution of isotope within the CNS. In 5/7 patients, isotope was visible throughout the spinal canal, and over the cerebral hemispheres by 24 hours. This was not achieved in 2 patients (No.2 and 5), and from myelographic studies was attributable to a spinal block from tumour in patient 2. In patient 5, isotope did not ascend above the basal cisterns. No cause was ascertained as both free flow of contrast was seen during myelography, and isotope during therapy.

Loss of isotope to blood was rapid. Both $^{125}$I and $^{131}$I were detected in blood minutes after injection. The concentration of both radiolabels increased with time, reaching a peak activity at approximately 24 hours, (Range 4-48 hours) before decreasing in an exponential fashion. Both peak activity in blood, and subsequent clearance of radiolabel were highly variable both between antibodies, and between patients. Table 2. (Mean T1/2 HMFG2; 56 hours, Range 45-65, Mean T1/2 UJ181.4; 74 hours, Range 45-90).
### TABLE 2.

**Pharmacokinetic data on patients administered antibodies.**

<table>
<thead>
<tr>
<th>Pat No.</th>
<th>MoAb.</th>
<th>Peak % Blood.</th>
<th>Blood T1/2 (hrs)</th>
<th>CSF T1/2</th>
<th>I%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UJ181.4</td>
<td>9</td>
<td>47</td>
<td>9</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>HMFG2</td>
<td>25</td>
<td>57</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>F8.11.13</td>
<td>25</td>
<td>70</td>
<td>NE</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>HMFG2</td>
<td>35</td>
<td>65</td>
<td>NE</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>UJ181.4</td>
<td>15</td>
<td>86</td>
<td>6</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>HMFG2</td>
<td>25</td>
<td>60</td>
<td>7</td>
<td>29</td>
</tr>
<tr>
<td>5</td>
<td>Mel.14</td>
<td>9</td>
<td>92</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>4C.6</td>
<td>26</td>
<td>36</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>6</td>
<td>UJ181.4</td>
<td>26</td>
<td>85</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>HMFG2</td>
<td>33</td>
<td>53</td>
<td>3</td>
<td>20</td>
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<td>7</td>
<td>UJ181.4</td>
<td>19</td>
<td>45*</td>
<td>6</td>
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<td></td>
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<td>46</td>
<td>45</td>
<td>6</td>
<td>36</td>
</tr>
<tr>
<td>16</td>
<td>FD32</td>
<td>42</td>
<td>74</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>UJ181.4</td>
<td>11</td>
<td>90</td>
<td>NE</td>
<td>NE</td>
</tr>
</tbody>
</table>

Where T1/2 refers to the biological half life of radiolabelled conjugate; Peak % blood, to the maximal percentage of injected dose present in blood at any time, I%, the estimated percentage of injected dose present in CSF, as determined by extrapolating the second clearance phase to the ordinate; NE, not estimated.

It was noted that in patients 2-7, (Crude data not available for patient 1), that there was a tendency for the non-specific antibody to be present in an unexpectedly high amount in serum, when compared with the tumour binding antibody. **Fig 10.1.**

To investigate whether this was due to different kinetic properties of the paired antibodies, or to delayed excretion of the binding antibody, Patient 16 was administered UJ181.4 as the non-specific antibody. The
Fig 10.1 Vascular clearance of $^{131}$I "relevant" and $^{125}$I "irrelevant" antibody after intrathecal administration in patients.

Graph shows the percentage of injected dose ($^{131}$I-$^{125}$I) in blood for different patients.
kinetic pattern was reversed, with the "specific" antibody present in early excess.

Clearance of isotope from the CSF.
The fall in activity from the CNS, followed biexponential clearance kinetics. This was reflected both in measurements taken with the external scintillation probe and in direct measurements by CSF sampling. The initial fall in suspended activity (CSF radioactivity) was rapid, with an estimated mean biological half life 8 hours (Range 3-10). The magnitude of the initial fall is reflected in the intercept with the ordinate of the second clearance phase. Less than 3% of the injected dose, is estimated to be present in CSF at the end of the first clearance phase. This estimate should be compared with the estimates of activity, using external probe measurements. In the three patients, studied in this way, considerably higher estimates of activity in the neuraxis were made. Table 3.

<table>
<thead>
<tr>
<th>Pat No.</th>
<th>131I External Probe</th>
<th>131I CSF Samples</th>
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</thead>
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<tr>
<td></td>
<td>$T_1/2_1$</td>
<td>$T_1/2_2$</td>
</tr>
<tr>
<td>1.</td>
<td>8</td>
<td>59</td>
</tr>
<tr>
<td>2.</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>4.</td>
<td>4</td>
<td>44</td>
</tr>
</tbody>
</table>

*Estimate of radioactivity present in thoracic segment of the neuraxis performed by Mr. R. Richardson, Medical
Physicist, Frenchay Hospital, Bristol. T1/2 refers to the biological half life of radiolabelled conjugate.

Several variables were probably influential in determining the observed kinetics in patients. These include the influence of tumour binding on the rate of isotope loss from the CNS as well as fluctuations in the quality of injected product. To interpret the kinetic data more fully, it was decided to investigate the kinetics of UJ181.4 and HMFG2 in the absence of CNS tumour.

A comparison of the pharmacokinetics of intrathecally administered monoclonal antibodies HMFG2 and UJ181.4.

STUDY DESIGN.

Both rabbits and marmosets were considered for use in this experiment. After a number of preliminary experiments, marmosets were chosen to model the pharmacokinetics of I/T monoclonal antibodies. This was because, a) marmosets appeared to tolerate intrathecal administration better than rabbits, b) it was easier to reliably inject isotope into the thecal sac of marmosets and c) neither HMFG2 or UJ181.4 was expressed on marmoset CNS as determined by indirect immunofluorescence.

12 marmosets were selected that had not been used for any previous experimental procedure. Three of the animals were chosen to examine the kinetics of HMFG2 and UJ181.4 following intravenous administration. The remaining nine were utilised to define the kinetics of the paired antibodies after intrathecal administration.
Monoclonal antibody HMFG2 and UJ181.4 were radiolabelled by the modified Chloramine-T technique with $^{125}\text{I}$ and $^{131}\text{I}$ respectively. The antibodies were radioiodinated to a specific activity of approximately $5\mu\text{Ci}/\mu\text{g}$. Both radiolabellings were examined by HPLC for the presence of aggregates and free iodine before administration. No preparation was accepted unless there were no appreciable aggregates or free iodine in the radiolabelled protein (Fig 10.2).

Animals underwent thyroid blockade in the standard manner. Three animals were injected intravenously with 100 ul of a solution containing 4 $\mu\text{Ci}$ of $^{131}\text{I}$ UJ181.4 and 5 $\mu\text{Ci}$ of $^{125}\text{I}$ HMFG2. The animals had serial blood samples taken at 30 minutes, 1, 2, 4, 6, 24 and 28 hours from injection. Time activity curves were generated for the two isotopes in the usual manner.

The remaining 9 animals were injected with 100 ul of a mixture of the isotopes intrathecally. The introduction of isotope into the subarachnoid space was monitored by scintigraphy of the marmoset spine at 1 minute and 1 hour after injection. An estimate of the administered dose of $^{131}\text{I}$ was made from measuring the full and empty syringe on the camera face.

Animals underwent sequential blood sampling until sacrifice at the time points 3, 6 and 24 hours from injection. At these time points marmosets were killed by excessive anaesthesia, and the cranium and vertebral column opened. The cerebral cortex and spinal cord was dissected out and a portion of both removed for gamma
counting. To limit any error associated with injection artefact, the spinal cord segment was always removed from the upper thoracic region.

RESULTS.

Vascular clearance of monoclonal antibodies.
Following intravenous administration of the paired labels, both monoclonals cleared in a biphasic manner. There was an initial rapid loss of activity from the vascular compartment of a mean of 48% of the injected activity of UJ181.4, and 50% of HMFG2. The rate of clearance for individual antibodies was similar between animals but different for the two antibodies. $^{131}$I UJ181.4 cleared at approximately twice the rate of HMFG2. The mean biological half life of UJ181.4 was measured as 18 hours (Range 18-20 hours; N=3) compared with a mean biological half life of 33 hours (Range 28-36 hours; N=3) for HMFG2.

Pharmacokinetics following intrathecal introduction of antibodies.
Animals were injected with between 10 and 19 uCi of each isotope. All animals except one, demonstrated clear flow of isotope up the spinal column. No extravasation of isotope was noted apart from the single animal. The results from this animal were discarded. Rapid emergence of both isotopes in the vascular compartment was documented (Table 4). $^{131}$I UJ181.4 reached peak levels at approximately 6 hours after
injection, when a mean of 24% of the injected dose was present in the vascular compartment. (Range 16-33%) This compared with a peak of 48% for HMFG2 at a similar time. The resultant difference in the rate of clearance of the two antibodies is illustrated in Fig.10.3

TABLE 4.

Percentage of injected dose present in vascular compartment with time.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>%HMFG2</th>
<th>%UJ181.4</th>
<th>Difference.</th>
<th>HMFG2-UJ181.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>3.0</td>
<td>2.3</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>0.04</td>
<td>28.0</td>
<td>25.2</td>
<td></td>
<td>2.8</td>
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<tr>
<td>0.06</td>
<td>24.0</td>
<td>18.0</td>
<td></td>
<td>6.0</td>
</tr>
<tr>
<td>0.08</td>
<td>0.2</td>
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<td>0.08</td>
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</tbody>
</table>

A comparison of the clearance of the two monoclonals from the CNS was made by measuring the specificity ratio of the monoclonals with time. The specificity ratios
Fig 10.2 HPLC of radiolabelled UJ181.4 and HMFG2, administered to marmosets.

Fig 10.3 Vascular clearance of $^{131}\text{I}$ UJ181.4 and $^{125}\text{I}$ HMFG2, following simultaneous intrathecal injection. Graph demonstrates the % of injected dose ($^{131}\text{I}-^{125}\text{I}$) present in blood with time.
Fig. 10.4 Median specificity ratios of $^{131}$I UJ181.4 to $^{125}$I HMFG2 in cerebral cortex and spinal cord of marmosets. Specificity ratio; % of injected dose (relevant antibody)/ % of injected dose in blood (relevant antibody) : % of injected dose (irrelevant antibody)/ % of injected dose in blood (irrelevant antibody).
Fig 10.4
demonstrated a trend for $^{131}$I persistence with time, which was most marked for the spinal cord. Fig.10.4.

**DISCUSSION.**

This approach to therapy depends on differences achieved in dose delivery to tumour and CNS tissue. Several interesting features with relevance to this issue arise out of the results in Chapter 12. Firstly, the clearance of suspended radioactivity (i.e. that circulating within the CSF of patients) is rapid. By 24 hours less than 1% of the injected dose is available in CSF for non-specific dose delivery. In contrast, scintigraphy demonstrates that isotope has accumulated within the neuraxis at sites of known disease. This is reflected in the difference between measurements made by the external scintillation probe and direct CSF sampling. The data indicates that a high proportion of the radioactivity is rapidly bound to tissue elements within the CNS. Tissue resection data from marmosets suggests that some of the observed tissue binding for UJ181.4 may result from cross-reactivity with normal CNS elements, as by comparison with HMFG2, UJ181.4 is preferentially retained within the CNS. It was not possible to quantitate the binding, as difficulties were encountered in getting a reliable injection of radioactivity into the thecal space. This is reflected in the large variation of injected dose present in blood 0.03-0.09 hours after injection (Table 4). The phenomenon of UJ181.4 binding in CNS, is presumed to reflect specific tissue binding to low levels of antigen expression below the threshold of detection by indirect
immunofluoresence. This is partially substantiated by data from Dr R. Mosely (Bristol Brian Tumour Research Group-Personal Communication), who has shown that UJ181.4 binds specifically to mature human brain in a sensitive radiobinding assay. The property is not confined to UJ181.4 and is a feature of several other antibodies raised against neuroectodermal tissue.

Whilst tumour and tissue binding of antibody may influence the vascular kinetics of radiolabelled antibody, data from the marmosets once again demonstrates that in the case of UJ181.4 and HMFG2, the major influence on vascular kinetics is a difference in catabolic rate. Although both antibodies are of the same isotype, UJ181.4 cleared from blood at approximately twice the rate of HMFG2. This suggests that for these antibodies, other determinants, such as susceptibility to proteases, are important in determining clearance. (See Chapter 13 for full discussion). Some additional data is available which suggests that UJ181.4 may be less stable than other antibodies. In the course of radiolabelling, UJ181.4 was noted to aggregate. This was true both in the tracer studies and in the Phase I investigation (See Chapter 11). Examination of serum by S300 by column chromatography in these patients, demonstrated that isotope was associated with a large number of additional low molecular weight species. This was in contrast to serum activity profiles in patients administered other radiolabelled antibodies. (Drs. A. Davies and Dr R. Mosely-Personal Communication). A similar phenomenon was
observed in marmosets when serum was examined by HPLC. The pattern was not consistent, and varied from batch to batch of antibody. The behaviour of the antibody is reminiscent of that observed for UJ13A. (Chapter 5), and like UJ13A has been associated with a reduction in yield and immunoreactivity. Following the strategy pursued in Chapter 5, recloning has produced antibody of higher yield and immunoreactivity. However, the stability of the antibody requires further characterisation.

In the interim, another monoclonal antibody M340 has been characterised. This antibody, was raised against medulloblastoma tissue, and has similar tissue specificity to UJ181.4. By Ouchterloney, M340 is of the IgG1 isotype, and is readily purified from ascites by Protein A affinity chromatography in yields of >3mg/ml. This antibody radiolabels easily, maintaining an immunoreactive fraction in excess of 70%, and is stable in vivo. Consequently this antibody is currently being used in studies requiring an antibody of neuroectodermal specificity.

As a consequence of the data generated in this chapter and that in Chapter 11, several conclusions have been drawn. Firstly, CNS radiation dose is chiefly delivered by activity bound to tissue. The pattern of isotope distribution suggests that this radioactivity is largely associated with tumour, which is partially mediated through a specific effect (Tumour binding study-Patient 1/See Chapter 11). However, there may be a
significant non-specific component, as tumour localisation has been observed in patients administered antibody with a low immunoreactive fraction. Non-specific irradiation of brain and spinal column is enhanced by antibodies which bind antigens expressed at low levels in CNS tissue. Such factors may be important in determining differential late toxicity between conjugates.
CHAPTER 13.
Discussion.

The advent of monoclonal antibodies as agents for site-specific drug delivery has aroused considerable interest. Despite major research efforts in a number of institutions, their contribution to therapy has been small. Many of the factors limiting progress are reflected in the data presented in this thesis.

The intravenous administration of $^{131}$I UJ13A results in a relatively slow clearance of the radionuclide from blood. (Chapter 3). This finding has two major implications for tumour specific irradiation. Firstly, vascular retention of isotope generates a high non specific tissue radiation dose; secondly, isotope fails to adequately concentrate in tumour. These factors are accentuated by the murine origin of many monoclonals. A significant proportion of the injected dose is cleared by the reticulo-endothelial system, which increases the element of non-uniform total body irradiation, and further limits the availability of conjugate for tumour dose delivery. (Chapters 3, 6 and 7). Repeated administrations of the monoclonal antibody result in sensitization of the host and additional reticuloendothelial sequestration of conjugate (Chapter 6).

These problems place in question the role of monoclonal antibodies in the treatment of both neuroblastoma and other tumours. Any further development
must concentrate on improving relative tumour uptake, whilst reducing whole body irradiation.

Reducing whole body irradiation.

One method of decreasing the level of total body irradiation is to increase the rate of catabolism of the carrier system. Several factors have been elucidated which govern the rate of clearance of monoclonal antibodies.

Antibody class.

To reduce the likelihood of antibody mediated therapeutic effects, an early decision was taken to choose antibodies of the IgG\textsubscript{1} isotype. However, it is possible to select for antibodies with more favourable pharmacokinetics. The catabolic rate of homologous immunoglobulin, certainly appears to be influenced by antibody subclass. Fahey has shown that across the class spectrum of murine immunoglobulin, IgM is cleared most rapidly, followed by Ig\textsubscript{A}, 2b, 1 and 2a (186). In man, Morrel et al. found little difference in clearance rate within the IgG subclasses apart for IgG\textsubscript{3}, which had a significantly shorter circulation time (187). This work has been endorsed by other authors, including Spiegelberg and Fishkin, who demonstrated both increased catabolism of the IgG\textsubscript{3} subclass and variation within subclasses of myeloma proteins (188). Increased catabolism of IgG\textsubscript{3} is not believed to result solely from characteristics in the Fc fragment, but is also a feature of the Fab portion of
the molecule (189). Other structural characteristics probably contribute, and include increased susceptibility to proteolysis.

Thus antibodies of IgG3 isotype, might be of particular value in radiation targeting, and an attempt could be made at initial screening of hybridomas, to select for clones secreting this isotype. A disadvantage in using the IgG3 isotype is the reported instability and poor solubility of some IgG3’s in common buffering systems. This makes them more difficult to work with, although this has been successfully achieved in the case of the anti GD2 monoclonal antibody 3F8 (See Chapter 1, and (86)(87)).

**Antibody fragments.**

A reduction in whole body radiation dose may also be achieved by using antibody fragments. This has been illustrated in this thesis, and in the work of many other authors, and is due not only to changes in antibody size, but also to the loss of the Fc component. Several studies have demonstrated that the Fc portion of the immunoglobulin species is largely responsible for controlling both rate, and site of protein catabolism. This is most elegantly illustrated in the study of Arend WP and Silverblatt FJ, (190) who studied the serum disappearance and whole body clearance of homologous immunoglobulin fragments in rats.

To reduce the effect of molecular weight on distribution, the authors compared these parameters for
the Fab and Fc fragments (approx 50KDa). Both the Fab and Fc fragment cleared rapidly from the circulation during the initial phase, although the rapid fall in activity was maintained for longer with the Fab fragment. A rapid clearance pattern was maintained during the second clearance phase of the Fab fragment. This was not seen for the Fc fragment, where the biological half life of the second clearance component mirrored that of the whole IgG.

These features were partially explained by differences in the renal handling of the conjugate. This was established by the authors comparing the clearance characteristics of the Fab and Fc components in nephrectomized animals. No change was seen in the clearance characteristics of the Fc component in anephric rats. Although those animals receiving the Fab fragment had a similar initial rate of loss as the group receiving Fc, subsequent clearance was prolonged.

The features highlight the importance of the renal mechanism for loss of the Fab monomer. The Fc component appears to slow the rate of antibody catabolism. Subsequent studies have demonstrated that the Fc component binds specific receptors on tissue macrophages (191)(192) so this results in a shift in protein excretion away from the renal tract and towards the reticuloendothelial system.

The importance of the shift in pattern and rates of metabolism on targeting are considered in the work of Covell et al. (152) who have studied the pharmacokinetics
of a murine monoclonal antibody and its fragments in mice. Using a mathematical approach the group have constructed a compartmental model of antibody kinetics. The antibody studied, MOPC-21 had no known binding sites in the host species, the mouse. The experimental data confirms that the rate of clearance of antibody is inversely proportional to protein size. At the lower end of the scale the Fab fragment clears 35 x faster from whole body as the whole immunoglobulin, whilst the F(\(ab'\))\(_2\) fragment cleared at 17 x the rate. The study supports the importance of the kidneys which account for 73.4% of the total Fab catabolism. The intact immunoglobulin is principally metabolized through the gut, with this route accounting for a similar proportion of the total catabolism.

