Strategies to Improve Cancer

Radioimmunotargeting

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ABSTRACT

Radioimmunotherapy (RIT) and radioimmunolocalisation (RIL) are developing and promising technologies to diagnose and treat tumours by use of radiolabelled antibodies targeting tumour specific antigens. The major reason why RIL and RIT not are efficient enough, is the comparatively low accumulation of radiolabelled antibodies in the tumours. Irrespective of the antigen - antibody system used, the maximal tumour uptake in humans is often limited to below 0.1 % of the total injected dose, with significant radionuclide remaining in the blood pool and extravascular fluid.

In the present thesis, the following putative improvement techniques for radioimmunotargeting have been evaluated in an experimental model using HeLa cell-xenografted nude mice: 1) Repetitive, simultaneous targeting of different antigens, 2) Removal of non-targeting antibodies using secondary antiidiotypic antibodies, 3) Preinjection of unlabelled antibody to remove shedded antigen and 4) Use of fractionated antibody administration. By use of multiple injections of mixtures of two different $^{131}$I-labelled monoclonal antibodies targeting placental alkaline phosphatase (H7) and cytokeratin 8 (TS1), respectively, a significant tumour growth inhibition compared to controls, was obtained. In the treated group, a negligible increase in tumour volume was seen compared to the control group, in which a 20-fold increase was observed. Quantitative determinations of volume densities of viable tumour cells, necrotic cells and connective tissue demonstrated no significant differences in the relative proportions between the groups, indicating that the irradiation caused decelerated growth. Using hybridoma technology, monoclonal antiidiotypic antibodies were generated against both TS1 and H7. The in vitro and in vivo effects of these antibodies, αH7 and αTS1, were investigated. Both these antiidiotypes were found to generate stable complexes with the radiolabelled idotype antibody, as revealed by gel-electrophoresis and autoradiography. Using biosensor technology (BIAcore, Pharmacia) the interactions were followed in real time and the association rate-, dissociation rate-, and affinity constants between the reactants were determined. In vivo, the antiidiotypes promoted a rapid dose dependent clearance of the $^{125}$I-labelled idiotypes with a decrease in total body radioactivity and concomitant dramatic increase in non-protein bound $^{125}$I excreted in the urine. The syngeneic monoclonal antiidiotypic antibody αTS1, was furthermore evaluated as a secondary clearing antibody at radioimmunolocalisation. Injection of αTS1 in a molar ratio of 0.5-0.75:1 to TS1, 24 hours after the $^{125}$I-labelled TS1 improved the tumour to normal tissue ratio 2-3 fold. This was due to a decreased level of total body radioactivity as well as a slight decrease in tumour-radioactivity. A model describing the kinetics of the involved components, i.e. the antigen, the idotype and the antiidiotypic was presented. It is concluded that high affinity monoclonal antiidiotypes can be used as tools to regulate the levels of idiotypic antibodies in vivo. This strategy, combined with preinjection of non-labelled idiotype antibodies, caused accumulated doses of 3 Gy to the tumour and 0.9 Gy to non tumour tissues as calculated for $^{125}$I-labelled antibodies (80 MBq/mg) by MIRD formalism based on repetitive quantitative radioimmunoscintigraphies. By approaching the maximal tolerated whole body radiation dose for mice (i.e. 6 Gy), it can be estimated that doses up to 20 Gy are possible to obtain following one single injection of labelled antibody. It was furthermore demonstrated that a single bolus injection of antibody is to be preferred, compared to exactly the same dose divided into three or ten fractions. Thus, not only the dose of radioactivity, but also the amount of antibody should be considered for fractionated RIT.

In summary, the thesis demonstrates that several techniques can be used to improve radioimmunolocalisation and to approach the proposed 70 Gy required to sterilise tumours at radioimmunotherapy.

Key words: cancer, monoclonal antibody, antiidiotypic antibody, radioimmunotargeting, placental alkaline phosphatase, cytokeratin, BIAcore.

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In summary, the thesis demonstrates that several techniques can be used to improve radioimmunolocalisation and to approach the proposed 70 Gy required to sterilise tumours at radioimmunotherapy.

Key words: cancer, monoclonal antibody, antiidiotypic antibody, radioimmunotargeting, placental alkaline phosphatase, cytokeratin, BIAcore.
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


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ABBREVIATIONS

Ab  Antibody
Ag  Antigen
BIA  Biomolecular interaction analysis
CDR  Complementarity determining region
CEA  Carcinoembryonic antigen
ELISA  Enzyme-linked immunosorbent assay
HAMA  Human anti-mouse antibodies
HCG  Human chorionic gonadotropin
I.D.  Injected dose
K_a  Affinity constant
k_a  Association-rate constant
k_d  Dissociation-rate constant
kDa  kilo Dalton
LESA  Liposome entrapped secondary antibodies
LET  Linear energy transfer
mAb  Monoclonal antibody
MIRD  Medical international radiation dose
PA  Primary antibodies
PLAP  Placental alkaline phosphatase
RES  Reticuloendothelial system
RIL  Radioimmunolocalisation
RIS  Radioimmunoscintigraphy
RIT  Radioimmunotherapy
SA  Secondary antibodies
T1/2  Half-life
TAA  Tumour associated antigen
TPA  Tissue polypeptide antigen
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1. INTRODUCTION

1.1 Historical Notes

The first attempt to treat cancer by use of antibodies dates back to the turn of the century. Already in 1895, Hericourt and Richet observed clinical responses by treating patients suffering from osteogenic sarcomas with xenoantisera prepared from donkeys or dogs (Hericourt and Richet 1895a, Hericourt and Richet 1895b). In 1906 Ehrlich (Ehrlich and Herter 1904) introduced immunotherapy, using specific molecules, as a potential new approach to reach pathological targets in vivo, and antibodies were later referred to as "magic bullets". These very early attempts were not successful in curing patients, and the properties, specificity and purity of the antibodies were unknown. Most importantly, however, a new principle was launched.

Despite these early reports, no real improvements were seen until the mid 1950s, when Pressman reported evidence for selective anti-tumour antibody accumulation in tumours, using purified radiolabelled polyclonal antibodies (Pressman 1953, Pressman and Kornfeld 1957). This led to anticipation of "magic bullets", and there was a period of renewed interest for antibody-based therapy of cancer, although the early investigators were careful in pointing out the many difficulties that had to be overcome. The delay in further development until the 1970s was to a great extent caused by the difficulty in finding a suitable animal model for studying the localisation of human tumours as well as due to problems to develop antibodies against human tumour antigens. In the early 1970s, human choriocarcinomas and colon carcinomas growing as xenografts in hamster cheek pouches, were targeted by radiolabelled antibodies against human chorionic gonadotropin (HCG), and carcinoembryonic antigen (CEA), respectively (Quinones et al. 1971, Primus et al. 1973, Goldenberg et al. 1974). In these experiments, which were the start of the modern "magic bullet" era, the localisation efficiency of affinity-purified specific antibodies was documented.

Perhaps the largest impact in the field of cancer radioimmunotargeting was made by Köhler and Milstein (Köhler and Milstein 1975), who in 1975 described the generation of monoclonal antibodies (mAbs) from hybridoma cell lines. The possibility to produce mAbs against defined tumour associated antigens is essential and compulsory for the large number of strategies investigated to improve and establish radioimmunotargeting as a cancer treatment modality today.
1.2 Oncofetal Antigens

Tumour-associated antigens (TAAs), or tumour markers, are molecules produced by, or associated with tumour cells which can be used as biochemical indicators of specific malignancies (For a review, see Sell 1990). TAAs are usually defined by the use of antibodies and are classified irrespectively of their biochemical properties. Oncofetal antigens are TAAs normally expressed during embryogenesis and present only in trace amounts in normal tissues. High amounts in adult tissues, however, may be a consequence of a malignant transformation. A large number of TAAs have been identified, some of which are found in several types of tumours. CEA is one of the most extensively studied TAAs, and is often expressed in colorectal or gastrointestinal cancers, but also in malignant tumours from the lung, liver, pancreas and breast (Gold and Freedman 1965, Hammarström et al. 1981, Hedin et al. 1982a, Hedin et al. 1982b). For a review of the use of tumour markers in Sweden and their properties, see Stigbrand 1992 (Stigbrand 1992).