Whilst this analysis would support the use of fragments on the basis of a reduction in tissue cumulative irradiation, the kinetic analysis highlights additional factors. Of the three delivery systems, the Fab fragment has the greatest transcapillary flux. The F(\(ab'\))\(_2\) fragment and IgG reside for the most part in the plasma compartment, whilst for the Fab fragment the converse is true. The data supports the hypothesis that, the smaller the species the more readily it exits from the vascular compartment. This apparent advantage of the smaller species needs to be "traded off" against the faster whole body clearance. The model examines this issue by considering the net number of cycles of antibody per gram tissue through the interstitial and cell
associated spaces, before excretion. If one considers the data for the carcass, whole immunoglobulin cycles 8 x more often than the Fab fragment through the interstitial space. As the rate in whole body clearance increases, a point is reached at which the impact of transcapillary flux becomes important. From the values obtained for this antibody, the Fab fragment cycles through the interstitial space in the carcass 2 x more often than the \( F(ab')_2 \) fragment. The data suggests that if the affinity of the \( F(ab')_2 \) fragment and the Fab monomer are the same, then the Fab fragment has an advantage over the \( F(ab')_2 \) fragment.

However, it should be noted that the model takes no account of valency of antibody/antigen interaction. This adds a further dimension to the analysis. The Scatchard analysis of UJ13A binding, indicated that the binding was both high affinity and linear over a limited number of experiments (Chapter 5). The linearity of the plot does not shed any light on whether the binding is univalent or bivalent over the conditions studied. Consequently, there may be a substantial difference between the affinity of the Fab monomer and the avidity of the \( F(ab')_2 \) dimer (193).

The impact of these parameters on tumour dose delivery may be more complex than first apparent. The issue is summarised in the analysis of Weinstein et al, who has looked at the relationship between antibody affinity and tumour penetration by immunoglobulin (194). In a theoretical consideration that considers tissue
penetration by antibody as a process of diffusion, the author has represented antibody percolation through tumour by one dimensional Cartesian geometry. Using Fab fragments with different forward rate constants (Kf), the author predicts that increasing the Kf results in a lower tumour penetration. The implication of the study is that a more uniform dose deposition may result from lower affinity antibodies.

Clearly the issues are complex and may be summarized as follows. The theoretical considerations would suggest that cumulative tissue radiation dose is reduced significantly by the use of antibody fragments. However their rapid whole body clearance reduces the opportunity for tumour targeting. In the context of the MOPC-1 antibody, the kinetics favour the use of the Fab fragment over the F(ab′)2. This does not take account of the strength of the antibody/antigen interaction where species with lower forward rate constants may favour a more uniform dose distribution. These factors require separate analysis.

The mathematical models give useful insights into how we might proceed in our evaluation of both fragments and choice of other available monoclonal antibodies. Unfortunately, the analysis outlined by Covell et al cannot be applied retrospectively to our kinetic data. However, if the F(ab′)2 fragment of UJ13A proves to be stable in vivo, then any future comparative studies could be constructed to generate appropriate information for this form of analysis. In the absence of a suitable
F(ab')_2 fragment the models would appear to encourage the investigation of the Fab monomer. Further experimental work is required to assess whether the limited cycling of the fragment through the interstitial spaces will further compromise tumour uptake.

Modification of the biodistribution of whole immunoglobulin.

An alternative approach is to modify the biodistribution of whole immunoglobulin by shifting the distribution away from radiosensitive tissues. As has already been stated, marrow toxicity is the usual dose limiting toxicity. Provided there is no bone marrow involvement by tumour, radiation of the marrow cavity is largely attributable to vascular carriage of isotope. Techniques developed to increase "signal to noise" ratio in immunoscintigraphy, by increasing vascular clearance may be of value. In 1982, Begent et al. reported on the use of liposomally entrapped second antibody, to increase clearance of radiolabelled anti-tumour antibody (195). In this study, patients with carcinoma of the colon or rectum, were administered radiolabelled, polyclonal goat anti CEA. After 24 hours, liposomally entrapped horse, anti-goat immunoglobulin was administered. This produced an accelerated clearance of the initial antibody from blood. The commensurate increase in hepatic uptake of isotope, following liposome administration, appeared to be short lived, and is in keeping with our own data on clearance of immune complexes from liver. (Chapter 6).
Bradwell et al. pointed out in a subsequent letter to the Lancet, that the same effect could be achieved by second antibody alone (196). This has proved to be the case in subsequent work by Begent et al. (197) and may prove to be a useful therapeutic as well as diagnostic manoeuvre.

Other methods for modifying the biodistribution of the monoclonal antibodies, include deglycosylation and Fc receptor blockade. The basis of these strategies are clear. Sugar residues on antibodies are thought to be the major sites through which Fc interaction takes place (192). Deglycosylation is relatively easily achieved by growing hybridomas in medium containing Tunicamycin. This antibiotic inhibits glycosylation by blocking the dolichol-dependent, asparagine linked glycosylation pathway. Resulting antibodies are less able to induce ADCC, bind to Fc receptors and activate complement.

However, whilst these strategies may reduce the reticuloendothelial accumulation of radiolabelled conjugate, they are unlikely to result in any major benefit within the context of targeted radiotherapy, as vascular carriage of isotope may be prolonged.

**Improving tumour dose delivery.**

Several factors are amenable to change which have the potential to increase tumour radiation dose.
Improving the immunoreactivity of the radiolabelled protein.

Current routine methods of radiolabelling rely on covalent coupling of isotope to some amino acid, e.g. tyrosine, lysine or aspartic/glutamic acids. In our own studies the use of the Chloramine-T technique inserts iodine into tyrosine residues. This may result in the coupling of isotope into amino acids within the hypervariable region, with a commensurate diminution in immunoreactivity. In addition, the reaction processes of oxidation and reduction may in themselves contribute to biological damage. A reduction in antibody affinity, secondary to damage from radiolabelling, has been shown to adversely affect tumour uptake of conjugate in several studies (198)(199).

A significant criticism of our early studies, is the choice of assay system to monitor biological activity (Chapter 3). In our later studies on the intrathecal administration of radiolabelled antibodies, this deficiency has been corrected. Accumulated data from repeated radiolabellings of antibodies indicate that UJ13A retains approximately 80% immunoreactivity after radiolabelling. (Personal communication: Dr R Mosely, Brain Tumour Research Laboratory, Frenchay, Bristol.) Variation around this level of immunoreactivity is observed, and lies in the range 60-90%. Other monoclonal antibodies appear to be more vulnerable to iodination techniques. For example the monoclonal antibody UJ181.4,
consistently retained only 20% of its biological activity after radiolabelling. This occurred despite attempts to radiolabel with the iodogen method instead of the Chloramine-T.

An approach to the problem, is to site specifically label the monoclonal antibodies. The aim of the techniques under investigation, are to consistently place the radionuclide away from the antigen combining area. These methods are still under development, but are available both for iodine and other radioisotopes of interest.

Much of the current interest lies on site specific labelling of immunoglobulins by heavy metals, such as Indium (In), and Yttrium (Yt). The approach takes advantage of the oligosaccharide moieties sited distal to the hypervariable region. After oxidation to aldehydes, these carbohydrates may be utilised for site specific linkage to a radionuclide. In a study of the IgM antibody HPCM 2, Rodwell JD et al. described a method for site specific labelling, and then compared the affinity of the labelled species with unmodified antibody (200). No reduction in antibody affinity was observed after radiolabelling.

A research programme is currently underway, to produce and evaluate site specifically labelled UJ13A. $^{111}$In has been successfully linked to UJ13A, and is ready for evaluation as a scintigraphic agent.
Altering the radionuclide.
The discussion of site specific monoclonal antibody modification, comments on interest in other radionuclides rather than iodine. $^{131}$I is relatively unsatisfactory for immunoscintigraphy and therapy. Iodine 131 is a mixed gamma and beta emitter. Approximately 2/3 of the energy released during radiactive decay is carried by photons. This energy is highly penetrating and contributes little to the tumour dose. However, the gamma emissions do contribute to the whole body irradiation and hence toxicity of the patient, and to the irradiation of their family and health care team. With regard to scintigraphy, the energy of the photon emission is higher than is optimal for the crystals used in most modern gamma cameras. Heavy collimation is required to reduce scatter in the scintigrams and poorly defined images often result.

The case for the use of alternative isotopes in therapy is made by considering both the work of Vaughan and of Humm. The first of these authors has emphasised the short fall in tumour dose delivery by $^{131}$I (201). Vaughan constructed a mathematical model which allowed calculation of whole body and tumour dose after intravenous administration of antibody conjugated to $^{131}$I. As with all such mathematical modelling the conclusions are dependent on the many assumptions involved in its construction. The authors surveyed the literature on antibody uptake, and based on this data, assumed a
maximum tumour uptake of 0.005% of the injected dose /g of tumour at 27.3 hours.

Kinetic parameters are probably more variable than the variation in maximal uptake of isotope in tumour, and for the purpose of the model whole body clearance of isotope was assumed to be monoexponential, with a biological half life of 3 days. These values are not substantially different from those published for a variety of monoclonal antibodies.

Using these parameters, the authors estimated that delivering a tumour dose of 6000 rads was accompanied by a whole body radiation dose of 1700 rads. Clearly, this level of whole body irradiation is unacceptable. A therapeutic advantage might be obtained by using different isotopes with more favourable targeting characteristics. These have been summarised by Humm as isotopes which decay to safe daughter products, with half lives lying between 6 hours and 4 weeks, and with low yields of penetrating radiation (61). Such isotopes may be identified from the classes of alpha and beta emitters.

The alpha emitters have the apparent advantage of releasing particles of limited range (50-90um), with a high linear energy transfer ratio. There are a relative shortage of suitable isotopes in this class as the elements frequently decay to unstable daughter products. These tend to diffuse away from antibody, as the energy produced in alpha recoil frequently ruptures the bond linking isotope to antibody. Of the available compounds,
both Bismuth and Astatine are under investigation. Both radionuclides have been successfully conjugated to produce a stable radiolabelled antibody (202)(203)(204).

The beta emitters decay by electron loss. The lower particle mass results in higher tissue penetration by the particle. The range of the particle emission varies for different isotopes and may lie between less than 200um to greater than 2mm.

The impact of physical characteristics on tumour radiation dose is illustrated by the theoretical analysis of Humm (61). The author considered two tumour spheroids of different diameters, 200 and 1000 um respectively. By assuming a uniform distribution of radionuclide within the spheroids, Humm calculated the weight of radiolabelled immunoglobulin, required to deliver a tumour dose of 8000 rads for five different isotopes. The analysis included $^{131}$I, the pure beta emitter $^{90}$Yt and the alpha emitter $^{211}$At. A clear theoretical advantage emerged for the use of the alpha emitter: by a factor of $2 \times 10^{-2}$ when compared with $^{90}$Yt, and a factor of $2 \times 10^{-4}$ for $^{131}$I.

Whilst illustrating the effect of differences in energy emission, this calculation does not take account of particle range. The distribution of isotope within solid tumours is not uniform, and this results in the areas of negligible isotope deposition. The shorter the range of the emitted particle, the smaller the dose to these "cold" areas. This would suggest an advantage in the use of the longer range, high energy beta emitters in
the treatment of larger tumour masses. This sort of consideration would favour the use of $^{90}\text{Yt}$ in treatment of larger tumour deposits whilst alpha emitters may be more suitable for the therapy of small volume disease.

Discussions of the advantages of changing the radionuclide should be tempered by knowledge of the biodistribution of the conjugate. The discussion has already highlighted tissues which receive higher radiation doses, because of the metabolism of the conjugate. Larger radiation dosage to tumour may be bought at high radiation exposure to other organs.

This problem may be accentuated by mechanisms for metabolizing free isotope. The heavy metals enter different metabolic pathways to iodine, shifting the biodistribution of free isotope away from thyroid to other tissues. This is illustrated by data on the biodistribution of both $^{111}\text{In}$ and $^{90}\text{Yt}$.

$^{111}\text{In}$ is a pure gamma emitter, and of no interest as a therapeutic agent. However, its physical characteristics are highly suitable for immunoscintigraphy. Despite improved methods of conjugation, the radiolabelled antibody is frequently unstable in vivo. It is postulated that the free isotope interacts with transferrin, and is deposited in the liver (205). Certainly, numerous studies have underlined the high hepatic accumulation of isotope (206)(207). As well as increasing radiation dose to liver and surrounding tissues, hepatic deposition of isotope in this context,
diminishes the sensitivity of the imaging technique for
detecting liver metastases (208).

A similar disadvantageous shift in biodistribution is
seen with Yttrium. The element derives from the same
periodic grouping as calcium and strontium.
Biodistribution data obtained in mice, indicates that the
isotope $^{88}\text{Yt}$ is handled in a manner similar to other
alkali earths, with accumulation of $^{88}\text{Yt}$ in bone[11].
This may be of particular significance in shifting
toxicity towards marrow. Bone marrow toxicity may be
accentuated at time of high osteoblast activity, e.g.
under the physiological conditions of growth, or
pathological conditions of bony destruction or
remodelling.

These considerations illustrate the advantages of
certain isotope/antibody combinations. Theoretical
considerations regarding likely toxicity and efficacy,
whilst useful must ultimately be tested in clinical
studies. The combination of an isotope such as Yttrium
to a monoclonal antibody such as UJ13A, has energy
characteristics that are far superior for targeting
tumour than $^{131}\text{I}$. Production of $^{90}\text{Yt}$ UJ13A is certainly
feasible and presents a logical progression in attempting
radiation delivery in neuroblastoma.

**Dose scheduling.**

A strategy for increasing the therapeutic ratio within
current constraints, has been highlighted by Wheldon et
al. Using the same biological model as Vaughan, the
author has extended the analysis to the clinical situation of minimal residual disease. Wheldon fails to demonstrate any significant advantage of $^{131}$I monoclonal antibody in this setting but suggests that by changing the therapeutic strategy to high dose therapy and bone marrow rescue, a clinical benefit may ensue (150).

Wheldon compared tumour radiation dose delivery by conventional total body irradiation (TBI) regimens with repeated administrations of $^{131}$I monoclonal antibody. He looked at the substitution of 200 rad TBI fractions in a "typical" 6 x 200 rad regimen, by an equivalent amount of targeted radiation.

For a standard whole body dose of 1200 rads the tumour dose increased from 1200 rads (no targeted component), to between 1600 and 2100 rads for tumours of 200um when all fractions were given as targeted irradiation. Increasing the size of the tumour was estimated to improve the therapeutic ratio, as more of the beta irradiation is absorbed within the tumour. At tumour diameters of 1cm$^3$, radiation dose was estimated to increase to 3684 rads.

These computations take no account of any changing kinetics due to anti mouse Ig response. Obviously the magnitude of this influences the practical application of the technique. However strategies are available to overcome these problems. A further point in favour of this approach to therapy, is that the model does not take account of the radiobiological effect of declining dose rate. As the proportion of targeted radiotherapy
increases within the regimen of TBI, the model overestimates the toxicity of 1200 rads on late responding tissues.

**Strategies for reducing the immune response.**

The strategies for circumventing the immune response are four fold. They involve the use of immunosuppressive agents such as cyclosporin, the use of antibody fragments, a change towards the use of human monoclonal antibodies or humanization of murine immunoglobulins by production of chimeras.

The use of antibody fragments has, already been discussed in detail, and the strategy investigated in this thesis (Chapter 6). Little work has been undertaken on the use of immunosuppressive agents and generation of the immune response in targeted therapy although the use of cyclosporin is currently being evaluated at the Charing Cross Hospital, London (Personal communication from Dr R. Begent).

An obvious strategy for reducing the immune response to the monoclonal antibody, is the production of human monoclonal antibodies. A variety of techniques have been developed to generate human antibodies. These include the production of mouse-human hybrids, human-human hybrids, Epstein-Barr virus (EBV) transformation, and fusion of EBV transformed specific B-lymphocytes with a malignant cell line.

The production of antibodies from mouse-human somatic cell hybrids, was first demonstrated by Schwaber and
Cohen in 1973 (210). The technique produces antibodies of mixed phenotype but has been adopted with some success to produce human antibodies in useful amounts (211)(212). These interspecies hybridomas grow rapidly and produce up to 15ug of immunoglobulin per ml of spent culture medium (213). The major problem encountered in their use is instability in the karyotype. There is a tendency for the human chromosomes to be preferentially segregated with a resultant loss in expression of the human phenotype.

Human-human hybridomas do circumvent this problem. The practical limitation on this technique is the availability of human fusion partners. Human myeloma cell lines are difficult to establish in culture, and those that have been successfully cloned are not always widely available. This has encouraged the use of EBV+, B cell lymphoblastoid lines as fusion partners (214)(215). Whilst the lymphoblastoid cell lines grow rapidly, they are poor secretors of immunoglobulin. Concentrations of less than 1ug/ml may be anticipated in the spent culture medium (216).

The other aspect of human-human fusions is the selection of immunized B lymphocytes. Clearly human subjects cannot be immunized against antigens of interest, nor are splenocytes readily available for fusion. Other sources of B lymphocytes have been successfully fused to produce human monoclonal antibodies. These include circulating peripheral blood lymphocytes, bone marrow and tonsil (218). This change
in source does not adequately address the problem of immunization status of the lymphocytes. Research is underway to antigen prime immunocompetent cells in vitro and progress in this direction should increase the range of human monoclonal antibodies available for clinical evaluation.

To hasten the process, other workers have concentrated on the production of genetically engineered monoclonal antibodies. Techniques have been established for the production of murine-human chimeric antibodies (219)(220). Workers in the field have fused murine variable region exons from existing murine hybridomas, with human constant regions. The result is a chimeric antibody which retains the antigenic specificity of the original murine monoclonal, but has a human constant region.

This approach may be useful in facilitating repeat administrations of the antibody although anti-idiotypic antibodies will undoubtedly emerge, and still limit the number of therapeutic applications of any chimeric monoclonal antibody.

Moreover, clinical data is required on vascular persistence of modified antibodies. It may be anticipated from studies on the rate of degradation of homologous immunoglobulin, that human and humanized monoclonals will circulate for long periods. This may render the antibodies useful as biological response modifiers and carriers for other cytotoxic agents, but may limit their use as carriers of radionuclides.
The relevance of these strategies to the treatment of neuroblastoma.

In justifying the continued exploration of targeted therapy in neuroblastoma, the need for alternative therapeutic strategies in this disease was emphasised. The rationale for continuing research in this direction, requires re-evaluation in the light of information generated in the thesis, and from other workers in the field.

Targeted radiotherapy as a treatment option in neuroblastoma.

The two major factors justifying clinical optimism in this area, are the apparent radiosensitivity of this neoplasm, and the availability of two classes of relatively specific delivery systems.

The relative merits of the radiopharmaceutical mIBG were emphasised in Chapter 9. A preliminary examination of the kinetic data, both in animal models and patients, suggests that the compound has several properties which favour radiation targeting. Although, the tissue resection data has not been subjected to compartmental analysis, it is clear that a major transcapillary flux of isotope occurs, and that in spite of a more rapid whole body clearance of isotope when compared with immunoglobulin, the loss is not as rapid as occurs with the Fab fragment. This implies a longer residence time in the extravascular space, giving a greater opportunity for radiation targeting.
Two mechanisms are available for intracellular transfer of mIBG (221). The mechanism most likely to limit tumour accrual of isotope, is the active amine transport system (162). Other factors are probably important in facilitating mIBG accrual in tumour cells, and may include both the amine storage properties of neurosecretory granules and other cytoplasmic storage mechanisms. Although there is little published data on this aspect of mIBG accrual in neuroblastoma tissue, it might be expected that these highly specialized mechanisms may vary in a heterogenous tumour population (223)(224). As with the theoretical consideration of monoclonal antibodies, absolute levels of tumour isotope accrual are important. This area has been investigated by Dr J. Moyes at the Royal Marsden Hospital (Personal communication), in a study of resected neuroblastoma tissue. Patients going to theatre, for resection of their primary tumour have been administer tracer amounts of mIBG. Tumour has been resected and examined by gamma counting for presence of radioactivity.