The use of tumour markers as diagnostic tools for malignant diseases or as follow up parameters after treatment, rely on a more or less extensive shedding of these antigens into the circulation. As targets for antibody localisation, the shedding of TAAs and the heterogeneity in expression on the tumour cells represent aberrations which will be discussed later. Placental alkaline phosphatase and cytokeratins are the two TAAs which have been used in the present investigation.

1.2.1 Placental Alkaline Phosphatase (PLAP)

The family of human alkaline phosphatases consists of at least four different enzymes, i.e. the tissue non-specific, the intestinal, the placental and the germ cell alkaline phosphatases. The amino acid sequences have been deduced from the cDNA sequences of all four isozymes (Weiss et al. 1988, Henthorn et al. 1988, Berger et al 1987, Kam et al 1985, Millán 1986, Millán and Manes 1988). The dimeric placental isozyme, PLAP appears in several allelic forms, which can be attributed to the combination of several alleles (Beckman et al. 1966, Beckman and Beckman 1969, Donald and Robson 1974). Altogether, six common and at least 15 rare phenotypes can be recognised (Robson and Harris 1965, Robson and Harris 1967, Donald and Robson 1974). The molecular weight of PLAP dimer is 128 kDa. The highly stable dimer resists heat denaturation at 65°C (McComb et al. 1979). Moreover, PLAP is a plasma membrane protein anchored by a glycan-phosphatidylinositol (GPI) moiety. (Jemmerson and Low 1987, Howard et al. 1987, Low and Saltiel 1988, Ogata et al. 1988). PLAP lacking the GPI-tail after purification, is water soluble (Makiya 1992).
PLAP is normally expressed by the syncytiotrophoblasts in the placenta during pregnancy from the 12th week of gestation and increases steadily in serum concentration until parturition (Holmgren et al. 1978, De Groote et al. 1983). In healthy adults trace amounts can be detected in tissues such as testis, lung, liver and intestinal mucosa (Hirano et al. 1987), whereas smoking is known to induce falsely positive elevated PLAP serum levels (Fisken et al. 1989). The first time PLAP, i.e. the Regan isoenzyme, was observed as a tumour associated antigen was in 1968 by Fishman et al, who found increased levels of this isozyme in the circulation, of a patient with a broncogenic carcinoma (Fishman et al. 1968a). Later, similar results were obtained involving other carcinomas and these early observations contributed to establish the concept "oncofetal antigens" (Fishman et al. 1968a, Fishman et al. 1968b). Besides being studied as a TAA and a potential target for radioimmunotargeting, PLAP has also been extensively studied as a putative immunoglobulin-G (IgG) receptor involved in the placental IgG transport from the mother to the fetus during the pregnancy (Makiya et al. 1992, Makiya and Stigbrand 1992a, Makiya and Stigbrand 1992b). PLAP is used clinically as a tumour marker for seminomas (Lange et al. 1982, Hirano et al. 1987, Wahren et al. 1987).