The results suggest that there is considerable heterogeneity for isotope accrual within a tumour, and that there is a similar variation between patients. However in 2/5 patients, there has been a log gain in isotope uptake over that typically seen in patients receiving whole immunoglobulin. It may be possible to further enhance tumour uptake of isotope by pharmacological blockade of mechanisms governing amine efflux. There is some preliminary evidence suggesting
that this may be mediated through calcium channel blockers (225)(226).

The strategies outlined by Wheldon for clinical use of monoclonal antibodies, equally apply to mIBG. The advantages in tumour dose delivery should be accentuated by a promise of higher tumour uptake, and lower whole body irradiation at comparable doses of radionuclide.

Disadvantages in the use of mIBG, are its lack of flexibility. The intrinsic specificity of the molecule may result in some neuroblastoma cells not accumulating isotope. Secondly, there is a limited range of radionuclides that may be conjugated to the compound. At present it would seem possible to extend the range of therapeutic options to the other halides such as $^{125}$I and $^{211}$At.

Considerations pertinent to the use of alpha emitters conjugated to immunoglobulin, are equally relevant to mIBG. In fact, the wider range of normal tissues accumulating the compound may extend the toxicity (Chapter 8,9). $^{125}$I is of theoretical interest, as the decay pattern includes significant emission of Auger electrons (227). These are capable of producing lethal ionizing events, but over a very short range (228). To produce a significant event it is necessary to localize the isotope to within 50 nanometers of DNA (229).

Further work is required, to precisely locate mIBG within the cellular structure, before further comment can be made on the likely outcome of this therapeutic strategy.
Monoclonal antibodies continue to promise a more flexible approach to tumour targeted therapy. In the context of neuroblastoma, there are a wide range of monoclonal antibodies directed against a variety of antigens, available for study. It is unlikely that any of the antibodies will be sufficiently exceptional to significantly improve tumour levels of isotope in bulk disease. Clearly, further evaluation of the fragments and new radionuclide combinations are necessary, before final conclusions can be made. Some therapeutic gain is likely by appropriate dose scheduling, and this may be enhanced by the use of fragments or human/humanized antibodies.

Characteristics which may limit the use of humanized monoclonal antibodies for targeting isotopes, may render them suitable as biological response modifiers, or for carrying new agents, such as toxins. In view of the problems encountered in gaining tumour access, it is unlikely that any of these alternative therapeutic strategies will be suitable for treating large amounts of disease. The place for clinical evaluation of their efficacy should be in minimal residual disease.

Development of intrathecal tumour targeting.
The apparent success of the intrathecal approach to radiation targeting of leptomeningeal tumour illustrates the potential of site specific delivery with monoclonal antibodies. In this specialized situation, the constraints placed on the technique by vascular barriers
is eliminated. Other features of importance may be a lower dilution factor, and the absence of any initial reticuloendothelial interactions. The priority of this research, is to look at ways of extending the therapeutic range of the technique.

The issues may be summarized as defining the range of clinical situations in which the delivery system may be of benefit, and to establish which therapeutic agents may be of value.

Extending the therapeutic range.

CNS permeability to monoclonal antibodies.

Infiltration of the meninges by leukaemic cells and carcinomas, has been well characterised. Histological examination of central nervous system, indicates that tumour growth begins on the pia arachnoid, and infiltrates into the brain parenchyma as the disease progresses (117)(118). To extend the use of monoclonal antibodies in this clinical context, it is necessary to define the anticipated therapeutic range of what may simply be a "topical" treatment.

Histological examination of the leptomeninges, reveals a two layered structure: the pia and the arachnoid. The pia intimately invests the brain and spinal surface, whilst the arachnoid membrane defines the opposite border of the sub arachnoid space. Nabeshima et al. studied the structure of the two membranes by electron microscopy in a variety of mammals (233). The author concluded that there were no cytological criteria
for separating pia and arachnoid. However, some

differences were noted in structure at an organizational
level. The pia consists of a layer of cells with long,
thin processes extending outwards. In places there is
evidence of direct contact between the pial cells and the
parenchymal surface of the brain. An anatomical
continuum between the sub arachnoid space and brain, is
suggested by an absence of tight junctions between cells
of the pia, and the presence of patent clefts in the
marginal astrocytes of the brain and spinal column.
These clefts were noted to be continuous with the
subarachnoid space and the perineuronal space.

Whilst the cells of the arachnoid membrane were
cytologically similar to those of the pia, they are
organized to form a continuous layer. Contact between
the arachnoid cells in apposition to the dura mater, is
made through tight junctions.

In an earlier study, Brightman and Reese had reported
a similar leptomeningeal structure in a variety of lower
vertebrates (234). They tested the functional integrity
of the leptomeninges by infusing horseradish peroxidase
into the subarachnoid space, ventricular CSF and blood.
The authors noted that horseradish peroxidase readily
entered the interstitial spaces of the brain, when it had
been administered into CSF. The parenchymal penetration
of the dye was not as great when administered into the
sub arachnoid space, as when administered intra-
ventricularly. This was felt to be possibly due to a
lower number of open channels in the leptomeninges
compared with the ependyma. Intravenous administration of the HRP did not result in CNS penetration.

A variety of other studies support penetration of proteins across the leptomeninges, and document migration of macromolecules through extracellular space (235)(236). It would appear that transfer of molecules across the space occurs by bulk flow, as no difference in rates of penetration have been reported for molecules of differing molecular weight. In the absence of a major CNS lymphatic network, the data suggests that the CNS extracellular space serves an important accessory transport function, and probably acts as a conduit for fluid to move from the ventricles into the sub arachnoid space and pre-lymphatic channels.

Whilst further data is required on CNS penetration by immunoglobulin, it is reasonable to postulate that a proportion of intrathecally administered immunoglobulin will penetrate the extracellular space and reach sub pial extension of tumour. Whether this will occur in sufficient amounts to effect therapy, requires elucidation.

Other therapeutic options.
A functional transport system within the CNS, both through CSF circulation and intraparenchymal flow, offers a potential to explore a variety of other therapeutic conjugates. As indicated in the Introduction to this thesis, there are a range of therapeutic modalities that may be linked to monoclonal antibodies.
The agents available may be placed in two groups: those that are proven to have activity against the tumours of interest, and those that offer the potential of new cytotoxic agents. In the first category are the drug-antibody conjugates.

A variety of chemotherapeutic agents have been defined with activity against either primary or metastatic tumours of the CNS. Drug efficacy is sometimes limited by problems of transfer across the blood brain barrier, which has led to interest in regional delivery of chemotherapeutic agents. Whilst regional delivery may improve the therapeutic levels of the free drug, toxicity may be troublesome. The intrathecal administration of drug-antibody conjugates should increase the therapeutic index of these cytotoxic agents.

The techniques for linking high ratios of drugs to immunoglobulin species have rapidly evolved. By simple covalent binding of drug to antibody it has proven possible to produce conjugates with a drug:antibody ratio of 10:1. At molar ratios of 2:1, Takhashi has reported on clinical experience with covalently bound mitomycin C and neocarzinostatin in colorectal cancer (237).

In this study, 41 patients with colorectal cancer were administered drug conjugate prior to surgery. Immunoperoxidase techniques on resected tissue, indicated that the immunoglobulin was bound to areas of tumour and not to normal tissue. Of the patients with metastatic tumour, 3/8 had CT demonstrable regression of hepatic
metastases. Patients with lung and peritoneal metastases did not experience any therapeutic effect. Once again, this result probably reflects a privileged site for antibody delivery.

The use of a variety of intermediaries and spacers has significantly increased the molar ratio of drug to immunoglobulin, without losing antibody activity and inducing steric hindrance. Using the monoclonal antibody SSEA-1 and a carbodiimide coupling reaction, Shen and colleagues have produced a methotrexate conjugate containing an average of 45 molecules of drug:1 of immunoglobulin (238). This conjugate has been prepared without loss in affinity for its antigen and has proven cytotoxic in vitro.

A large number of other methods are available to link a variety of other drugs and it would seem appropriate to explore this area in more detail within the context of leptomeningeal tumour.

Similar potential exists for the use of new therapeutic agents, such as the plant and bacterial toxins. Many of these compounds have proven too toxic for use as free agents. Methods have been evolved for their conjugation to immunoglobulins, and the conjugates have proven to be active in vitro (239). Preclinical studies have indicated that the compounds are stable in a range of animal species (240)(241) but an appropriate clinical scenario is required for their development. Whilst the CNS would appear as an ideal compartment for their evaluation, the anticipated potency of the toxins,
requires a cautious approach. The potential of the technique, has been illustrated in the report of Zovickian and Youle. Having demonstrated in vitro, the potential of an anti transferrin receptor monoclonal antibody-ricin immunotoxin against human glioblastoma and medulloblastoma (242), the authors have looked at a ricin immunotoxin against leptomeningeal L2C leukaemia in guinea pigs (243). The immunotoxin was administered intrathecally, in animals previously administered the L2C leukaemia. The normal course of events is intrathecal spread of the tumour line, followed by death of the animal within 15-20 days. Intrathecal introduction of the immunotoxin within 24 hours of injection of tumour cells, produced a median extension in survival of 5.5 days, and corresponded to an estimated 2-3 log tumour cell kill.

To extend this approach into clinical practice, antibodies of high tumour specificity are necessary and extensive preclinical toxicology is required to assess the importance of low levels of antigen expression on normal tissue. With these reservations, it would appear that continued exploration in this area could yield a group of new and exciting therapeutic agents.

In Summary.

Monoclonal antibodies have given the area of targeted therapy, a major impetus. As clinical experience has accrued, the initial optimism that surrounded their development as therapeutic agents has palled. This has
resulted in a more realistic appraisal of how antibodies might be used and modified.

This thesis suggests that in the field of neural tumours, some room for optimism persists. The intrathecal administration of antibodies may prove an effective therapeutic strategy for a selected group of tumours.

For systemic tumours, such as neuroblastoma, antibodies are unlikely to make a dramatic contribution to clinical management. It is probably realistic to expect some therapeutic advantage from targeted radiotherapy, in the context of minimal residual disease. This contribution from monoclonal antibodies should not be dismissed. Neuroblastoma, represents one of the few diseases in which there are two classes of compounds, capable of radiation delivery. This offers a unique situation in which to study and extend the concept of targeted radiotherapy, using delivery systems with very different properties.
REFERENCES.


A Pilot Study of $^{131}$I Monoclonal Antibodies in the Therapy of Leptomeningeal Tumors

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A pilot study was performed to investigate the toxicity and therapeutic effect of radiolabeled antibody administered intrathecally in patients with leptomeningeal tumors. Five patients who failed conventional therapy received between 11 mCi and 40 mCi of radiolabeled antibody. The choice of antibody varied depending on the immunophenotype of the tumor. Therapy was well tolerated generally, with minimal acute toxicity. Four of five patients achieved an objective response to treatment that has been sustained for a period varying from 7 months to 2 years. No clinical signs of chronic toxicity have been observed in patients 1 and 2 years after therapy.


The use of antibodies to target therapeutic agents to tumors is a concept that was pioneered in 1957 by Pressman et al.¹ The development of monoclonal antibodies has led to recent progress in this field. However, an encountered difficulty has been the low amount of antibody gaining access to bulk disease.² As a result, some investigators think that monoclonal antibody guided therapy should be used in the treatment of low volume, diffuse disease.⁴

When a tumor is confined to a single body compartment, a therapeutic advantage may be gained by direct administration of antibodies into the body cavity. This approach has been investigated in tumors with pericardial, pleural, and peritoneal extensions.⁶ The subarachnoid space is a unique body cavity that can be used for investigation of this technique. The cerebrospinal fluid (CSF) has its own circulation, and tumors with a propensity for leptomeningeal spread are often disseminated diffusely and radiosensitive highly.⁷⁻⁹

To investigate the feasibility of antibody administered intrathecally for targeting radiotherapy, a pilot study was performed on patients with relapsed leptomeningeal tumors. The study objectives were to investigate the biodistribution of radiolabeled antibody, to seek evidence of the specificity of the approach, and to monitor toxicity and therapeutic effect. They were accomplished by studying the pharmacokinetics of tracer amounts of radiolabeled antibody administered intrathecally, and by intrathecal injection of larger doses of tumor binding antibody.

Materials and Methods

Antibody Selection

Patients who had either relapsed or possessed a persistent leptomeningeal tumor despite adequate conventional therapy were eligible for study. The immunoreactivity of each patient's tumor was reviewed with a panel of monoclonal antibodies. A single antibody was chosen for radiation targeting. Selection was based on the isotype of the antibody, its immunoreactivity with the patient's tumor, and its lack of reactivity with normal central nervous system (CNS) components.

To study targeting specificity, a second control antibody was selected. This antibody was matched for isotype with the "specific" antibody, but was not immunoreactive with either the patient's tumor or normal CNS tissue.

Both antibodies were radiolabeled by a modified chloramine-T technique¹⁰ to a specific activity of be-
TABLE 1. Summary of Data on Patients With Leptomeningeal Tumors

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Tumor</th>
<th>Prior therapy</th>
<th>Clinical status at referral</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pineoblastoma</td>
<td>Subtotal resection; Cranial irradiation 4500 cGy</td>
<td>Comatose with diffuse leptomeningeal spread</td>
</tr>
<tr>
<td>2</td>
<td>Lymphoma</td>
<td>Subtotal resection; Craniospinal irradiation 3000 cGy; Boost to tumor site 1500 cGy VAC, IT Mtx prednisone</td>
<td>Diffuse leptomeningeal spread; multiple cranial nerve palsies, cervical block</td>
</tr>
<tr>
<td>3</td>
<td>Teratoma</td>
<td>Resection; craniospinal irradiation 3500 cGy; 5500 cGy to posterior fossa CPDD/VP-16 ifosfamide</td>
<td>Diffuse leptomeningeal spread; cervical block</td>
</tr>
<tr>
<td>4</td>
<td>Primitive neuroectodermal tumor cerebellum*</td>
<td>Resection; cranial irradiation 5500 cGy; 1500 cGy to spinal block T8-L1.</td>
<td>Multiple spinal deposits, paraparesis</td>
</tr>
<tr>
<td>5</td>
<td>Melanoma</td>
<td>Resection</td>
<td>Diffuse leptomeningeal deposits</td>
</tr>
<tr>
<td>6</td>
<td>Ependymoma</td>
<td>Resection; Craniospinal irradiation; vincristine</td>
<td>Mild cauda equina symptoms</td>
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VAC: vincristine (2 mg), doxorubicin (40 mg), and cyclophosphamide (1 g); IT mtx: intrathecal methotrexate; CDDP: cisplatin (33 g/m²); VP-16: 150 mg/m²; ifosfamide: 6 g/m².

* Initial diagnosis: neuroblastoma.

tween 5 and 15 μCi/μg of protein. The specific antibody was radiolabeled with radioactive iodine (131I) and the control antibody with 125I. Free iodine was separated from the radiolabeled products by column chromatography using Sephadex G25 (Pharmacia, Uppsala, Sweden), equilibrated with phosphate-buffered saline (PBS) (PBS/1% human plasma protein fraction). Before injection into patients, the preparation was passed through a 22-μm Millex filter to ensure sterility.

Contaminating free iodine in the preparation was assessed by instant thin layer chromatography or precipitation with 10% trichloroacetic acid. Aggregated protein was estimated by either centrifugation at 10,000 × g for 30 minutes by Sephacryl S300 (Pharmacia) column chromatography. Preservation of biologic activity was established using an indirect immunofluorescence assay on frozen tissue sections both before and after radiolabeling.

To reduce the potential of radiolytic damage, monoclonal antibodies were administered within 8 hours of radiolabeling.

**Patient Preparation**

A full clinical assessment of disease was performed. It included a neurologic examination, CSF cytology and biochemistry, myelography, a computerized axial tomography (CAT) scan of the brain and spinal cord, full blood count, liver function, and thyroid function estimates.

For 72 hours before isotope administration, all patients received 0.3 ml of Lugol's iodine aqueous solution three times daily. Adults received an additional 80 μg of liothyronine daily. Thirty minutes before conjugate injection, patients were tested for hypersensitivity to mouse protein by intradermal injection of 10 μg of mouse immunoglobulin in 100 μl of PBS.

**Tracer Study**

Specific (11 to 60 μg) and control (22 to 55 μg) monoclonal antibodies were administered intrathecally simultaneously to four of the five eligible patients (Tables 1 and 2). The route of administration was direct lumbar puncture in Patient 4 and lumbar reservoir in the remaining three patients.

To follow the clearance kinetics of both antibodies, blood was sampled at frequent intervals from 5 minutes to 5 days. CSF was withdrawn from patients with indwelling reservoirs at 30 minutes: Thereafter, at 4, 12, and 24 hours and daily. Urine collections every 24 hours were established on the day of injection and continued throughout the study period.

Additional information on the clearance of the 131I radiolabeled monoclonal antibody was provided by sequential scintigraphy. Scans were performed on an International General Electric 400T gamma camera with a medium- or high-energy collimator linked to a DEC PDP computer with Gamma 11 software.

Anterior, posterior, and lateral views of the head, and anterior and posterior views of the abdomen and thorax, were obtained at 10 minutes, 4 hours, and daily where possible until radioactivity in the neuraxis had fallen to background levels.

**Therapeutic Study**

A thyroid blockade was maintained and patients received a short course of oral dexamethasone com-

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Results

Patient Recruitment

Six patients were enrolled in the study. These patients represented a diverse group of tumors. As a result, a variety of monoclonals were used (Tables 1 and 2).

One patient (Patient 6) underwent a diagnostic study only. This patient, with a treated ependymoma, was referred because of mild neurologic deterioration. Myelography showed equivocal changes that could be interpreted as resulting from previous surgery. The tracer study did not demonstrate any focal accumulation of radionuclide within the CNS, and the tracer kinetics were complicated by the extravasation of a portion of the injected protein. This patient did not receive therapy. No further neurologic deterioration occurred. At 6 months after the tracer study, there is no evidence of active disease.

Patient 3, a child 3 years of age, did not undergo a tracer study. The child was believed to be too ill and was treated on the basis of immunohistologic evidence of UJ181.4 expression on the primary tumor, and prior radioresponsiveness.

No patients were excluded because of preexisting sensitivity to mouse protein.

Quality of the Injected Product

Full quality control data were available for most patients studied (Table 2). A good match was obtained between control and specific antibodies in all patients, except Patient 4. This patient was injected with a preparation containing 9% aggregated material in the specific...
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**Therapeutic Study**

A thyroid blockade was maintained and patients received a short course of oral dexamethasone com-
antibody, compared with 2% in the control. The quality of the radiolabeled protein in the therapeutic administration was satisfactory for all patients (Table 2).

**Biodistribution of the Monoclonal Antibodies**

The circulation of radionuclide within the CSF pathways was followed by scintigraphy. The tracer study of Patient 6 illustrates that in the absence of a tumor, radionuclide ascends the spinal column rapidly. Radiolabel was visualized in the basal cisterns at 4 hours, and was demonstrated over the cerebral hemispheres and in the ventricular system at 24 hours (Fig. 1).

Patient 5 had a ventricular reservoir. Radiolabel was detected in CSF withdrawn from this site 5 minutes after a lumbar injection.