1.2.2 Cytokeratins

The complex composition of morphologically distinct filaments in the cytoplasm of all eucaryotic cells includes essentially three types of filamentous structures, i.e. microfilaments, microtubules and intermediate filaments (Moll et al. 1982). The cytokeratins form a multigene family related biochemically to intermediate filaments and appear as an intracellular network of filaments. It has been shown that the cytokeratin filaments are directly connected to the plasma membrane, the nucleus and the cell organelles (including the endoplasmatic reticulum and cytoplasmatic vesicles) (Wedrychowski et al. 1986), which indirectly contributes to the structural integrity of cells. Using two-dimensional gel electrophoresis, at least twenty types of cytokeratin polypeptides can be identified (Moll et al. 1982, Sundström and Stigbrand 1994). Epithelial cells can be characterised by its content of cytokeratin polypeptides, since the expression pattern varies with the type of epithelium. In the case of malignant transformation of epithelial cells, the specific expression pattern of cytokeratins is generally preserved (Moll et al. 1982, Quinlan et al. 1985). Therefore cytokeratins can be used as tumour markers, especially in cases of tumours not being easily classified, such as undifferentiated metastases (Osborn and Weber 1982, Osborn and Weber 1986). The most abundant cytokeratins in carcinomas are cytokeratins 8, 18, and 19 which are found in simple epithelia (Moll et al. 1982) reflecting that most of these carcinomas are derived from this tissue type. Cytokeratins as putative tumour markers were initially described as tissue polypeptide antigen (TPA) (Björklund and Björklund 1957) in attempts to
find a common antigen for carcinomas. TPA has been suggested to be a cytokeratin-derivative or a degradation product of cytokeratins (Weber et al. 1984, Ochi et al. 1985, Mellerick et al. 1990). However, it is now regarded as a molecular complex containing cytokeratins 8, 18 and 19 (Sundström and Stigbrand 1994).

Like several other TAAs, cytokeratins may be released into the circulation, where they can be quantified using immunoassays (Björklund and Björklund 1957, Sundström and Stigbrand 1994). There are several immunoassays available for different types of cytokeratin. Increased serum levels of these antigens have been observed in patients with ovarian-, colonic- and pancreatic carcinomas (Ikarashi et al. 1985, Sundström and Stigbrand 1990).

1.2.3 The "Optimal" TAA for Radioimmunotargeting

An optimal tumour antigen for tumour targeting would ideally be tumour-specific, i.e. synthesised by malignant cells exclusively. Furthermore it should be expressed by all cells in large amounts with a homogeneous distribution within the tumour. No release to the circulation or shedding from the tumour is to be preferred. The property of internalising the targeted antibody may affect the residence time in the tumour. Moreover, highly immunogenic tumour antigens facilitate the production of high affinity antibodies which can be used as "magic bullets". Unfortunately, no tumour antigens are known which fulfil all these criteria. However, as discussed below, use of several target antigens combined with one or several improvement strategies may overcome the absence of a single "optimal" tumour antigen for radioimmunotargeting.

1.3 Experimental Models

There are major differences between experimental models and the clinical cancer complexity (Wessels 1990). The relevance of one chosen experimental model in comparison with a clinical situation must therefore be carefully considered. The two main groups of tumour experimental models for radioimmunotargeting are multicell spheroids and human tumour xenografts in mice.

1.3.1 Multicell Spheroids

Multicell spheroids are in vitro maintained clusters of non-vascularized cultured tumour cells which are used to simulate micrometastases during their avascular phase of
growth (Sutherland 1988, Bardiès et al. 1992, Lund-Johanssen et al. 1992). The concentration of oxygen and nutrients vary as a function of distance across the spheroid, similarly to a tumour growing in vivo (Knox 1995). This model system is of little use for studies of parameters such as pharmacokinetics of antibodies, potential radiation effects on the vasculature, tumour vascularity or tumour blood flow (Buchsbaum 1995), which are all factors that affect the tumour localisation in vivo. The potentially defensive role of normal host cells is also neglected in this model. On the other hand, the model can be useful to compare effects of different radionuclides, and furthermore to study in detail some parameters influencing the targeting of tumour cells, such as the relationship between antigen and antibody concentration, and antibody affinity. Along the same lines, Langmuir et al. have compared the targeting efficiency of two mAbs binding the same antigen but with different affinities (Langmuir et al. 1992), and Carlsson et al. have studied penetration and binding properties of different mAbs, using multicell spheroid models (Carlsson et al. 1989).

1.3.2 Human Tumour Xenografts

A variety of animal models and a large number of histologically distinct cancers have been used to study radioimmunotargeting in vivo. For reviews of animal models in general and of radioimmunotherapy (RIT), see Denekamp (Denekamp 1992) and Buchsbaum (Buchsbaum 1995), respectively. As compared to the multicell spheroids, animals xenografted with human tumours represent a model for studying RIT "one step closer" to the clinical reality. The major differences between animal models and patients consist of absence, or lower amounts, in animals of cross-reactive antigens in normal tissues which may react with mAbs (Buchsbaum 1995). Differences in the body volume and its effects on antibody distribution, the concentration of antibody, the extent of tumour load and tumour cell diversity also differ (Buchsbaum 1995). There may be differences in tumour cell cycle and tumour volume doubling times, which might affect radioresponsivness (Buchsbaum 1995). Moreover, host immune responses differ between animals and patients, especially when genetically immunodeficient nude or SCID mice are used as hosts, although minor immune responses can be detected also in these models (Stigbrand et al. 1996). Normal tissue toxicity and pharmacokinetics and immunogenicity of the antibodies also contribute to the differences between man and other species. There are, however, in many cases situations in which results achieved in experimental models are valuable also in a clinical context. Comparisons of the biodistribution between different antibody derivatives i.e., Fab’, (Fab’)2 and ScFv antibodies performed in animals show also clinically relevant. Moreover, studies on toxic effects of new radionuclides, targeting potential of antibodies, physiology of tumour uptake, use of radiosensitizers to protect the bone marrow, as well as dosimetric calculations are examples of studies in animals which may be clinically relevant. It is important to remember that the
concentration of radioactivity achieved in tumours (mCi/g) in animal studies is similar to that achieved in tumours in humans, as discussed by Buchsbaum (Buchsbaum 1995).

1.4 Antibodies

Immunoglobulins, or antibodies, are glycoproteins present in the serum and tissue fluids of all mammals. They are produced by plasma cells which develop from precursor B lymphocytes after encountering a foreign molecule, an antigen. Five classes of immunoglobulins can be identified in higher mammals, i.e. IgG, IgM, IgA, IgE and IgD. The IgG molecule, which is depicted in Fig. 1, may be considered as the typical and basic antibody structure.

Fig. 1. Ribbon presentation of a murine monoclonal antibody determined by X-ray analysis. The heavy chains are shown in yellow and blue, the light chains in red. The Fc stem of the molecule projects towards the viewer and assumes an asymmetric, oblique orientation with respect to the Fabs. Reprinted with permission from authors and Nature. From: Harris L., Larson S., Hasel K., Day J., Greenwood A. & McPherson A., 360, 369-372, 1992, Nature, Macmillan Magazines.
This molecule has two pairs of heavy and light chains, linked by inter-chain disulphide bonds. Both the light and the heavy chains comprise constant and variable regions. There are two antigen binding sites (Fab) per molecule, harbouring the complementarity-determining regions (CDRs) located in the variable regions. This part of the IgG molecule exposes a pronounced sequence variability, by which the diversity in binding properties is generated. The constant domains of an antibody, i.e. the Fc part, present variability as well; allotypic variations refer to genetic variation within one species with different alleles at a given locus, whereas isotypic variation refers to differences in the genome coding for the isotype of an antibody, i.e. the heavy chain subclass (Turner 1989). Also, there are regions within the constant part which differ between species. Antibodies may thus be referred to as syngeneic or xenogeneic, reflecting whether they are administered to the same or to another species, respectively, as compared to the host of production.

1.4.1 Monoclonal and Polyclonal Antibodies

The immune response in a host to an antigen normally includes both T-cell mediated- and humoral effector mechanisms. The antibody response is normally polyclonal, i.e. several independent plasma cells are synthesising antibodies to a defined structure of the antigen, the epitope. Antibodies, which bind to the same epitope, may expose similar binding surfaces but may not necessarily have the identical amino acid sequence.