Three patients (Patients 2, 3, and 4) had a spinal block demonstrated by myelography. This was dealt with, in the case of Patient 3, by dividing the therapeutic dose into two parts and giving approximately 50% by the

**Table 3. Pharmacokinetic Data on Patients Receiving Radiolabeled Antibody**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Antibody</th>
<th>Peak activity in blood*</th>
<th>Blood T diagnostic (hr)</th>
<th>Therapy (hr)</th>
<th>CSF T T1 (hr)</th>
<th>T2 (hr)</th>
<th>Tumor T (hr)</th>
<th>WB T (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UJ181.4</td>
<td>9</td>
<td>47</td>
<td>62</td>
<td>9</td>
<td>38</td>
<td>253</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>HMFG2</td>
<td>25</td>
<td>57</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>F8-11-13</td>
<td>25</td>
<td>70</td>
<td>74</td>
<td>NE</td>
<td>43</td>
<td>NE</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>HMFG2</td>
<td>35</td>
<td>65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>UJ181.4</td>
<td>15</td>
<td>86</td>
<td>85</td>
<td>6</td>
<td>47</td>
<td>NE</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>HMFG2</td>
<td>25</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Mel 14</td>
<td>9</td>
<td>92</td>
<td>62</td>
<td>NE</td>
<td>NE</td>
<td>127</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>4C6</td>
<td>26</td>
<td>36</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>FD32</td>
<td>NE</td>
<td>Significant extravasation</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
</tbody>
</table>

NE: not estimated; T: half-life of radiolabeled conjugate; CSF: cerebrospinal fluid; T1: first (fast) clearance component; T2: second (slow) clearance component; WB: whole body; UJ181.4: specific antibody; HMFG2: control antibody.

* Peak activity equals maximal percentage of injected dose present in the blood.
lumbar route and 50% intraventricularly. Patients 2 and 4 received limited external beam irradiation to the site of the block. This was successful in establishing CSF flow. With the exception of Patient 3, all patients demonstrated adequate circulation of the therapy dose of $^{131}$I monoclonal antibody.

During the tracer study, activity cleared from the neuraxis in a biexponential manner. Clearance of the $^{131}$I labeled antibody from the thoracolumbar region of the neuraxis was estimated using the gamma camera. The rate of loss was variable between patients. It fell between 5.2 and 17.3 hours for the first half-life (mean, 10 hours [$n = 4$]), and between 16 and 56 hours for the second half-life (mean, 41 hours [$n = 4$]).

Direct CSF sampling in two patients confirmed the biphasic clearance of both the $^{131}$I and $^{125}$I radiolabeled antibodies from the CSF pathways (Table 3). Variation in the clearance rate of the control and specific antibody was observed in the same patient. In patients with adequate CSF sampling, a trend was observed for the control antibody to clear faster than the specific antibody. This was reflected in the CSF half-lives, the magnitude of the initial loss of isotope from the CSF, and the rate of loss of isotope to blood.
Radiolabel appeared in the vascular compartment within minutes. The proportion of injected dose in the blood rose steeply. It peaked at between 20 and 45 hours (mean, 29 hours \( n = 8 \)), and then fell in a monoeponential manner. There was no substantial difference between the times at which control and specific antibodies reached peak activity in the blood. However, in all patients the specific antibody reached a lower peak activity than the control antibody. Subsequent clearance of the specific antibody also was slower than the control antibody in Patients 2, 4, and 5.

The difference between the rates of clearance of the control and specific antibodies are illustrated graphically in Figure 2. In Patients 2 and 4, an initial preferential loss of \( ^{131}I \) over \( ^{125}I \) was observed. This loss was small and unsustained.

In Patient 1, it was possible to demonstrate that the retention of specific antibody within the CSF was a specific phenomena. After injection of control and specific antibodies, CSF was sampled sequentially. The cellular component was separated by centrifugation, and the ratio of specific to control antibody was counted in the cell pellet and supernatant. Over 3 days, the ratio of \( ^{131}I/\text{UJ181.4} \) (specific antibody) to \( ^{125}I/\text{HMFG2} \) (control antibody) reached 10:1 in the cell pellet compared with 1:1 in the supernatant.

**Therapeutic Study**

**Toxicity:** Acute toxicity within the CNS was confined largely to a mild to moderate inflammatory reaction. Toxicity is summarized in Table 4. Only Patient 1 was symptomatic substantially.

The biodistribution of both the diagnostic and therapeutic antibody administrations was not different substantially. Clearance from the whole body was monophasic and similar between patients (Table 3). Despite the measures outlined for thyroid blockade, thyroid uptake of isotope was a persistent problem. Radiation dose estimates for this organ have not been performed.

Patients 1 and 2 have reached postmortem 2 and 1 years, respectively, from the administration of radiolabeled antibody. Multiple sections through the brain, spinal cord, and leptomeninges were examined for histologic evidence of radiation damage. There were no histologic abnormalities attributable to radiation damage in either the parenchyma or vasculature of the brain or spinal cord. In Patient 2, some fibrotic changes were identified in the cerebral leptomeninges. The changes were patchy and consisted of a loose meshwork of collagen deep to the layer of arachnoid cells. This meshwork contained a mixture of fibroblasts, macrophages, and scattered chronic inflammatory cells (Fig. 3).

**Therapeutic Response**

Four of five clinical responses have been achieved and are detailed below.

**Patient 1:** This patient had disseminated pineoblastoma and was cachectic (weight, 45 kg), confused, and incontinent at referral (Karnofsky index, 30). He had sharp root pain when he moved and visual failure due to optic atrophy. Other clinical signs were gross muscle wasting, severe meningism, extensor plantar responses, absent tendon reflexes, and a right-sided weakness.

Computed tomography (CT) scanning showed ventricular dilatation, and myelography indicated multiple filling defects along the spinal canal. The CSF cell count was raised at 158/mm\(^3\). The CSF protein level was elevated at 2.37 g/l and the glucose level was reduced at 0.2 g/l. Cellular morphologic and immunohistologic studies yielded results consistent with a recurrence of pineoblastoma.

**Table 4. Toxic Effects Associated With Intrathecal Administration of Radiolabeled Antibody**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Therapy</th>
<th>Acute toxicity</th>
<th>Chronic toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24 mCi</td>
<td>Increased nuchal rigidity with CSF leukocytosis</td>
<td>Chronic elevation of CSF protein but no histologic evidence of radiation damage to cord or meninges</td>
</tr>
<tr>
<td>2</td>
<td>40 mCi</td>
<td>None</td>
<td>Chronic elevation of CSF protein and fibrosis of leptomeninges</td>
</tr>
<tr>
<td>3</td>
<td>11 mCi</td>
<td>Asymptomatic pyrexia 37.5°C, CSF leukocytosis, peripheral neutrophilia</td>
<td>Not applicable*</td>
</tr>
<tr>
<td>4</td>
<td>40 mCi</td>
<td>Asymptomatic pyrexia 37.5°C, peripheral leukocytosis</td>
<td>None apparent*</td>
</tr>
<tr>
<td>5</td>
<td>40 mCi</td>
<td>Asymptomatic relative peripheral neutrophilia</td>
<td>None apparent*</td>
</tr>
</tbody>
</table>

I: iodine; CSF: cerebrospinal fluid. * No postmortem examination performed.
A dose of 24 mCi of $^{131}$I/UJ181.4 was administered by lumbar injection. Patchy uptake of isotope was seen throughout the spinal column. Accumulation of conjugate was striking particularly around the left optic nerve (Fig. 4). The estimated half-life of the conjugate at this site was 253 hours.

On the third day after administration of 24 mCi of $^{131}$I/UJ181.4, a progressive improvement in clinical status was documented. The patient became lucid, pain free, and was able to gain weight. Bladder control was reestablished. The clinical response was accompanied by a fall in CSF cell count and protein level to normal, and a rise in CSF glucose level. This response was maintained for 22 months without further therapy.

During this time, the patient functioned with a Karnofsky index of 60 and gained 12 kg of body weight. CSF cell count and glucose level remained normal, but the CSF protein level remained elevated. Magnetic resonance imaging (MRI) 6 months after therapy suggested some residual neoplastic disease with lesions posteriorly at C1–C2 and an irregular area in the medulla. A clinical relapse occurred at 22 months. The patient did not receive further treatment at the request of his family.

Patient 2: A clinical response also was documented in
Patient 2. This patient had a 2-week history of weakness and clumsiness in the right arm and leg, and a mild expressive dysphasia. A CT scan showed a left posterior frontal tumor that was resected subtotally. Histologic examination of the tumor indicated that it was an anaplastic tumor with a strong stromal component. Immunohistologic study indicated a diagnosis of B-cell lymphoma. Clinical staging indicated that the tumor was confined to the CNS, and the patient received 3000 cGy to the neuraxis with an additional 1500 cGy to the tumor site. Within 5 weeks of the CNS irradiation, the right-sided weakness progressed and the patient became incontinent. Despite treatment with cyclophosphamide, vincristine, and doxorubicin, intrathecal methotrexate, and oral steroids, a further progression of clinical signs occurred. At referral, the patient was cachectic, confused, and unable to walk unaided. In addition to neck stiffness, there were palsies of the third, fourth, sixth, and seventh cranial nerves. There was a bilateral decrease in palatal sensation and a negative gag reflex. Examination of the patient’s limbs showed a generalized weakness that was worse on the right. CT scanning indicated an area of contrast enhancement around the brain stem and fourth ventricle. A spinal block was apparent at C7–T2. CSF cell count was elevated at 83/mm^3. Twenty percent of the cells were undifferentiated by conventional cytology and immunoreactive with antibodies B1 and F8-11-13. CSF glucose level was reduced at 0.7 mmol/l. CSF protein level was raised at 2.25 g/l. Local irradiation was given to the cervicothoracic block. All chemotherapy was discontinued for 6 weeks before antibody administration. The cell count within the CSF remained elevated at 372 mm^-3 on the day of antibody therapy. The CSF cell count and glucose and protein levels returned to normal 4 days after injection. Normal cell count and glucose level were maintained, but the CSF protein level rose to 1.83 g/l at 6 weeks after therapy. A resolution of all clinical signs occurred apart from the patient’s seventh lower motor neurone (LMN) facial nerve palsy. The patient’s Karnofsky index improved from less than 30 to 60 at 3 months. The patient remained clear clinically of CNS disease for 1 year, but relapsed extracranially. Further chemotherapy was unsuccessful in controlling systemic disease, and the patient died 1 year after antibody therapy. At postmortem, small islands of subependymal lymphoma were identified near the Foramina of Monroe and in the meninges adjacent to the medulla. No evidence of tumor was seen on the spinal meninges or on the cerebral surface.

Patient 4: This patient had a midline posterior fossa tumor that was classified originally as a primary intracranial neuroblastoma. Whole-brain irradiation was administered at a dose of 5500 cGy to a level of C2. Two years after diagnosis, tumor dissemination occurred within the spinal canal. At referral, multiple tumor deposits were imaged throughout the neuraxis. A CT spi-
nal scan suggested widening of the entire cord above T9. MRI demonstrated irregularities of the lower cervical cord and broadening of the entire thoracic cord. Myelography indicated a spinal block at T9 with multiple filling defects below. A dose of 1500 cGy were delivered locally to T8-L1. No additional therapy was administered. Immediately before therapy, the patient walked with the aid of a frame. Clinical signs indicated a mild spastic weakness in the right leg with loss of joint position sense in the toes and ankle, and a patchy sensory loss.

After administration of 40 mCi of $^{131}$I UJ181.4, localization of the radiolabeled antibody was demonstrated at the site of the primary disease (posterior fossa), thoracic, and lumbar spine (Fig. 5). The effective half-lives of conjugate at these sites were not quantified. The patient made a sustained clinical recovery. At 2 months after therapy, the patient was able to ride a bicycle. No clinical signs of disease activity were elicited. CSF cell count and glucose and protein levels remained normal for 7 months after therapy. At 7 months, a single tumor deposit was demonstrated in the thoracic region. This was treated successfully with local irradiation, and the patient remains in remission at 16 months from antibody administration.

**Patient 5:** This patient had disseminated melanoma within the CNS. She experienced general malaise, nausea, and seizures. A CT scan demonstrated a right-sided enhancing lesion adjacent to the insular of the Sylvian fissure. This was removed by stereotactic guided craniotomy. At the time of operation, widespread involvement of the cortical pia by tumor was noted. A repeat CT scan of the head was performed. This showed a persistent tumor at the operative site and a small metastasis in the region of the right quadrigeminal plate. Myelography indicated filling defects at the base of the lumbar sac and at T12-L2. CSF cell count was normal at the time of therapy. After administration of 40 mCi of monoclonal antibody Mel 14, localization of the conjugate was demonstrated both in the lumbar spine and in the cerebral cortex (Fig. 6). The effective half-life of the radiolabeled antibody at these sites was 127 hours. Repeat CT scanning demonstrated clearance of parenchymal tumor deposits (Fig. 7). This patient remained in clinical remission for 8 months before there was a rise in CSF cell count. At 12 months, the patient died with progressive disease.

**Patient 3:** This patient had a tumor of mixed cellularity. The tumor expressed vimentin, UJ181.4, and cytokeratin markers. Despite a transient improvement in well being, no evidence of tumor localization was demonstrated either on scintigraphy or autoradiographic examination of the CSF cytospin preparation. Clear evidence of progressive disease was detected 1 week after therapy, and the patient died at 4 weeks.

**Discussion**

Malignant meningitis remains a devastating clinical condition. Advances in chemotherapy and radiotherapy have had little impact on the course of this disease. Mean survival for extracranial tumors metastasizing to the leptomeninges is 2 to 3 months $^{12-14}$ whereas for a
primary neural tumor such as medulloblastoma, mean survival is between 12 and 13 months.\textsuperscript{15,16}

Although many of the tumors metastasizing to the leptomeninges are radiosensitive, adequate radiotherapy is frustrated often by the risk of neural radionecrosis. This is a problem particularly when neuraxis irradiation was given as part of the initial therapy. Intracavity administration of the meninges with a radionuclide such as \textsuperscript{131}I has the advantage of restricting radiation dose delivery to a depth determined by the range of the predominant radiation emission.\textsuperscript{17}

This strategy has been investigated by intrathecal administration of colloidal gold to patients with leukemia and medulloblastoma. The development of this approach was curtailed when it was observed that the colloidal tended to pool in the sacral sac resulting in a high radiation dose to the cauda equina.\textsuperscript{18,19} To some extent, these problems have been overcome by using larger infusion volumes and gravity to assist the flow. This technique is still being investigated particularly for the treatment of meningeal leukemia.\textsuperscript{20,21}

Radiolabeled monoclonal antibodies offer the potential to deliver radiotherapy in a specific manner, provided the initial distribution of the radionuclide is uniform. The distribution of the radiolabel within the CSF pathways has been followed by scintigraphy. In the absence of a spinal block, the pattern of CSF circulation of isotope mirrors that seen in \textsuperscript{131}I albumin cisternography.\textsuperscript{22,23} The biexponential clearance of isotope from the CSF follows broadly that predicted by \textsuperscript{131}I albumin,
and radiolabeled protein is transferred rapidly to the vascular compartment.

However, the rate of clearance of antibody from the vasculature is more rapid than that observed for albumin, and is in keeping with observations on the vascular loss of antibody after intravenous administration. Rapid clearance of unbound antibody from both CSF and blood favors targeting by reducing the total nonspecific radiation dose to the whole body and the CNS. At the doses of radiolabel administered during this study, no bone marrow toxicity was observed.

To establish beyond a doubt that tumor radiation dose delivery is mediated through a specific effect is difficult because tumors could not be biopsied for analysis. The demonstration of preferential excretion of the control antibody from the whole body was encouraging, and may indicate the effect of tumor binding on the specific antibody. In the first patient studied, there is no doubt that the preferential retention of the tumor binding antibody within the CNS was a specific phenomena. However, the magnitude and consistency of the difference is surprising. In the absence of further data on the ratio of specific to nonspecific antibody in tumor tissue, the assumption that matched antibodies clear from the CNS at a similar rate requires testing. This is being studied currently in a primate model.

Further indirect evidence supporting a specific effect is supplied by scintigraphic data. Localization of radiolabeled antibody to tumor sites has been observed, and the retention of radiolabel at these sites has been prolonged. These observations probably do not represent vascular pooling of isotope because the half-life of conjugate at tumor sites is in excess of that observed in the blood.

The remaining objectives of this study were to look at the toxicity of the technique and to detect evidence of therapeutic effect. The administration of radiolabeled albumin into the thecal space has been associated with aseptic meningitis. A similar mild inflammatory reaction has been the principle complication of intrathecal antibody administration. All febrile reactions were self limiting, and only in one patient was there an increase in nuchal rigidity after therapy. The chronic elevation of protein levels in two patients may be attributable to a variety of factors, including occult disease or toxic damage. Both of these patients reached postmortem. A careful search was made of the CNS for chronic radiation damage, and in one case there was some histologic abnormality. In this case, the fibrotic changes observed in the cerebral leptomeninges may have resulted from previous therapy. Therefore, it is not possible to determine if radiolabeled antibody was implicated in these changes. Toxicity data from animal studies are required to investigate this problem.

Figs. 7A and 7B. CAT scans of the brain of Patient 5 with contrast enhancement, before and after the administration of 40 mCi of $^{131}$I Mel 14. (A) Contrast enhancing lesions at the (a) operative site and in the (b) region of the insular of the Sylvian fissure. (B) Comparable cut showing loss of contrast enhancing lesions.
Lastly, the clinical response data observed in this group of patients was encouraging. It was established previously that, despite a variety of therapeutic maneuvers, the prognosis for malignant menigitis is dismal. A single injection of radiolabeled monoclonal antibody has produced a sharp clinical improvement that has been sustained without additional therapy in four of five patients. Clinical response was accompanied by clearance of malignant cells from the CSF (two of two patients) and complete clearance of imageable disease in Patient 4. A partial clearance of disease was documented in Patient 5. The survival of this group of patients was 22 months, 12 months, 4 weeks, 16 months, and 12 months, respectively. These results were achieved with minimal acute toxicity and a mean bed occupancy of the radioisotope suite of 5 days.

The encouraging results of this pilot study suggest that this form of therapy is worth further investigation, both at an experimental level and in larger clinical trials. It is important particularly to seek further evidence of the specificity of the technique and to determine what proportion of radiation delivery is due to a simple "bath" effect. Additional questions include the therapeutic effect of monoclonal antibody alone, the optimal size of tumor deposits for therapy, and the best available isotope. At a clinical level, provided chronic toxicity proves to be acceptable, this method of radiation delivery should be interesting particularly for the control of meningeal leukemia, lymphoma, and medulloblastoma. This Phase I study has been established to evaluate 131I/UJ181.4 in the therapy of relapsed medulloblastoma.

REFERENCES


The biodistribution and pharmacokinetics of meta-iodobenzylguanidine in childhood neuroblastoma


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Abstract. MIBG is generating considerable interest for the treatment of neuroblastoma. This study has investigated the biological variation in handling of the compound in children with neuroblastoma. The biodistribution of the compound has been characterised in children undergoing tracer administrations of 123I and 131I-mIBG. Estimates of hepatic and whole body radiation dose delivery have been made. The results indicate substantial interpatient variation in hepatic dose delivery. This organ may be critical in some patients undergoing targeted radiotherapy with mIBG.

Key words: MIBG - Neuroblastoma - Dosimetry - Pharmacokinetics - Biodistribution

The sympathetic ganglion blocker guanethidine relies for its therapeutic effect on the ability to concentrate in neurosecretory granules. Analogues of guanethidine have been synthesised which share this property, and are also easily radiolabelled with isotopes of iodine. In particular, the analogue meta-Iodobenzyguanidine (mIBG) has found use as a radiotracer for both normal and abnormal tissue derived from the autonomic nervous system. The first tumour to be imaged in this way was phaeochromocytoma where benign and malignant tissue has been detected with a high degree of sensitivity and specificity (Shapiro et al. 1985). Sufficient uptake of mIBG into tumours has been achieved to undertake therapy by targeting 131I to phaeochromocytoma (Sisson et al. 1984).

Neuroblastoma shares with phaeochromocytoma the ability to actively accumulate mIBG into neurosecretory granules. The use of the compound as a targeting agent in neuroblastoma is attractive for a number of reasons. Firstly, conventional therapy has made little impact on the outcome of stage IV disease (Shafford et al. 1984). Secondly, the tumour is highly radiosensitive (Deacon et al. 1985). However, disease is often widely disseminated making application of external beam therapy difficult without substantial systemic toxicity. The use of targeted radiotherapy has the theoretical advantage of high tumour dose delivery with low systemic toxicity.

Few model systems are available to study the uptake of mIBG into neuroblastoma cells either in vivo or in vitro.

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The cell line SK-N-SH has been reported to take up mIBG but the origins of this line are controversial. Whilst originally classified as a neuroblastoma the line is now believed to be from a patient with a neuroepithelioma (F.F. Proceedings 1985). In the absence of adequate models to study mIBG uptake into neuroblastoma, the biodistribution and pharmacokinetics of the compound have been investigated in children with stage III or IV neuroblastoma imaged with mIBG.

Materials and methods

131I-mIBG was purchased from Cis and Mallinckrodt. 123I-mIBG was prepared in the laboratory according to the method of Wieland et al. (1980). Between 1 and 2 mg mIBG were added to 370 MBq 123I supplied in a dried form from Harwell and 100 μl glacial acetic acid was added to this mixture followed by 3–5 mg ammonium sulphate powder. The bottle was sealed and heated for 45 min at 140°–160° C in a glycerol bath after which 1.5 ml 0.005 M acetate buffer was added (pH 4.1) and free iodine removed from the preparation following passage through a Cellex-D ion exchange column. Radiolabelled mIBG was sterilised by passage through a 0.22 μm Millex filter and collected in an evacuated vial. The radiochemical purity of the preparation was determined by thin layer chromatography using a 3:1 mixture of propan-1-ol and 10% ammonium hydroxide.

Patient studies. Seventeen children with variable tumour burden were entered into the study with informed parental consent and ethical committee approval. To effect thyroid blockade, children received 0.3 ml Lugol’s iodine 3 times daily for a period of 72 h before, and 1 week after, injection of radiolabelled mIBG. Each patient received a standard tracer dose of mIBG, 20 MBq 131I-mIBG was given to children, irrespective of body weight (specific activity 74 MBq/mg). For patients receiving 123I-mIBG, a dose of 10 μg/kg body weight was administered amounting to between 20 MBq and 185 MBq (specific activity 370 MBq/mg).

The early biodistribution of the compound was studied in five patients by a dynamic acquisition series; mIBG was injected intravenously, with the patient lying supine on the camera face. Thirty sequential 1 min views of abdomen and thorax were collected. Static acquisition scans were ob-
contain on all patients, these were obtained on at least 3 occasions from 4–72 h after the administration of conjugate. Multiple images at each time point were collected, consisting of anterior and posterior views of abdomen and thorax, right and left lateral views of head and neck to include right and left upper limbs, and posterior views of lower limbs and pelvis.

Time activity curves were generated for liver and heart by region of interest studies with appropriate background subtraction. In 8 patients, clearance of isotope from blood was measured by repeated blood sampling, 30 min to 48 h after mIBG administration. Total blood activity was calculated using this information and applying Nadler tables for blood volume estimation.

In patients undergoing scintigraphy with $^{131}$I conjugates, whole body retention was measured using a shielded scintillation probe, positioned at 2 m from the umbilicus. Measurements on individual patients were made immediately after injection and at 24, 48 and 72 h. Urine collections were also made on these patients to assess the rate of loss of isotope by this route. Estimates of organ and whole body dosimetry were performed using the Medical Internal Radiation formalism.

To correct for organ mass the dose (mGy/MBq) was calculated from the following equation: $D = A \times S \times 70 / W \times 1/d$, where $A$ is the cumulated activity, $S =$ absorbed dose fraction, $W =$ weight of the patient, $d =$ administered dose. In Patients undergoing $^{123}$I scintigraphy, an estimate of radiation dose was made assuming the compound had been radiolabelled with $^{131}$I.
isotope in salivary tissue, myocardium, and liver. Images study consistently demonstrated a high concentration of Accumulation of counts within the liver occurred rapidly fell to 75% of this peak level 30 min after injection of the was small, and did not account for the vascular loss. Direct observation period the activity or counts remained con­ over 30 min. Maximal counts were seen in the 1st min and of the injected dose excreted by this mechanism (range bladder activity was seen. The magnitude of this early loss was highly variable between patients with a mean value of 45 h (12 observations, range 8–145 h). The calculated dose delivery to the liver from $^{131}$I-mIBG lay between 0.2 and 4.6 mGy/MBq with a mean value of 1.5 mGy/MBq ($n = 12$) (Table 1).

Whole body clearance of isotope followed biexponential clearance kinetics and paralleled the urinary excretion of isotope. The first phase of loss was rapid and insufficient data from whole body retention measurements was available to quantity this accurately. The 2nd clearance component from the whole body is slower with a biological half life of between 19 and 45 h (mean value 35, $n = 7$, Table 2). This component is the most influential on whole body dose and if taken alone provides an estimated whole body dose of between 0.2 and 0.5 mGy/MBq of administered isotope (mean value 0.4 mGy/MBq, $n = 7$).

Results

Thin layer chromatography of mIBG revealed two bands. The major band represented mIBG ($r_f = 0.75$) whilst the other material running at the solvent front was free iodine. Following a bolus intravenous injection of radiolabelled mIBG, activity cleared from the blood following biexponential clearance kinetics. The first clearance component was very rapid with between 1.5% and 10% of the injected dose remaining in blood at 60 min (mean 4.4%, $n = 8$, Table 3). Subsequent loss of isotope was monoexponential over the assay period of 48 h. The biological half life of this clearance component was variable between patients and lay between 9 and 130 h. The early biodistribution of the compound was followed using the dynamic acquisition series. Within the first 30 min radiolabel was observed to be excreted by the kidney (Fig. 2), and isotope was demonstrated to accumulate rapidly in the bladder. This phase of renal filtration was of short duration and after 15–20 min no further increase in bladder activity was seen. The magnitude of this early loss was small, and did not account for the vascular loss. Direct measurement of urine activity at 3 h indicates only 13% of the injected dose excreted by this mechanism (range 11%–26%).

Additional loss occurs due to selective organ uptake. Accumulation of counts within the liver occurred rapidly over the 1st 5 min (Fig. 3). During the following 25 min observation period the activity or counts remained constant. Counts measured over the heart fell at a linear rate over 30 min. Maximal counts were seen in the 1st min and fell to 75% of this peak level 30 min after injection of the conjugate (Fig. 1).

Scintigrams performed after the dynamic acquisition study consistently demonstrated a high concentration of isotope in salivary tissue, myocardium, and liver. Images at 4 h also showed mIBG uptake in the lung which was not clearly seen in the lower third. This uptake was transient and no radioactivity above background level was observed by 24 h. Normal left adrenal was visualised on only one occasion, and the presence of activity in bowel was variable between patients. No uptake was demonstrated in the spleen, bone or bone marrow above background.

Local accumulation of isotope into abnormal areas was visualised by 4 h, these were most clearly seen at 24 h and 48 h, due to better tissue to background ratios. In 19 scans mIBG correctly identified 73 tumour sites, 3 areas of focal accumulation were seen in which it was not possible to image tumour by X-ray, ultrasound or CT scanning. These areas were all at sites of primary disease. None of these patients demonstrated any evidence of active disease by conventional assessment 2 years after the abnormal mIBG scan. MIBG did not image known disease on two occasions, these sites consisted of a small cervical lymph node and one bone marrow infiltrate. Because of the difficulty of accurately estimating tumour volume in our series, it was not possible to measure radiation dose delivery at these abnormal sites.

However, later clearance of isotope was determined for both liver and whole body. The biological half life in liver was highly variable between patients with a mean value of 45 h (12 observations, range 8–145 h). The calculated dose delivery to the liver from $^{131}$I-mIBG lay between 0.2 and 4.6 mGy/MBq with a mean value of 1.5 mGy/MBq ($n = 12$) (Table 1).

Discussion

A large number of tumour sites have been shown to accumulate mIBG in this study. This supports the recent interest in developing $^{131}$I-mIBG to treat neuroblastoma. To establish a basis for therapy, it is necessary that the biodistribution of the compound be defined in a paediatric population. In this study it has been possible to identify the major sites of isotope retention and quantify the clearance of mIBG in children with neuroblastoma. The initial biodistribution of mIBG has been broadly similar between patients. There is good evidence that the isotope is rapidly distributed throughout the body with only 4.4% of the injected dose remaining within the vascular compartment at 60 min. The magnitude of early loss is not accounted for by renal filtration. Although a number of organs have been identified as organs of uptake it seems likely that loss occurs into an additional second space. It is probable that mIBG rapidly equilibrates with the extravascular compartment.

The study has highlighted the importance of the renal route for excretion of mIBG. However, over the range of renal function studied, (EDTA clearance 60–160 ml/min per 1·73 m$^2$) radiation dose estimates to liver and whole body
Table 1

<table>
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<tr>
<th>Patient no.</th>
<th>Isotope</th>
<th>Weight (kg)</th>
<th>EDTA clearance (ml/min per m²)</th>
<th>A₀</th>
<th>% injection dose</th>
<th>T₁ Biol (hours)</th>
<th>Dose (mGy/MBq)</th>
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<tr>
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<td>45.0</td>
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<td>8.0-145.0</td>
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</tbody>
</table>

Uptake and clearance of mIBG from liver

Weight: – is the weight of the patient in kg.
EDTA clearance: – is the estimated creatinine clearance of the patient from ⁵¹⁷⁷-EDTA
A₀: – is the estimated uptake of mIBG in liver at time 0, expressed as a percentage of the injected dose
T₁ Biol: – is the estimated half-life of mIBG in liver in hours
Dose: – is the estimated radiation dose delivery to liver from 37 MBq ¹³¹I-mIBG
N.E. = Not estimated

Table 2

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>A₀</th>
<th>% injected dose</th>
<th>T₁ Biol (hours)</th>
<th>Dose mGy/MBq</th>
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</thead>
<tbody>
<tr>
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<td>33</td>
<td>0.5</td>
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</tr>
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<td>40</td>
<td>19</td>
<td>0.2</td>
<td></td>
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<tr>
<td>7</td>
<td>68</td>
<td>28</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>63</td>
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<td>0.4</td>
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<tr>
<td>14</td>
<td>55</td>
<td>33</td>
<td>0.5</td>
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<tr>
<td>15</td>
<td>68</td>
<td>45</td>
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<tr>
<td>16</td>
<td>56</td>
<td>36</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Mean Range</td>
<td>58</td>
<td>34</td>
<td>0.4</td>
<td>0.2-0.5</td>
</tr>
</tbody>
</table>

Clearance of mIBG from whole body

A₀: – is the % of dose remaining in whole body at time zero, if the second component of the biexponential clearance curve is extrapolated to the origin (see text)
T₁ Biol: – is the estimated biological half-life of the second clearance component
Dose: – is the estimated radiation dose to whole body from 37 Bq ¹³¹I-mIBG

Estimates of radiation dose to whole body were of the order of 0.4 mGy/MBq. This appears to be of the order estimated from animal data (see Communication). In contrast, the estimated radiation dose to liver of 1.5 mGy/MBq (range 0.2-4.6 mGy/MBq, n=12) was significantly higher than anticipated. Patients exhibited considerable variation both in the percentage of injected dose sequestered in liver, and in its subsequent rate of clearance. Potential factors that might influence hepatic uptake of mIBG include metastatic involvement of liver, and prior exposure to hepatoxic chemotherapy. It was not possible to establish a correlation between these factors and liver dose in this study. The magnitude of the liver dose suggests that this may be the critical organ in some patients undergoing targeted therapy. If a relationship can be established between the behaviour of low specific activity mIBG in tracer studies and the high specific activity mIBG used in therapy (1110 mBq/mg), the radiation dose to radiosensitive organs could be planned.

References

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Late sequelae of foreign body inhalation

A multicentric scintigraphic study *

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Clinica Pediatrica IV dell’ Università di Torino, Torino (Italy)

Chairman: A. Piepsz

Abstract. A multicentre European study on foreign body inhalation has been organized by the Pediatric Task Force covering both the SNME and the ENMS. Among the 100 cases which could be collected in a 2 year period, about 40% of scintigraphic abnormalities were found 6 months after relief of the foreign body. The frequency of these defects was clearly related to the time interval between aspiration and removal. Factors like the localization of the foreign body and the site of the early scintigraphic defects could be related to the further scintigraphic outcome, but this could not be proven statistically. Similarly, the absence of bronchial tree inspection after removal of the foreign body seemed to be associated with more permanent scintigraphic lesions. Finally, it is obvious that a large number of patients demonstrated scintigraphic alterations 6 months or more after removal of the foreign body, although the chest X-rays were considered normal.

Key words: Foreign body - Lung - Child - Radioisotopes

Foreign body (FB) inhalation occurs commonly in childhood. The diagnosis is generally based on anamnestic data, clinical manifestations and indirect radiological signs, such as atelectasis or emphysema (Caffey 1972). It is well known that scintigraphy may strongly contribute to the diagnosis, by detecting, with a high sensitivity rate, a perfusion or ventilation disturbance as a consequence of the bronchial obstruction (Mussa et al. 1981; Mantel and Butennandt 1986; Leonidas et al. 1973; Papanicolaou and Treves 1980). It has been suggested (Chiorazzi et al. 1974) that the scintigraphic defects may persist for some time after relief of the obstruction. As far as we know, no study at the present time has shown the relationship between the long term scintigraphic alterations and the other clinical data. This was the aim of the present multicentre study, organized by the pediatric task group of the European societies of nuclear medicine (SNME and ENMS).

Materials and methods

Questionnaire. A questionnaire was sent to all members of both European societies. The criteria for entrance into the study was any patient in whom a foreign body was extracted and who subsequently underwent lung scintigraphy. For each patient, one copy of the questionnaire had to be filled in. After identification of the center, hospital and town, a short history of the patient was requested, followed by several questions related to the foreign body itself: estimation of the delay between inhalation and relief, nature of the foreign body and method of relief. Attention was paid to the fact that inspection of the airways was or was not performed after extraction of the FB. Intercurrent infections, which could have altered the scintigraphic data after relief of the obstruction, had to be mentioned. The last two pages were devoted to the description of the different chest X-rays and scintigraphies performed, the type of radotracer used for either perfusion or ventilation had to be mentioned. The results of the chest X-rays and scans were expressed in terms of normality, abnormality and localization of the lesions in one or more pulmonary lobes. Owing to the type of questions, a considerable bias in the answers seemed relatively improbable and therefore the different centers would only return the questionnaire. The copies of the questionnaire were distributed after the European meeting in Helsinki in August 1984 and the patient list was closed in August 1986. Chi-square tests were used for statistical calculations.

Results

Table 1 summarizes the number of patients enrolled in the study. Exactly 100 patients, distributed among 11 European centers, were recorded. The highest incidence of FB was observed between 1 and 2 years, and 80% of the cases were less than 4 years old. The localization of the FB is represented in Fig. 1 and the nature of the FB in Table 3: peanuts were the most common FB seen. The methods of re-
Therapeutic Application of Radiolabeled Monoclonal Antibody UJ13A in Children With Disseminated Neuroblastoma

L. Lashford,1 D. Jones,2 J. Pritchard,1 I. Gordon,1 F. Breathnach,3 and J. T. Kemshead*2

ABSTRACT—Dosimetric data from UJ13A scanning studies using 131I are presented for children with stage IV neuroblastoma and primary brain tumor. The data demonstrate a large variation among patients in dose delivery to vulnerable organs and tumors. Against this background, a phase I toxicity study is under way with escalating amounts of conjugate administered to patients who have stage IV neuroblastoma. Major toxicity has been confined to bone marrow aplasia and necessitates bone marrow harvest prior to therapy. Specific problems encountered include altered kinetics during therapy following tracer studies and adequate dose delivery in large tumor masses.—NCI Monogr 3:53-57, 1987.

In 1983, a monoclonal antibody of the IgG1 subclass, UJ13A, was described; it recognizes a surface antigen expressed on the majority of cells of neuroectodermal origin (1). The antibody binds to a range of both normal and malignant tissues of neural derivation, including neuroblastoma, retinoblastoma, and primary brain tumor. Work with this agent in neuroblastoma has resulted in use of the antibody in in vitro diagnosis of tumor type (2). It has also been used in a cocktail of antibodies for removal of tumor cells from bone marrow harvested for autologous transplant (3). In addition, UJ13A has been applied to the in vivo radioimmunolocalization of primary and metastatic disease (4). The extension of the antibody's use into targeted radiation therapy has been supported by several observations.

1) Localization of the antibody into neuroblastoma xenografts in mice is specific (5).
2) When the antibody is radiolabeled with 131I, ablation of the tumor xenografts can be achieved (4).
3) Evidence from cell culture studies and xenografts supports the proposition that neuroblastoma is a highly radiosensitive tumor (6).

Prior to embarking on a therapeutic study using radiolabeled antibodies, dosimetric data were collected from tracer studies in children with the disease. The results of this study and the effects of therapy in 5 patients are described.

METHODS

Antibody Preparation

The UJ13A was prepared by the method of Allan et al. (1); it was radiolabeled with 131I by the chloramine-T method (7). Separation of the radioiodinated antibody from free iodine was achieved by Sephadex-G25 gel filtration in the presence of 2% purified human plasma protein. Prior to injection into patients, the antibody was sterilized by passage through a 0.22-μm Millipore filter. Aliquots of solution were routinely checked for free iodine by precipitation with 10% trichloroacetic acid and for aggregation by centrifugation at 10,000 g for 30 minutes.

Tracer Studies

Children with either neuroblastoma or primary brain tumor were entered in the study with ethical committee approval and informed parental consent. Each patient received 0.3 ml of Lugol's iodine solution three times daily for 72 hours prior to administration of 131I-labeled UJ13A and for 1 week after radioscintigraphy. Sensitivity to antibody was assessed by an intradermal injection of 10 μg of protein 30 minutes prior to iv administration. A bolus of the radiolabeled protein (10 μg/kg) was given iv (specific activity, 8–16 μCi/μg).

Imaging was performed with a Scintag-Berthold gamma camera fitted with a medium-energy collimator. A dedicated Informatek computer was used in the analysis of scans. Children received sequential static scans at variable times over a period of days following injection. In all cases, a minimum of three scans were performed to provide information on organ half-life. Dosimetry calculations were performed by the standard techniques using the medical internal radiation dosimetry method on single posterior views. Blood clearance data were obtained by repeated sampling over a period spanning 30 minutes to 48 hours after injection; an LKB ultragamma counter was used.

Therapeutic Studies

All children entered in the study had stage IV neuroblastoma and had relapsed or failed to respond to conventional therapy. The tumor in each patient was known to bind UJ13A in vitro. Thyroid blocking and skin testing were performed as for tracer studies. The children received a dose of 131I, which was escalated from 35 to 55 mCi until toxicity was observed. When toxic levels were reached, the dose was reduced for the next 2 patients.

Sequential blood sampling was performed to determine blood half-life, and sequential static scans were performed.
Evidence of toxicity was sought by examination of blood for hematologic parameters and liver function (SGOT, SGPT, alkaline phosphatase, and bilirubin).

Radioiodinated UJ13A consistently retained biologic activity, as measured by an indirect immunofluorescence assay on frozen sections of neuroblastoma tissue. In general, the injected compound contained <5% free iodine and was <5% aggregated (mean, 4.47%; range, 1%-6.5%). None of the children had an abnormal skin test, and thyroid uptake was not observed. Dosimetric data were available for uptake into the liver in 10 patients (mean dose to liver, 2.97 rad/mCi ± 1.85 sd), into the spleen in 9 patients (mean dose, 8.90 rad/mCi ± 5.28 sd), and into the whole body from blood in 9 patients (mean dose, 0.57 rad/mCi ± 0.29 sd) (fig. 1, table 1). Difficulties arose in evaluating tumor dosage because of the often intimate association of the tumor with the liver and spleen. However, reliable data were available for 7 tumor sites identified in 6 patients. The mean dose to tumor sites was 37.50 rad/mCi ± 23.00 sd (range, 18.4-88.0).