In 1975, Köhler and Milstein described the procedure to generate mAbs using the hybridoma technology (Köhler and Milstein 1975), which is depicted in Fig. 2. By immunising rats or more commonly mice, an enrichment of antigen specific plasma cells can be generated. After reaching a significant polyclonal serological response in the host, nucleated cells from the spleen can be fused with a mouse myeloma cell line and cultured in a medium in which only hybrids, but not myeloma cells, survive. Spleen cells die spontaneously in culture media after some days. All wells containing growing hybridoma cells are tested for antibody specificity. Cells in positive wells are cloned. A hybridoma clone constitutes a group of cells, a clone, in which all cells are identical and produce the same antibody.
Fig. 2. Schematic presentation of the hybridoma technology according to the advent by Köhler and Milstein (Köhler and Milstein 1975). The antigen used for immunisation of the Balb/C mouse displays a number of immunogenic epitopes, which induce proliferation and IgG synthesis in different lymphocytes. By fusing such individual lymphocytes with myeloma cells, hybridoma cells can be generated with specificity against a single immunogenic epitope. From: Campbell A., Monoclonal antibody and immunosensor technology, 1991, Ed: P. van der Vliet, 1991. Reprinted with permission from Elsevier Science.

By immunising rats or more commonly mice, an enrichment of antigen specific plasma cells can be generated. After reaching a significant polyclonal serological response in the host, nucleated cells from the spleen can be fused with a mouse myeloma cell line and cultured in a medium in which only hybrids, but not myeloma cells, survive. Spleen cells die spontaneously in culture media after some days. All wells containing growing hybridoma cells are tested for antibody specificity. Cells in positive wells are cloned. A hybridoma clone constitutes a group of cells, a clone, in which all cells are identical and produce the same antibody.
Chemical cleavage of an intact immunoglobulin into fragments retaining the hypervariable regions, can be achieved by controlled proteolysis, although the conditions vary with immunoglobulin class, subclass and species (Liddell and Cryer 1991). Following the classical discovery by Porter (Porter 1959) that antibodies can be cleaved into Fab and Fc components, protocols using papain and pepsin to generate both bivalent F(ab')$_2$ and monovalent F(ab') antibodies are now available (Liddell and Cryer 1991). In Fig. 3, the principles involved in producing these fragments, as well as enzyme cleavage sites are depicted. The significance of using fragments in radioimmunotargeting is discussed below in the "General Discussion" section.

A more recently developed approach to produce antibodies, bypassing the hybridoma technology, is the phage display technique (Smith 1985, Chiang et al. 1989, Winter and Milstein 1991). Antibody genes can be derived and cloned directly from hybridomas, B-cells or gene libraries and by gene fusion be inserted into vectors which enable protein synthesis in bacteria or phages. Today, selection of antibodies with desired specificity and affinity can be made from libraries in a complete in vitro selection procedure (Winter and Milstein 1991, Malmqvist 1994, Begent et al. 1996). The specificity in binding diversity is dependent and limited by the library size. New methods to expand the library size and the number of potential antibodies have been demonstrated by in vivo recombination of the genes coding for the variable fragments of both the heavy and the light chains (Waterhouse et al. 1993). The phage display process using combinatorial libraries does not rely on the immunisation of animals to stimulate antigen specific cells (Begent et al. 1996). Utilising the highly conserved framework of the antibodies upon which the antigen binding parts, i.e. the CDRs fold, and by introducing computerised binding-site modelling, fine adjustments of the antigen binding sites can be accomplished (Winter and Milstein 1991).