Phase I Study

To date, 5 patients have been recruited to the study. All of the patients were heavily pretreated with chemotherapy until the time of therapeutic administration of 131I-UJ13A. This chemotherapy consisted of 10 or 11 courses of OPEC in patients 1, 3, and 5 (8) (table 2). The remaining 2 patients received additional therapy in the form of low-dose, external beam, total body irradiation (two doses of 50 rad) and the drugs DTIC and doxorubicin. Patient 2 received these drugs because of relapse following 10 courses of OPEC. Patient 4 was changed to this therapy after six courses of OPEC, when clinical deterioration became apparent. In this patient, etoposide had already been substituted for teniposide because of an anaphylactic response on second exposure to the drug. All patients had failed to achieve a good partial response to conventional therapy, and all had evidence of metastatic disease. Patients 2, 4, and 5 were clinically deteriorating at the time the antibody conjugate was administered.

Acute Effects

The immediate effects from administration of 131I-UJ13A were minimal in 4 of the 5 patients. A mild pyrexia lasting 2–4 hours was observed in all patients, and 2 experienced nausea and vomiting. Patient 4, who had previously experienced problems with teniposide, developed a mild anaphylactic response to the therapeutic administration of 131I-UJ13A. This occurred despite a negative skin test and resulted in rapid clearance of the conjugate. A rise in the anti-mouse immunoglobulin titer was subsequently measured.

<table>
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<tr>
<th>Organ</th>
<th>No. of observations</th>
<th>Mean dose ± sd (rad/mCi)</th>
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</thead>
<tbody>
<tr>
<td>Whole body</td>
<td>9</td>
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</tr>
<tr>
<td>Liver</td>
<td>10</td>
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<td>Spleen</td>
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<td>8.90 ± 5.28</td>
</tr>
<tr>
<td>Tumor</td>
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</tr>
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</table>
Intermediate Effects

The principal side effect noted during therapy was bone marrow aplasia (table 2). Two of the patients experienced this problem, and in 1, it was irreversible. In patients 1, 4, and 5, there was no adverse effect on hematopoietic function.

Patient 1 received 35 mCi of isotope following a predicted whole body dose from tracer studies of 0.5 rad/mCi. This was confirmed during therapy with an estimated delivery of 0.65 rad/mCi.

Patient 4 received 50 mCi of isotope. Dosimetric calculation predicted a whole body dose of 0.77 rad/mCi, but the conjugate was rapidly eliminated following an anaphylactic response. Consequently, the patient received only minimal radiation therapy.

To circumvent this problem in patient 5, we did not perform any tracer studies prior to administration of 50 mCi of conjugate. At a whole body dose of 1.7 rad/mCi, there was no major toxicity to bone marrow.

The 2 patients who developed significant marrow aplasia both received 55 mCi of $^{131}$I-UJ13A. Patient 2 had received chemotherapy and total body irradiation in addition to OPEC. Tracer doses of antibody conjugate had predicted a whole body dose of 0.88 rad/mCi. Aspirated samples taken just prior to therapy showed no marrow involvement with disease. Six days after administration of $^{131}$I-labeled antibody, a rapid fall in blood count was observed; it resulted from hypoplastic bone marrow. Marrow function did not recover, and the patient died from advanced disease. Patient 3 and all subsequent patients had bone marrow harvested and purged by a separation technique using monoclonal antibody–magnetic microspheres. In the second patient to receive 55 mCi, we predicted a dose of 1.31 rad/mCi to the whole body. On day 6, the WBC and platelet counts dropped but recovered spontaneously within 5 weeks of therapy without the need to infuse the harvested marrow. This patient had bone marrow involvement with neuroblastoma at the time of $^{131}$I-antibody administration.

Hepatotoxicity

The tracer studies suggested that a significant dose would be targeted to the liver (mean uptake, 54% of injected dose). Nevertheless, no disturbance to liver function tests was observed in the first 4 patients. Patient 5 demonstrated a rise in liver function values, but this was also associated with a change to Australia antigen positivity.

Therapeutic Effects

Therapeutic benefit was clearly seen only in patient 3 (table 3), who had diffuse disease at the time of therapy, with bone marrow infiltrate and 2 small skull deposits. The patient showed clearance of bone marrow disease and radiological evidence of bony healing. This clinical state was maintained for a period of 8 months. The data from other patients exemplify specific problems associated with antibody therapy.

Patient 1, as the first patient in the phase I study, received only 35 mCi of $^{131}$I-UJ13A. Tumor uptake was determined, and tumor half-life was assessed as 74 hours. The patient was clinically well at the time of administration, despite active disease at two sites. No change in clinical status occurred after therapy, and the patient remained well for an additional 5 months. A subsequent attempt at therapy resulted in an extremely rapid clearance of the conjugate with an effective half-life in blood of 4 hours.

A similar effect was seen in patient 4, whose prior exposure to the antibody was confined to the tracer dose. An increased clearance of conjugate was seen on second exposure, and as a result of the rapid elimination, only a low dose of radiation was achieved.

To avoid sensitization, we did not give patient 5 a tracer dose. Marrow infiltration by UJ13A immunoreactive neuroblastoma was demonstrated just prior to therapy, but no uptake of the antibody was demonstrated in the large abdominal mass by gamma camera scanning after therapy. Patient 2 had similarly extensive abdominal disease, and a scan after therapy confirmed tumor uptake.
The immediate toxic effects of antibody infusion were mild and self-limiting, except in 1 patient who developed anaphylaxis associated with production of an anti-mouse immunoglobulin. The skin test performed was of no value in predicting this effect. This patient received no therapeutic benefit, which adds weight to the proposition that patients undergoing targeted radiation therapy should be excluded from tracer studies.

The major toxic effect of bone marrow aplasia gave rise to concern. This occurred at levels of 55 mCi with whole body dose deliveries of 0.88 and 1.3 rad/mCi. These levels compared with whole body doses of 0.55 and 0.77 rad/mCi in children who did not have this toxic effect. It is not clear whether it will become possible to recognize a group of patients who are more likely to experience this complication. Variation in the antibody kinetics of patients is as likely to influence the clinical response as is the total dose of antibody. Moreover, it is probable that prior treatment with certain combinations of therapy is likely to produce a more vulnerable stem cell. In an attempt to circumvent this problem, bone marrow is now harvested, purged, and cryopreserved prior to therapy.

Several general points emerged regarding the use of monoclonal antibodies as targeting agents. Despite a sound theoretical basis for the use of this antibody, adequate access to the tumor by whole immunoglobulin remains a problem. In addition, tumor bulk is likely to confer relative radioresistance, as illustrated by patients 2 and 3, who had similar kinetics and whole body dosage. Patient 2 had extensive abdominal nodal disease and received no therapeutic effect. Patient 3 had small tumor volume (two bony deposits in the vault of the skull and marrow infiltration). This patient had radiological evidence of healing and an 8-month remission.

Similarly, patient 5 was known to have UJ13A immunoreactive disease, as evidenced by bone marrow aspirates taken just prior to therapy. Radiolabeling of the antibody did not destroy its biologic activity, as determined by an indirect immunofluorescence assay on tissue sections. The patient had not been exposed previously to antibody, and the vascular half-life was 56 hours. Nevertheless, no antibody uptake at the tumor site was observed, and the patient received no therapeutic benefit. A similar phenomenon has been seen during tracer studies. Figure 2 shows comparable scans in a patient who had imaging first with UJ13A and then with the small molecular weight radiopharmaceutical mIBG. The tumor is clearly identified with mIBG, but a “cold area” remains on antibody scanning over the whole imaging period. This may be a func-

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Tumor sites</th>
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<th>Therapy</th>
</tr>
</thead>
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<td></td>
<td></td>
<td>Dose to tumor</td>
<td>Tumor half-</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>life (hr)</td>
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<td>2</td>
<td>Abdominal mass, Abdominal/pelvic lymph nodes</td>
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<td>Site 1 skull deposit, Site 2 skull deposit</td>
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<td>89</td>
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<td>Skull deposit</td>
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<td>84</td>
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<tr>
<td>5</td>
<td>Abdominal mass, Abdominal nodes, Bone marrow, Multiple bony sites</td>
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</tbody>
</table>

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Although different time points were chosen to illustrate difference in radiolabel uptake, images represent those seen at several time points examined over 48-hr period. Panel A: Isocontour profile of $^{131}$I-UJ13A 48 hr after administration of radiolabeled conjugate. Note negative "filling" effect in area of E scan. Panel B: Isocontour profile of $^{125}$I-mIBG 24 hr after administration. Note uptake in area E of scan. Area A, thyroid; B, heart; C, liver; D, spleen; E, tumor; and F, bladder.

section of molecular size alone; whether access can be improved by using antibody fragments is currently under investigation. The particular problems of the changing biodistribution on repeated exposure is similarly being assessed in animal models, both with the whole antibody Fab$_2$ and with Fab fragments.

Targeting therapeutic agents to tumor cells in vivo almost certainly involves a series of complex biologic interactions. A further understanding of the biology of the tumor and its vasculature can only increase the likelihood of successful targeted antibody therapy.

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Comparison of Pharmacokinetics of Radiolabeled Monoclonal Antibody UJ13A in Patients and Animal Models

D. H. Jones, L. S. Lashford, C. Dicks-Mireaux, and J. T. Kemshead

ABSTRACT—The antigen to which the monoclonal antibody UJ13A binds is expressed by normal primate brain and most tumors of neuroectodermal origin. The antibody has been used in radioimmunologic studies in patients with neuroblastoma and glioma, though the uptake of antibody into tumors is low. Animal models have been sought to allow definition of the parameters that influence tumor uptake. Animals include nude mice, in which xenografted human tumors can be studied, and marmosets, in which the primate response to exposure to murine antibodies can be investigated. This presentation reports the preliminary pharmacokinetic data from studies of mice and marmosets and compares the data with those obtained in patients.

At present, diagnosis of disseminated neuroblastoma in children relies to a large extent on a complex series of radiological investigations that is burdensome for the patients. These include chest x-ray, bone scans, liver scans, computed tomography, and ultrasound. It would be beneficial if these tests could be replaced by a single, reliable procedure, and to this end, many laboratories are investigating the feasibility of targeting specific radiopharmaceutical agents that demonstrate selective uptake into defined cancers. Falling into this category are reagents such as the polyclonal antisera to CEA (1); antisera to α-fetoprotein (2); antisera to ferritin (3); the guanethidine analogue meta iodobenzylguanidine (4); and a host of monoclonal antibodies showing reactivity toward a variety of cancers. In this laboratory, the potential of the monoclonal antibody UJ13A in this role is under investigation in animal models and in patients with neuroblastoma.

The UJ13A derived using fetal brain as an immunogen has broad specificity for normal human brain and neural tumors, including neuroblastoma, when assayed by indirect immunofluorescence on frozen tissue sections (5). Conjugate, which was radiolabeled and injected iv into immunocompromised mice xenografted with human neuroblastoma, localized specifically in tumor implants (6).

Similarly, when radioiodinated UJ13A was injected, conjugate localized in approximately 80% of known tumor deposits in patients with clinically diagnosed stage IV neuroblastoma examined by gamma camera scintigraphy (7). Serial scanning of these patients allowed estimation of the half-life of antibody in tumor and vital organs.

In an attempt to define and therefore optimize the parameters that influence uptake into tumor and other tissues, the pharmacokinetics of radiolabeled UJ13A have been investigated in animal models and the results have been compared to those found in patients. Areas under active investigation include the influence of blood clearance, circulating antigen, degree of tumor vasculature, and spontaneous or invoked anti-mouse immunoglobulin responses. This presentation reports our preliminary results in these comparative studies.

MATERIALS AND METHODS

Antibody

Ascitic fluid from hybridomas was applied to Protein A Sepharose (Sigma Chemical Co.), and resin was extensively washed in PBS (pH 7.4). The IgG bound to the column was eluted with 0.1 M citrate buffer (pH 3.0), and 0.75-ml fractions were collected in 0.4 ml of 0.75 M TRIS (pH 8.8). Antibody-containing fractions were pooled, dialyzed extensively against 10 mM ammonium bicarbonate, and lyophilized. Aliquots of antibody were weighed, dissolved in 0.1 M phosphate buffer (pH 7.4), and radiolabeled with 125I (Harwell, United Kingdom), 131I, or 133I (Amersham International, Amersham, United Kingdom) to specific activities of 8–16 μCi/μg of protein by the chloramine-T method (8). Radiolabeled protein was recovered in the void volume of a Sephadex G-25 column equilibrated in PBS containing 2% FCS for injection into mice; it was recovered in the human plasma protein fraction for injection into marmosets and patients. Radiolabeled antibodies were injected within 18 hours of preparation to minimize potential radiolytic effects.

Antibody was routinely chromatographed at 4°C on a column of Sephacryl S-200 (2.5×100 cm) equilibrated with PBS containing 2% FCS at a flow rate of 20 ml/hour; 2.5-ml fractions were collected for isotope estimation. Biologic activity of radiolabeled antibody was determined by indirect immunofluorescence using frozen tumor sections. Free iodine was assayed by trichloroacetic acid precipitation of antibody aliquots.

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ABBREVIATIONS: PBS = phosphate-buffered saline; FCS = fetal calf serum.
Patients

Ethical committee approval and informed consent were obtained for these studies. Clinical data on adults entered in the study have been described in full detail elsewhere (10). The study was comprised of 2 groups. In the first group, only tumor uptake data were acquired; these data have been described in detail previously (7). In the second group, data on tumor uptake and pharmacokinetics were obtained.

The second group was comprised of 6 patients (3 male and 3 female, aged 18 mo to 9 yr) who were treated for stage IV neuroblastoma by chemotherapy and surgery. All of the patients had returned to the Hospital for Sick Children for restaging and reassessment; 5 had had 4–6 months off treatment, and 1 had had no therapy for 2 years. The latter patient was considered to be in complete remission; in 3 others, the disease was static, and in 2, florid relapse was evident.

Patients were injected with 10–20 μg of ^131I-labeled UJ13A/kg, and serial blood samples were taken for weighing and radioactivity assessment. Gamma camera scintigraphy was also performed with anesthetized animals. Posterior images only were recorded with a dedicated Elscint computer. Regions of interest were drawn over approximately the same liver areas on static scintigrams performed several times during the 72 hours after injection. Counts were normalized for areas and for time, before subtraction of normalized background counts. Data were plotted on semilogarithmic graph paper, and half-life was calculated.

Marmosets

Marmosets were housed in the animal facility for a minimum of 4 weeks before use. Animals were anesthetized with halothane, nitrous oxide, and oxygen and were maintained under anesthesia for iv injection of 10–20 μg of radioiodinated UJ13A/kg. On withdrawal of blood samples, aliquots were weighed and radioactivity was assessed. Data were expressed as percentage of injected dose per gram of blood, and counts were corrected for physical decay to time of injection (t=0).

Gamma camera scintigraphy was also performed with anesthetized animals. Posterior images only were recorded and analyzed with a dedicated Elscint computer. Regions of interest were drawn over approximately the same liver areas on static scintigrams performed several times during the 72 hours after injection. Counts were normalized for areas and for time, before subtraction of normalized background counts. Data were plotted on semilogarithmic graph paper, and half-life was calculated.

RESULTS

Antibody Preparations

The UJ13A antibody was one of a series of hybridomas resulting from the immunization of mice with human fetal brain. After screening of hybrids for anti-neuroblastoma activity, UJ13A was selected because it was found to bind to all neuroblastoma tissue sections and cell lines tested. Titration of ascites fluid showed anti-neuroblastoma activity at dilutions >1:100,000.

The UJ13A IgG was prepared from ascites fluid by protein A chromatography as described previously and was radiolabeled with isotopes of iodine by the chloramine-T method. On chromatography on S-200, the protein profile showed no appreciable aggregation (<5%) following radio-labeling (fig. 1). Titration of radiolabeled antibody on frozen sections of neuroblastoma and comparison with equivalent dilutions of unlabeled antibody showed no loss of biologic activity as a result of radio-labeling.

Mouse Studies

The UJ13A was radiolabeled with isotopes of iodine and injected into nude mice bearing human neuroblastoma xenografts. An irrelevant antibody, FD44, known not to bind to neuroblastoma, was also radiolabeled and injected into a control group of xenografted mice, and animals from both experimental and control groups were killed at 24-hour intervals after injection. The radioactivity in resected tissues was counted and expressed as counts per minute per gram.

Ratios of UJ13A to FD44 are shown in table 1. They demonstrate the selective uptake of UJ13A into xenografts only; ratios in other tissues were 0.9:1 to 1.1:1, with the exception of spleen, which consistently gave a ratio of 1.5:1. Ratios of UJ13A to FD44 remained relatively constant during the 10-day period of study.

Expressed as a percentage of the injected dose per gram of tumor, the uptake into tumor xenografts is shown in table 2. Maximal uptake occurred at 24 hours following injection and fell thereafter. Uptake was shown to be independent of tumor size. The UJ13A antigen is not expressed by any mouse tissue, and the uptake of UJ13A was therefore considered low, prompting investigation of the pharmacokinetic distribution of radiolabeled UJ13A in mice.

Disappearance of antibody from blood was extremely rapid and followed a biphasic exponential decay profile (fig. 2). Approximately 80% of the injected antibody was removed from circulation within 30 minutes of injection. The first half-life was only 0.25 hours, while the second was considerably longer at 45 hours. Uptake of antibody into tissues such as tumor, liver, spleen, kidney, muscle, brain, or lung was not at levels sufficient to account for such rapid clearance, and it was concluded that antibody reequilibrated between intravascular and extravascular
space extremely rapidly. It is possible that this reequilibration would limit the availability of antibody in circulation, which in turn would limit its availability for uptake into tumor.

Uptake of antibody into the liver was significant, reaching a maximum of 13% per gram 1 hour after injection and falling steadily thereafter to 3% per gram by 24 hours, with half-life calculated as 53 hours. This observation suggests that the reticuloendothelial system plays a significant role in the biodistribution and metabolism of the antibody.

**Patient Studies**

Radioimmunolocalization of tumors known to express UJ13A antigen was attempted in 2 groups of patients, children being treated for stage IV neuroblastoma and adults with primary brain tumors. The adults had had 99mTc scans prior to injection of radiolabeled antibody to demonstrate blood-brain barrier disruption in the area local to the tumor site.

Following injection of radioiodinated UJ13A, serial blood samples were taken to allow determination of the rate of clearance of antibody from blood. As with mice, the blood clearance in both children (fig. 2) and adults showed biphasic profiles, which are described by the calculated half-lives shown in table 3. The similarity in the first half-lives for both children and adults demonstrates that levels of antibody in circulation did not fall below 50% per gram until at least 12 hours after injection. This contrasts markedly with the observations in mice; only 20% per gram of the antibody injected in mice remained in circulation after 30 minutes.

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**Table 1.** Ratios of uptake of specific (UJ13A) to nonspecific (FD44) monoclonal antibody into neuroblastoma xenografts and normal tissues in nude mice*  

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>7:1</td>
<td>1:1-20:1</td>
</tr>
<tr>
<td>Liver</td>
<td>1:1</td>
<td>0.9:1-1.2:1</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.5:1</td>
<td>0.7:1-2:1</td>
</tr>
</tbody>
</table>

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* Radiolabeled UJ13A and FD44 (known not to bind to neuroblastoma) were injected via tail veins into separate groups of nude mice with human neuroblastoma xenografts. Animals were killed at 24-hr intervals, and tissues were dissected, weighed, and counted for radioactivity. Expressed as counts per minute per gram, ratios of uptake of UJ13A to FD44 in tissues were calculated for tumor, liver, and spleen. In all other tissues, ratios were 0.9:1-1:1.

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**Table 2.** Uptake of radiolabeled UJ13A into neuroblastoma xenografts in nude mice*  

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Uptake (% injected dose/g of tumor)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
<td>Median</td>
<td>N</td>
</tr>
<tr>
<td>24</td>
<td>9.1</td>
<td>0.8-20.6</td>
<td>6.0</td>
<td>3</td>
</tr>
<tr>
<td>96</td>
<td>7.9</td>
<td>1.4-26.0</td>
<td>6.1</td>
<td>7</td>
</tr>
<tr>
<td>120</td>
<td>6.6</td>
<td>2.3-21.0</td>
<td>4.8</td>
<td>9</td>
</tr>
<tr>
<td>168</td>
<td>5.1</td>
<td>1.3-16.6</td>
<td>2.45</td>
<td>6</td>
</tr>
</tbody>
</table>

* Uptake was assessed by killing animals at daily intervals after injection. Tumors were resected, weighed, and counted for radioactivity.
In children, it was not possible to accurately assess the uptake of antibody into the tumor, but this assessment was possible in adults when tumors were resected shortly after administration of radiolabeled antibody. The data obtained from 7 patients show that resected tissue contained, maximally, <0.005% of the injected dose (table 4). Few other published studies show tumor uptake from resected tissue, but the data of Mach et al. (11) support the extremely low degree of uptake we observed in tumors in patients. This suggests that uptake of the same order of magnitude could be expected in neuroblastoma sites in children. From the limited number of studies performed, it appears that uptake in xenografted tissue in mice is a gross exaggeration of the uptake to be expected in patients.

The half-life of antibody uptake into tumors was assayed in patients by serial gamma camera scintigraphy.

**Table 3.—Biphasic clearance of $^{131}$I-UJ13A from blood of patients**

<table>
<thead>
<tr>
<th>Patients</th>
<th>$T_{1/21}$ (hr) Mean Range Median</th>
<th>$T_{1/22}$ (hr) Mean Range Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children</td>
<td>2.4 1.0-3.7 2.4</td>
<td>33 18-63 32</td>
</tr>
<tr>
<td>Adults</td>
<td>2.9 0.2-8.5 2.1</td>
<td>59 30-104 59</td>
</tr>
</tbody>
</table>

*Patients were injected with $^{131}$I-UJ13A, and blood samples were withdrawn at set times following injection. Samples were weighed and counted for radioactivity; counts were corrected for physical decay to time of injection (t=0). Half-lives were calculated from semilogarithmic plots of counts per minute per gram of blood vs. time.

**Table 4.—Uptake of $^{131}$I-UJ13A into brain tumors**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Time resected (days)</th>
<th>% injected/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0.0043</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>0.0018</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>0.00073</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>0.0015</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>0.00036</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>0.00062</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>0.00058</td>
</tr>
</tbody>
</table>

*Patients presenting with primary brain tumors (No. 1, malignant meningioma, and No. 2-7, gliomas) were injected with $^{131}$I-UJ13A prior to surgery. At surgery, resected tumor was separated into necrotic tissue and viable tissue; the viable tissue was weighed and counted for radioactivity.
DISCUSSION

By virtue of its longer half-life in tumor than in any other tissue, UJ13A has proved itself a valuable diagnostic reagent for the localization of disseminated tumor sites in children with neuroblastoma (7). Nonspecific uptake in liver and spleen necessitates analysis of late rather than early scintigrams for visualization of focal uptake into tumor. Although uptake to tumor is sufficient for diagnostic purposes, initial studies indicate that the amounts delivered to primary masses are insufficient for therapy. More research on a broad front is needed to define the parameters that influence uptake of antibody into tumor to optimize tumor uptake and minimize uptake into other tissues.

Data obtained from studies with the nude mouse xenograft model show little similarity to data obtained from patients. The nude mouse model has been used, however, to demonstrate the specificity of uptake of UJ13A into neuroblastoma xenografts as a prerequisite to patient studies. Blood clearance of injected radiolabeled UJ13A was much more rapid than that observed in patients; up to 80% of injected antibody was removed from circulation within 30 minutes of injection. Nevertheless, a mean uptake of 9% of the injected dose per gram of tumor (median, 6%) was found in tumors resected 24 hours after injection. At this level of uptake, a therapeutic response has been obtained, with xenografts first shrinking and then disappearing on repeated injection of 131I-UJ13A (6).

Uptake into neuroblastoma in children is much more difficult to quantify because the tumor is often surgically removed during the course of chemotherapy. Gliomas, however, possess surface densities of UJ13A antigen similar to those in neuroblastomas, and glioma patients have received 131I-UJ13A several days prior to resection of tumor (10). Uptake per gram of tumor can thus be accurately assessed, and serial gamma camera scanning allows half-life to be calculated. At best, uptake of only 0.005% of the injected dose was observed, a value comparable to values obtained by injection of specific antibodies into colorectal carcinoma patients prior to resection of tumors (11). Thus, while data are not available for neuroblastomas in patients, extrapolation of the data presented here suggests that values approximately 1,000-fold less than those for the xenograft model could be expected. There are several possible explanations. Anti-mouse response and circulating antigen may limit antibody binding to tumor; UJ13A antigen is completely absent from all mouse tissues. It is also likely that there is a difference in vascular supply to xenografts and in situ tumors. These two possibilities cannot be studied in mice, which necessitates a search for a more suitable animal model.

Initial studies with marmosets indicate favorable comparisons in the handling of antibody by children and marmosets; this is reflected in the similar clearance rates of antibody from blood and liver. We have thus embarked on a study of the effect of molecular size on blood and liver clearance rates by comparing serial gamma camera scintigrams and blood samples after injection of radioiodinated UJ13A, IgG, Fab2, and Fab molecules. Repeated injection of the antibody and its fragments will also allow comparison of their relative immunogenicity by analysis of serum samples for anti-mouse activity. Future experiments involve investigation of the effects of circulating antigen on the biodistribution of radiolabeled antibody. Because the antigen is present in marmoset brain, levels of circulating antigen can be manipulated and correlated with changes in the biodistribution of radiolabeled antibody. Because the antigen is present in marmoset brain, levels of circulating antigen can be manipulated and correlated with changes in the biodistribution of radiolabeled antibody. Because the antigen is present in marmoset brain, levels of circulating antigen can be manipulated and correlated with changes in the biodistribution of radiolabeled antibody. Because the antigen is present in marmoset brain, levels of circulating antigen can be manipulated and correlated with changes in the biodistribution of radiolabeled antibody. Because the antigen is present in marmoset brain, levels of circulating antigen can be manipulated and correlated with changes in the biodistribution of radiolabeled antibody. Because the antigen is present in marmoset brain, levels of circulating antigen can be manipulated and correlated with changes in the biodistribution of radiolabeled antibody.


table 5

<table>
<thead>
<tr>
<th>T1/2 (hr)</th>
<th>Mean</th>
<th>Range</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroblastoma (8 patients, 9 sites)</td>
<td>79</td>
<td>18-110</td>
<td>84</td>
</tr>
<tr>
<td>Glioma (4 patients)</td>
<td>179</td>
<td>95-288</td>
<td>166</td>
</tr>
</tbody>
</table>

* Patients with tumors expressing antigen were injected with 131I-UJ13A and scanned over periods of several days (3 days for neuroblastoma; ≤ 16 days for glioma). Half-life of radiolabeled antibody in tumor was calculated from normalized regions.

Marmoset Studies

Groups of 3 marmosets were used in each study and were, of necessity, maintained under halothane anesthesia for the duration of the procedures. Animals were injected with radioiodinated UJ13A, and serial blood samples were taken to assess blood clearance. As with mice and patients, loss of antibody from blood followed a biphasic curve (fig. 2). The first half-life of 4.8 hours was very similar to that observed in both children and adults but very different from that observed in mice. The second half-life was 16 hours. Levels of circulating antibody were thus maintained at >50% per gram for about 8 hours, comparable to that observed in patients. The half-life of antibody in liver was assessed by serial scintigraphy on a gamma camera, as it was in patients. Initial studies suggest that mean half-life is about 12.5 hours, which compares favorably with that in children but again is very different from the 53 hours observed in mice.

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In order to determine the problems relating to antibody use in cancer diagnosis and therapy, more data from patient studies will be required before such questions can be answered. In view of these findings, a cautious and thorough approach to targeted radiation therapy using monoclonal antibodies needs to be taken.

REFERENCES


REPEATED EXPOSURE OF NON-HUMAN PRIMATES TO MONOCLONAL ANTIBODY AND FRAGMENTS: PHARMACOKINETIC STUDIES AND THEIR IMPLICATIONS FOR TARGETED THERAPY.

Lashford L.S. MRCP. (1,2) Elsom G. (1) Clarke J. MSc. (1) Gordon I. FRCR. (2) Kemshead J.T. PhD. (1)

(1) Imperial Cancer Research Fund, Oncology Laboratory, Institute of Child Health, London.
(2) The Hospital for Sick Children, Great Ormond Street, London.

INTRODUCTION.

The monoclonal antibody, UJ13A, recognises an antigen of pan-neuroectodermal origin and has been evaluated as both a scintigraphic agent (Goldman et al. 1984) and for targeting radiotherapy in childhood neuroblastoma (Lashford et al. 1987). The biodistribution of the radiolabelled antibody has been established in patients (Lashford et al. Submitted), and a phase 1 toxicity study of the 131-I labelled conjugate undertaken.

Two problems have been identified which limit the use of the antibody as an immunolocalising agent. Firstly, despite supporting evidence of immunoreactive disease, uptake of radiolabel at large tumour sites has been poor. Secondly, prior exposure to the antibody may significantly alter pharmacokinetics and hasten clearance of the conjugate.

Experience with other monoclonal antibodies suggests that the changes in pharmacokinetics may be due to the production of anti-mouse immunoglobulin (Ig) response in the patient (Schroff R. et al. 1985). Human anti-mouse Ig response have been reported to both the Fc protein and the
idiotypic domains of the molecule. Which type of response is elicited may be dependent on the dose and course of antibody administration. In developing UJ13A as a radiation delivery system it is important to determine whether rapid elimination of therapeutic amounts of antibody is a result of an anti-mouse Ig response, and if so to which part of the molecule the response is generated.

To address this question we have employed a non human primate model, the common marmoset. In this species, we have demonstrated that the pharmacokinetics of antibody handling mirror those seen in children (Jones D. et al. 1987). Marmosets have been given doses of radiolabelled antibody (Ig and Fab) proportional (on a weight basis) to those given to children. This strategy circumvents the ethical problems associated with undertaking these studies in children relapsing with metastatic neuroblastoma. Detailed studies have been undertaken on the biodistribution of the conjugates by direct venous sampling and gamma camera scintigraphy.

METHODS.

Protein Preparation.

A single batch of monoclonal antibody was prepared from ascites using protein A Sepharose. The material was concentrated to 1 mg/ml and aliquots of 100ug were stored at -20°C until required for use.

Fab monomer was prepared from whole antibody by a papain digestion using a 10:1 Wt:Wt ratio of antibody:papain. After 2 hours incubation at 37°C the reaction was terminated with 500mM of iodoacetamide and the resulting digest diluted to 10mls using distilled water at 4°C. Contaminating Fc chains were removed by Amicon filtration using a Ym 30 filter. The buffering solution was exchanged for 10mmol ammonium bicarbonate in the filtration unit, and the preparation lyophilised and stored in 100ug aliquots at -20°C. SDS polyacrylamide gel electrophoresis was performed in an 8% gel, under reducing conditions, to check the purity of the preparation.
Radiolabelling.

Both UJ13A and its Fab fragment were radiolabelled with $^{123}$I supplied by Harwell. During the eight months of the experiment, the form in which this was supplied was altered. The radioisotope was consistently supplied in dried form, prepared from a solution containing sodium hydroxide. A significant fluctuation in the level of sodium hydroxide was initially observed, and this resulted in a variable radiolabelled product. To overcome this problem, $^{123}$I was later supplied dried from a solution containing a low sodium hydroxide concentration.

Using a modified chloramine T technique, protein was radiolabelled to a specific activity of 5μCi/ug of protein. Free iodine was removed using a Sephadex G25 column equilibrated with phosphate buffered saline containing 1% human plasma protein fraction (PBS/1% HPPF).

The biological activity of both preparations was determined before and after radiolabelling by an indirect immunofluorescence assay. Biological activity was defined by the lowest titre at which maximal fluorescence was observed. Free iodine in the radiolabelled product was measured by either trichloroacetic acid precipitation or thin layer chromatography in 85% methanol. Protein aggregation was determined by centrifugation of an aliquot of the preparation in PBS with 2% foetal calf serum at 10,000 x g.

Animal Studies.

Nine animals were entered into the study, 3 animals receiving whole UJ13A, 3 (Fab) monomer and a control group receiving free iodine. Prior to administration of the radiolabel, thyroid blockade was achieved by adding 3 drops of Lugol's iodine solution to a liquid feed. Under Halothane /Nitrous oxide and oxygen anaesthesia, all animals had 0.5mls of blood withdrawn and stored as plasma. Experimental animals were then injected with 10ug/kg. $^{123}$I/UJ13A (Ig) or $^{123}$I/UJ13A (Fab) dissolved in 300ul of sterile PBS. Control animals received 0.3ml of a solution of free $^{123}$I. All administrations were followed by a further injection of 0.2mls of 0.9% saline to flush the syringe and needle. Repeat administration of Ig,
Fab or free 123I were performed on 3 further occasions 4 – 8 weeks apart.

Measurement of the injected dose was performed by counting the syringe and needle on the gamma counter before and after injection. The early biodistribution of the conjugate was studied using the gamma camera. A dynamic acquisition series was commenced at the moment of injection in 15 one minute frames. In order to estimate early organ uptake, measurements of counts in a standard region of interest were taken and expressed as a ratio of injected dose. Information on organ half life was obtained by sequential static scans from 1 hour to 48 hours after injection. The frequency and duration of counting was determined by the rapidity of fall of organ counts to background level.

To generate a time activity curve for blood, intermittent venous sampling was undertaken during the time period 30 minutes to 24 hours. In addition to plasma stored before each exposure to radiolabelled conjugate, plasma was also sampled and stored at approximately 4 weeks after injection. This was stored at -70°C until assayed for presence of anti mouse Ig.

### Preparation of Rabbit anti-Marmoset Ig and Measurement of an anti-mouse Ig in Marmoset sera.

Ig was purified from the sera of marmosets by ammonium sulphate precipitation. The Ig was emulsified in complete Freunds adjuvant. 2mg of the protein preparation was injected subcutaneously at multiple sites in rabbits. Injections were repeated at monthly intervals using Ig emulsified in incomplete Freunds (x5). Rabbit anti-marmoset Ig was affinity purified using an affinity column of marmoset Ig coupled to CNB activated Sephadex 4B. After dialysis and concentration to 1mg/ml the purified Ig was radiolabelled to a specific activity of 1ug protein/100uCi iodine.

For assay of an anti-mouse Ig response in marmosets, mouse Ig (monclonal antibody UJ13A) was coated onto 96 well vinyl plates (Dynatech). Serial dilutions of control and test sera were added to the wells. After a 30 minute incubation plates were washed using PBS/10% bovine serum
albumin and 100,000 cpm of rabbit anti-marmoset Ig added. Following a further 30 minutes incubation plates were washed and isotope bound determined using a LKB Ultra-gamma counter.

RESULTS.

Fab Fragments and Radiolabelled Antibodies.

A single band of approximately 50kd was observed on SDS polyacrylamide gel electrophoresis of the Fab preparation of UJ13A (Fig.1).

Fig.1. SDS polyacrylamide gel electrophoresis of preparation.

Channel A, M,Wt. Markers
B, IgG1 - UJ13A
C, Fab - 2hr digest
D, Fab - 4hr digest
E, Fab - 6hr digest

Whole Ig produced a single band of approximately 155,000 Mwt. Both were shown to be biologically active. Using standard neuroblastoma tissue a 1.0 mg/ml solution of whole UJ13A had a titre of 1/4,000 and an equivalent concentration of the Fab preparation titred to 1/2,000.

A reduction in biological activity was generally observed after radiolabelling. In addition, the amount of aggregated protein and free iodine contaminating the injected product, varied significantly. This was directly attributable to the fluctuation in sodium hydroxide content of the 123I over the period of the experiment.

Direct Venous Sampling.

On first administration of 123I UJ13A (Ig), isotope was cleared relatively slowly from the vascular compartment in a biexponential manner. Direct venous sampling demonstrated a fast
clearance component with a mean biological half life of 4.8 hours. The second component of the curve had a half life of 19.6 hours. Isotope in the blood remained attached to immunoglobulin as in excess of 98% of the counts were precipitable with 10% Trichloroacetic acid.

Coupling isotope to whole Ig dramatically changed its biodistribution and pharmacokinetics within the animal, as compared to the clearance of unconjugated 123I. Although a biphasic clearance curve for free iodine was suspected the first component was so rapid that it could not be accurately measured. Only 20% of the dose remained in the blood thirty minutes after injection. The second component of the clearance curve of isotope showed a mean half life of 6 hours. No significant change in the rate of clearance of unconjugated 123I was demonstrated at the second, third or fourth administration. The initial loss of 123I was estimated as 20% of injected isotope at 30 minutes for each exposure. Subsequent clearance had a biological half life of 5.5hrs, (range 5-5.8) 5.2hrs (range 4.4-5.7) and 5.7hrs (range 4.8-6.2) at sequential injections.

With each immunisation of 123I radiolabelled Ig, isotope cleared from the blood in an increasingly rapid fashion (Fig.2).

This was particularly marked for the first half life where sequential changes of 5, 0.3, 0.2 and 0.1 hours were measured (injections 2,3,4). This trend was similar for the second component where changes from 19.6 to 17.3, 14.4 and 11 hours were observed for successive administrations. Variability within the group became more marked with each injection of 123I UJ13A as indicated by the dispersion of counts around the mean.

The kinetics of 123I Fab clearance from the blood lay between those observed for whole Ig and free isotope. An initial mean half life of 0.26 hours was observed (n=3) with the second clearance component of 13 hours. With sequential administration of the fragment there was little change in the clearance kinetics. The scatter of the points around the mean remained small and did not change with increasing Fab exposure (Fig.3).
Gamma Camera Scintigraphy.

Dynamic acquisition studies were undertaken to provide information on organ uptake during the early phase after 123I Ig administration. Liver and heart were the only organs that were reliably identified on the scans, the latter reflecting conjugate in the vasculature. After standardisation for injected dose it was apparent that counts rapidly accrued in the liver during the first 5 minutes of observation, with a slower accumulation occurring during the next 8 minutes. Towards the end of the observation period a decline in activity was observed.

With successive exposures, dynamic acquisition studies showed that uptake of the radiolabel in liver increased with a quadrupling of counts between the first and fourth administrations. This increase in uptake was consistent within the group and independent of both the specific activity and degree of aggregation of the injected protein (Fig. 4).
marmosets on sequential exposures.

Data on animals given the UJ13A was lost due to computer failure. Observations on 2/3 animals are also incomplete due to difficulties encountered on repeated anaesthesia. However, static scans were obtained on all animals. Loss of isotope from liver was linear to 48 hours and was increasingly rapid with successive administrations of antibody (Table 1). Throughout the observation period loss of the radiolabel from whole body was monoexponential and became more rapid with each exposure to Ig.

Table 1.