1.4.2 Idiotypic and Antiidiotypic Antibodies

The sum of the unique epitopes within the variable regions of the heavy and the light chains, called the idiotopes, define an antibody’s idiotype (Kuby 1994). Antibodies which recognise unique idiotopes of other antibodies are defined as antiidiotypic antibodies (Fig. 4). Such antibodies may appear spontaneously as a response to the production of an idiotypic antibody (Kuby 1994); it should be kept in mind that primary idiotypic antibodies bear a mirror image of the antigenic epitope. The network hypothesis, which Jerne presented 1974 (Jerne 1974), predicts briefly, that the immunoglobulin expression might be regulated via a sequential cascade of such interactions between idiotypic and antiidiotypic antibodies. Manipulation of the immune system and its response using antiidiotypic reagents have been presented, and Jerne’s hypothesis has gained some support (Shoenfeld 1994).
Fig. 4. Jerne's Network Hypothesis. The antigen (Ag) seen to the left, can induce formation of an idiootypic antibody (Id). Against this idiootypic antibody, different antiidiotypic antibodies can be generated of which the upper one presents an antigen binding surface which compete with the original antigen, and can interact with the idiootypic antibody. From Taylor 1989, Reprinted with permission Gower Med. Publishing.

1.4.3 Anti-Antibodies, Recombinant- and Human Antibodies

Administration of foreign molecules to a host evokes immune responses, one of which is immunoglobulin synthesis. Endogenous antibody responses vary in magnitude and are related to factors such as immunogenicity and concentration of the antigen. As mentioned above, antibodies by themselves are immunogenic, particularly if they are polyclonal and xenogeneic. At radioimmunotargeting, such endogenous anti-antibody responses may interfere with the tumour localising antibody and appear following multiple injections as one of the major difficulties which has to be overcome. The induced antibody response may include anti-xenotypic, anti-isotypic, anti-allotypic, anti-metatypic and anti-idiotypic antibodies. The quantitative relationship between such populations of antibodies, varies most likely with the immunogenicity of the different epitopes in the individual antibodies. It seems feasible that anti-xenotypic responses dominate. The incidence of anti-antibody responses in patients have
been demonstrated to decrease with evolutionary proximity to the animal used for antibody production (Klein et al. 1995). By the discovery of the hybridoma technology numerous murine mAbs against a wide range of antigens have been produced. Such antibodies in humans inexorably evoke human anti-mouse antibodies, referred to as HAMA.

During the 1990s, recombinant techniques have been developed which enable the specific antigen binding sites, i.e. the variable regions of the CDRs of murine antibodies, to be transferred to the framework of human antibodies. In view of the difficulties of making human mAbs directly, mouse and rat antibodies have been "chimerized" by linking together murine variable and human constant regions (Winter and Milstein 1991). By grafting only the loops of the CDRs, the antigen binding sites alone could be transferred from the rodent to the human framework and "humanised" or "reshaped" antibodies can be made (Verhoeyen et al. 1988, Riechmann et al. 1988).

Recently, significant efforts have been devoted to develop human mAbs against TAA (Borrebaeck 1989, Hanna et al. 1991, Alonso 1991). The major problems have been to find sources of relevant activated B-cells, and to immortalise such B-cells into stable continuously growing cell lines (Klein et al. 1995). In vitro immunisation of B-lymphocytes obtained from normal peripheral blood has been accomplished with some success, but the most readily available source of B-cells has been found in patients immunised with their autologous tumour (Borrebaeck 1989, Klein et al. 1995). Although human mAbs have proved to possess lower immunogenicity in humans compared to either polyclonal, mouse monoclonal, "chimeric" or "humanised" antibodies, it must be kept in mind that endogenous anti-antibodies may nevertheless be raised. This indicates that "prospective" studies may be necessary to select antibodies with minimal CDR-immunogenicity from batteries of mAbs specific for a given epitope. Such procedures may be facilitated by computerised molecular modelling of the CDRs.