<table>
<thead>
<tr>
<th>Blood</th>
<th>$T_1$ (h)</th>
<th>$T_2$ (h)</th>
<th>Liver (h)</th>
<th>Whole body (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>15.6</td>
<td>10.6</td>
<td>10.1</td>
</tr>
<tr>
<td>2</td>
<td>0.3</td>
<td>17.4</td>
<td>8.8</td>
<td>10.0</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>12.5</td>
<td>6.9</td>
<td>8.1</td>
</tr>
<tr>
<td>4</td>
<td>0.1</td>
<td>10.8</td>
<td>4.26</td>
<td>7.6</td>
</tr>
</tbody>
</table>

Early dynamic acquisition images of animals given 123I Fab fragments of UJ13A showed uptake of isotope in the kidney as well as in liver. Maximal liver uptake occurred within the first 2 minutes and then plateaued throughout the observation period. Data was lost on one animal.
due to cardiac arrest at anaesthesia and for one
time point on another animal due to computer
failure during the dynamic acquisition scans. The
level of peak uptake in the liver was calculated
to be approximately 75% of that seen on 1st
exposure to whole immunoglobulin. This did not
increase on successive exposure of animals to
123I Fab (Fig. 5). A similar pattern of uptake was
observed in the kidney. During the time period 1–
15 minutes, no activity was observed in the
bladder.

Fig. 5. Uptake of 123-I UJ13A, Fab fragment into
liver and kidney of marmosets on sequential
exposures.

Static acquisition studies were performed up
to a time point of 6 hours. Scintigraphy was
discontinued after this time because of low count
rates, consequently late organ half lives could
not be obtained. The whole body clearance was
monoexponential and remained unchanged from
exposure to exposure. Mean effective half lives
of 6, 7.5, 5.3 and 5 hours were calculated for
injections 1–4 respectively.

The dynamic acquisition series on animals
receiving 123I alone support data obtained by
intravenous sampling showing a rapid loss of
isotope from the vascular compartment. Diffuse
homogenous activity was measured over the head,
thorax and abdomen. No organ could be identified
in the first 15 minutes of scanning with
distribution of isotope reflecting tissue mass
rather than specific organ uptake. By two hours a
region of activity was visible on the left upper
At later scans the activity identified in the left upper quadrant was diffusely distributed throughout the abdomen.

Clearance of the isotope from whole body was slightly longer than that observed for blood, with a mean half life of 6 hours (Range 5.5 - 6.5 hours). This did not change on repeated exposure of animals to isotope with mean body half lives of 6.5 (range 5.5-8) and 5 hours (range 4.8-5.2) for the 3rd and 4th exposures. All data was lost for the second exposure due to computer failure.

Screening of Sera for an Anti-Mouse Ig Response.

An anti-mouse Ig response was only noted in sera taken from animals receiving multiple injections of 123I UJ13A. An illustration of the change observed in sera are shown in Fig.6.

**Fig.6.** Rise in circulating marmoset anti-mouse Ig, on multiple administrations of whole UJ13A.

After the second injection of radiolabelled UJ13A, a four fold increase in 125I rabbit anti-marmoset Ig binding to plates preincubated with mouse Ig (UJ13A) and marmoset sera was noted over background levels (pre bleed scan). Levels of anti-mouse Ig increased on subsequent exposure of animals to UJ13A. In contrast no increase in binding 125I rabbit anti-marmoset Ig was later noted in sera from animals given multiple injections of either 123I Fab or 123I alone (control).
DISCUSSION.

Investigation of the biodistribution of UJ13A in patients has suggested that several problems limit the use of the antibody. Significant hepato-splenic uptake of isotope occurs which limits the bioavailability of antibody for tumour targeting, as well as resulting in a significant radiation dose to these organs. Additional organ radiation dose delivery is derived from the relatively slow vascular clearance of conjugate.

This study indicates that by utilising the Fab fragment of UJ13A the biodistribution of the conjugate is significantly changed. The initial rapid loss of conjugate from the vascular compartment suggests a more rapid equilibration of protein with the extravascular space. This feature coupled with more rapid clearance of isotope from whole body and other organs, lowers normal organ toxicity from equivalent levels of administered conjugate. In addition, the wider initial biodistribution of radiolabelled fragment may facilitate tumour uptake.

A similar therapeutic advantage may be seen with the Fab2 dimer of UJ13A. This was studied in the same model system, with results indicating a similar pattern of tissue distribution as documented for the Fab monomer. However it was not possible to obtain quantitative data on this fragment as preferential radiolabelling of a small molecular weight contaminant occurred. This contaminant was present in low amounts and was not detected in routine SDS gel electrophoresis.

The study also set out to determine whether the observed change in handling of UJ13A in patients at second exposure was the result of a circulating anti mouse Ig. It is clear from this study that repeat administrations of UJ13A produced increasingly rapid clearance of the conjugate from blood and organs. The initial vascular loss correlated with both hepatic sequestration and the strength of the anti-mouse Immunoglobulin. The magnitude of the immune response in influencing biodistribution appeared independent of other factors such as the degree of protein aggregation. Repeated injection of the
Fab fragment produced a stable biodistribution of conjugate and no anti mouse Ig activity. This data suggests that at this level of injected UJ13A, the generated anti mouse Ig is principally anti-Fc. It is likely that a stable biodistribution of the Fab2 dimer would also be obtained.

These results indicate that the use of fragments will circumvent some of the problems experienced in our early work. Provided that tumour uptake of fragments is satisfactory, further progress in the use of monoclonal antibodies to target radiotherapy may be achieved.

ACKNOWLEDGEMENTS.

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REFERENCES.


A COMPARATIVE STUDY OF THE BIODISTRIBUTION OF META-IODOBENZYLGUANIDINE (mIBG) AND THE MONOCLONAL ANTIBODY UJ13A IN PATIENTS AND ANIMAL MODELS.

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INTRODUCTION.

Two types of compound are available for radiation targeting in neuroblastoma. These are the monoclonal antibodies exemplified by UJ13A, and the small molecular weight radiopharmaceutical meta-Iodobenzylguanidine (mIBG). The monoclonal antibody UJ13A recognises a cell surface antigen expressed on tissue of neuroectodermal origin. In 1984, Goldman et al. demonstrated that radiolabelled UJ13A imaged both primary neuroblastoma and secondary deposits. In subsequent studies the work was extended to demonstrate that accumulation of UJ13A in human neuroblastoma xenografts was a specific phenomena, and that increasing the dose of radiolabelled 131-I UJ13A produced a temporary ablation of the xenografts (Jones D.H. et al.1985).

The radiopharmaceutical mIBG shares with UJ13A the ability to accumulate at tumour sites. mIBG is actively transported into neurosecretory granules and unlike UJ13A is deposited intracellularly (Buch J. et al.1985). A number of investigators have demonstrated that administration of 100-200mCi of this radiolabelled conjugate may produce a temporary response in
The success of either delivery system in future clinical trials is dependent on the balance struck between radiation delivery to tumour and to normal organs. To quantitate this latter aspect of radiation dose delivery, a comparative study of the biodistribution of both conjugates has been undertaken in non human primates (marmosets) and patients with stage IV neuroblastoma.

METHODS.

UJ13A was prepared by the method of Jones et al. The antibody was radiolabelled by the chloramine T technique, to a specific activity of between 8 and 16uCi/ug of protein. Free iodine was separated from the radiolabelled product by column chromatography using Sephadex G25 equilibrated with phosphate buffered saline/1% human plasma protein fraction (PBS/1%HPPF). Prior to injection into patients or marmosets the protein was passed through a 22um Millex filter to ensure sterility. An aliquot of the solution was routinely checked for free iodine by precipitation with 10% trichloroacetic acid. Protein aggregation was determined by either centrifugation at 10,000 xg in PBS containing 2% HPPF or by high performance liquid chromatography.

The immunoreactivity of the radiolabelled antibody was confirmed before and after radiolabelling using an indirect immunofluorescence assay.

131-I mIBG was purchased from Amersham. 123-I mIBG was prepared in the laboratory by the method of Wieland et al. 1980. 1-2mgs of mIBG were added to 10mCi of 123-I supplied in dried form from Harwell. 100ul of glacial acetic acid was added to this mixture followed by 3-5mgs of ammonium sulphate powder. The bottle was sealed and heated for 45 minutes at 140-160°C in a glycerol bath. 1.5mls of 0.005M acetate buffer was added (pH 4.1) and free iodine removed from the preparation by passage through a Cellex-D ion exchange column. Radiolabelled mIBG was sterilised by passage through a 0.22um Millex filter and collected in an evacuated vial. The radiochemical purity was...
determined by thin layer chromatography using a 3:1 mixture of propan-1 ol and 10% ammonia solution.

Animal studies.
To obtain a detailed understanding of the pattern of clearance of the two compounds from different organs, a quantitative assessment the biodistribution of both compounds was undertaken in marmosets.

12 marmosets were injected with 10ug/kg of 131-I mIBG (S.A.2.5uCi/ug), and then sacrificed at different time points. Immediately prior to death each animal was scanned using a Siemens gamma camera fitted with a 410keV medium energy collimator. Blood and whole organs were then removed and counted in a LKB ultragamma counter. In this way a relationship was established between counts measured on the gamma camera and counts in any organ.

Six further marmosets received sequential administrations of 131-I mIBG and 131-I UJ13A in doses of 10ug/kg over a three week period. The pharmacokinetics of the radiolabelled conjugate were studied by whole body scintigraphy at 1 minute, 20 minutes, 1,2,4,6,24 and 28 hours. Using the tissue resection data from the initial study, time activity curves were generated for organs of interest. Comparative organ radiation dose estimates from the two compounds for each animal, were undertaken by a comparison of the accumulated activity in organs of interest.

Patient studies.
Nine patients were entered into the study. All patients had completed chemotherapy as defined by Trial 1 of the European Neuroblastoma Study Group. Vincristine 1.5mg/m², Cis platinum 60mg/m², VM26 150mg/m², cyclophosphamide 600mg/m², 6-10 courses, randomised to high dose melphalan.

To shorten the time period of the scanning study, the first six patients with stage IV neuroblastoma were sequentially injected with 123-I mIBG (half-life of 123-I, 13.2hrs.) followed by 131-I UJ13A (half life of 131-I 8.04 days). To account for potential differences in isotope the following 3 patients were injected with 131-I mIBG
and 123-1 UJ13A. The early biodistribution of each conjugate was followed in a dynamic acquisition series of 30 x 1 minute frames of abdomen and thorax. To define the later clearance of isotope, sequential static images were obtained. These studies comprised scans of right and left lateral skull with the respective arm, anterior and posterior thorax, anterior and posterior abdomen and pelvis and posterior views of legs to include feet. All scans were obtained on at least 3 occasions over the time period 4 hours to 48 hours. Time activity curves were generated for the principle organs of uptake both for the time period 1-30 minutes and for the later clearance 4-48 hours.

Radiation doseage was calculated using the Medical Internal Radiation Dose formalism. The assumption was made that the principle radiation dose delivery to organs of interest came from a single monoexponential clearance component, and that this was adequately defined by estimates of organ uptake over the three time points between 4 and 48 hours.

RESULTS.

Marmosets.

Principle organs of uptake were defined as kidney and liver for mIGB and liver for UJ13A. The accumulation of mIBG in kidney was short lived, and no significant activity over background was detected in this organ by 1 hour. From the data provided by direct tissue resection, accumulation of isotope in kidneys accounted for 10% of the injected dose at 20 minutes (Range 8-11.6%, N=3). This fell to 0.52% by 4hrs (Range 0.36-0.64%, N=3). Loss of isotope through the kidneys after 1 hour was monoexponential with an effective half life of 7 hours. Examination of urine by thin layer chromatography demonstrated that the major excretory product was intact mIBG in both the fast and slow phases of urinary excretion (Fig 1).
Fig. 1. Thin layer chromatogram of administered mIBG compared with urinary excretion of mIBG and contaminants over time periods 0-20 minutes and 20-60 minutes, marmosets 1-free iodine, 2-mIBG, 3,4,5-larger molecular weight bands.

The impact of the initial urinary loss of mIBG was to produce a fast and a slow component to the clearance of mIBG from whole body. The initial rapid loss of isotope was sustained for a period of 1 hour, and accounted for 17% of the injected dose (Range 12-21%, N=6). Subsequent loss was monoexponential with an effective half life of 22.3hrs (Range 19-26.4hrs, N=6).

No early period of renal excretion could be identified for 131-I UJ13A. Clearance of this conjugate from whole body followed monoexponential clearance kinetics. The rate of clearance was both variable between animals and prolonged (Mean Teff: 67.4hrs, Range 52-81, N=5). Using these results to derive the accumulated activity in whole body from 1mCi of 131-I UJ13A and 1mCi of 131-I mIBG a mean of 3.8 times the whole body dose is delivered from UJ13A as from mIBG. (Range 2.6-5.3, N=5) (Fig. 2).

Fig. 2. Comparison of accumulated activity (uCi hrs) in whole body from 1mCi of 131-I UJ13A, 1mCi of 131-I low specific activity mIBG and 1mCi of 131-I high specific activity mIBG.

Both mIBG and UJ13A were initially taken up by liver before being cleared by this organ. For
UJ13A, uptake was maximal at 1 hour and subsequent clearance was monophasic. A similar period of isotope accumulation was demonstrated for mIBG, with maximal uptake of isotope at 1 hour; however subsequent clearance of the radiolabelled conjugate isotope followed biphasic clearance kinetics.

The contribution of this initial period of mIBG accumulation and loss to the total accumulated isotope in liver was small (Teff 1st component: Mean 2 hours, Range 1.2-3.6, N=6) (Fig.3). Estimates of accumulated activity in liver were therefore based on information provided by the influential second clearance component and compared with the monophasic time activity curve generated for UJ13A. Both the proportion of injected UJ13A removed by liver and its subsequent rate of clearance were significantly increased over mIBG. This resulted in an estimated radiation dose delivery from 1mCi of UJ13A to liver, of 6.4 times that delivered from 1mCi of 131-I mIBG (Fig.4).

Fig.3. Time-activity curves for low specific activity mIBG in liver of marmosets.

Fig.4. Estimated accumulated activity (uCi hrs) in liver of marmosets, from 1mCi of 131-I UJ13A, 1mCi low specific activity mIBG and 1mCi of high specific activity mIBG.
Patient studies.

The observations on the early biodistribution of UJ13A and mIBG were confirmed in the patients. Dynamic acquisition curves were generated for heart, liver, kidney and bladder following injection of mIBG. Similar curves were generated for liver, spleen and heart following intravenous administration of UJ13A. The heart curves suggest that the initial rate of loss of radiolabel from blood for both conjugates is similar (Fig 5). This is not consistent with the values obtained by blood sampling. By 1 hour, a mean of 95.6% of administered mIBG is lost from the vascular compartment (Range 90-98.5%, N=8). This compares with a loss of 32% of injected UJ13A (Range 12-44%, N=8) (Table 1).

Table 1.

<table>
<thead>
<tr>
<th>UJ13A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>4.6</td>
</tr>
<tr>
<td>5.0</td>
</tr>
</tbody>
</table>

Table 1.

The dynamic acquisition curves generated for UJ13A over heart, reflect the measured rate of vascular loss. The disparity between dynamic acquisition data and vascular sampling for mIBG, indicates that a period of myocardial uptake occured in all patients studied.

This evidence of myocardial uptake of mIBG was confirmed on the later static acquisition scans.

Principle sites of isotope accumulation were liver and salivary tissue for mIBG and liver and spleen for UJ13A. The uptake and clearance of isotope from liver was quantified for both conjugates. The mean biological half life for mIBG in liver was estimated as 27 hours (Range 8-66 hours, N=8) (Table 2). This compared with 22 hours for UJ13A (Range 11-39 hours, N=8).
Table 2. Clearance and Estimated Radiation Doseage to Liver, of 1 mCi of Administered Radionuclide.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Ao (%)</th>
<th>T(Biol) (hrs)</th>
<th>Dose rads</th>
<th>Ao (%)</th>
<th>T(Biol) (hrs)</th>
<th>Dose rads</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>66</td>
<td>0.7</td>
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<td>2</td>
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</tr>
<tr>
<td>4</td>
<td>3.8</td>
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<td>39</td>
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</tr>
<tr>
<td>6</td>
<td>2.6</td>
<td>13</td>
<td>0.6</td>
<td>5.2</td>
<td>20</td>
<td>1.9</td>
</tr>
<tr>
<td>7</td>
<td>3.8</td>
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<td>2.1</td>
<td>15</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>10.0</td>
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<td>3.8</td>
<td>11</td>
<td>1.1</td>
</tr>
<tr>
<td>9</td>
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<td>27</td>
<td>NE</td>
<td>NE</td>
<td>22</td>
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</tr>
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<td>7.0</td>
<td>32</td>
<td>NE</td>
<td>11.0</td>
<td>23</td>
<td>NE</td>
</tr>
</tbody>
</table>

Mean: 4.6 27 1.2 6.2 22 2.1

Ao is the estimated % of injected dose present in liver at time 0.
T(Biol), is the biological half life of the radionuclide in liver.
(hours), dose is the estimated radiation dose to liver from 1mCi of injected conjugate.
Figure 5. Dynamic acquisition of isotope in various organs.

5a - mIBG heart
5b - UJ13A heart
5c - mIBG liver
5d - UJ13A liver
5e - mIBG kidney
Estimates of maximal organ uptake were 4.7 for mIBG (Range 0.5-10%) and 6.2% for UJ13A (Range 1.5-11%). The mean radiation dose delivered to liver from these conjugates was estimated as 1.2 and 2.1 rads/mCi respectively (Table 2, see separate sheet).

DISCUSSION.

Whilst monoclonal antibodies and mIBG exploit relatively specific mechanisms for localising tumour deposits, additional specific and non-specific mechanisms operate in vivo which result in the conjugate accumulating in a variety of other tissues. The magnitude of these competing mechanisms limit both the bioavailability of conjugate for radiation targeting and set limits on size of the administered dose.

Bone marrow is the tissue most likely to limit escalation of the dose of radiolabelled conjugate. In the absence of tumour infiltration, this organ receives its principle dose delivery from blood. The results clearly show that a higher proportion of administered UJ13A remains in blood at any time point. It is likely that on a mCi for mCi basis UJ13A will be significantly more marrow toxic than mIBG. This hypothesis is supported by the whole body clearance data from marmosets, which suggests that whole body radiation dose is 3.8 times as great from 1mCi of UJ13A as from 1mCi of mIBG. The data is in accord with both our own observation of marrow toxicity after administration of 55mCi of 131-I UJ13A (Lashford L.S. et al. 1987) and observations from other investigators of marrow toxicity from 131-I mIBG at between 150 and 200mCi of injected conjugate (Hoefnagel C. & Voute T. 1986).

Further dose escalation is possible provided marrow rescue is available. It would seem likely that the next dose limiting tissue might be liver. The marmoset model demonstrates a clear advantage of mIBG over UJ13A. This advantage appears to be lost in our patients where no significant difference is observed in dose delivery between the two conjugates. Moreover in a larger series of patients scanned with either mIBG or UJ13A the trend is for mIBG to give a larger radiation dose.
Comparative Study of mIBG and UJ13A

The differences observed in marmosets are attributable to the higher uptake of UJ13A in liver and the more rapid clearance of mIBG. The difference in liver uptake is partly explained by the influence of splenic accumulation of UJ13A in patients, and the difficulty in making accurate measurements of total organ uptake in patients of varying sizes. Less easy to explain is the rapid clearance of mIBG from the liver of marmosets. Whilst it is possible that the difference between patients and animals reflects a physiological variation between lower and higher primates prior exposure to chemotherapy may also be an important factor.

Clearly, the factors that influence the metabolism of these compounds are complex. However, an understanding of the variables can only lead to a better appreciation of how to use these new delivery systems. At our current level of technology, mIBG is probably less likely to reach dose limiting toxicity than 131-I UJ13A. However, the potential to manipulate monoclonal antibodies by the preparation of fragments, and the possibility of using alternative isotopes, ensures that this technology remains an exciting possibility.

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