1.4.4 Affinity and Avidity of Antibodies

All antibody-antigen interactions are reversible. The affinity of a specific epitope for a single antigen-binding site of an antibody can be defined as the sum of the strength of all non-covalent interactions (Kuby 1994). The affinity constant \( K_A \), the association rate constant \( k_a \) ("on-reaction") and the dissociation rate constant \( k_d \) ("off-reaction") are all defined by the law of mass action:

\[
K_A = \frac{k_a}{k_d} = \frac{[\text{Ab} - \text{Ag}]}{[\text{Ab}] \times [\text{Ag}]}
\]

\( K_A \) is classically determined using Scatchard plots, based on several equilibrium dialyses with a constant antibody concentration but varying the concentration of the ligand, or
ELISAs (Friguet et al. 1985, Goldberg and Djavadi-Ohaniance 1993). The on- and off-rate constants describe the kinetic properties of a reaction, in contrast to the affinity constant which is a measure of the system at equilibrium (Malmquist 1994). Until recently, it has not been possible to perform or evaluate kinetic studies on antigen-antibody interactions. Furthermore, characterisation and comparisons of antibody reactivities and their kinetic properties have been difficult to obtain (Malmquist 1994). The biosensor based BIAcore™ system is a new methodology for performing kinetic analyses of antibodies in real-time (Karlsson et al. 1991, Malmquist 1993, Malmquist 1994).

The avidity of an antiserum is the strength by which multivalent antibodies bind to a multivalent antigen, and should be distinguished from the binding affinity between a single antigenic determinant and a single combining site (Steward 1989). As described above, IgG consists of two independent binding sites. Whether or not both these sites can be used simultaneously varies most likely between antibodies and should be dependent on parameters related to the antigen, such as density and sterical conformation. In the case of bivalent interactions, the affinity is considerably higher than the sum of the two monovalent affinities (Steward 1989).

1.5 Radiobiology of Radioimmunotargeting

Ionizing radiation causes detrimental molecular lesions in target molecules directly by primary ionization’s or indirectly by attacks from radical anions or cations derived from ionisation of water (Denekamp 1989, Hall 1993). One of the biological consequences may be chromosomal aberrations. Several types of DNA lesions are induced by ionizing radiation, including single strand breaks, various types of base damage and DNA-protein cross links. Perhaps the most important lesion is the double strand breaks (Denekamp 1989, Ahnström 1989). Although there is a repair capacity for double strand breaks, the repairs may not always be complete. Persisting lesions will then cause cell death and tissue malfunction.

In radioimmuno-targeted imaging of cancer, the choice of radionuclide is dependent upon its ability to be detected, and not to the biological effects they may exert. For therapeutic purposes, however, the entire concept relies on the molecular and biological damages induced by the ionizing radiation. As with radiotherapy, it is important to determine the dose absorbed by tissues for treatment planning and assessment of results (Fisher 1995). Irrespective of the biological factors associated with the antibodies, such as kinetics, tumour uptake, tumour retention and antibody internalization, RIT in contrast to conventional radiation therapy, is a continuously decreasing low-dose rate radiation treatment modality (Brady et al. 1988, Fowler 1990, Buchsbaum and Wessels 1995). The limits of normal tissue tolerance and the dose required to kill tumour cells using the low-dose rate radiation of beta-emitting radionuclides are not completely known (Breitz 1993). It has been calculated that the
decreasing low dose rate radiation of RIT is about 20% less effective than external beam therapy of 2 Gy fractions (Fowler 1990). A tumour that would require 60-70 Gy to be sterilised using conventional radiotherapy of 2 Gy fractions, would thus require 72-84 Gy at RIT. The maximal deliverable tumour doses by RIT have until recently been approximately 0.4 to 0.5 Gy/h during some hours for short-lived radionuclides (Williams et al. 1995). At such dose rates, only little killing can be expected from the β-component of cell killing and the major part of the sublethal damage may be repaired, particularly in normal tissues having a small α/β-ratio (Williams et al. 1995). In the last decade the analysis of radiation damage has involved modelling that contains two modes of cell kill: the LQ or linear quadratic model. It contains two components, one linear with dose and irreparable, the α term, and one which is quadratic with dose and is reparable with fractionation or low dose rate, the β term. The change in response to fractionation is defined by the α/β-ratio, which is large for tumours and acutely responding tissues and small for late reacting tissues (Hall 1993). However, it has recently been observed experimentally that single or multiple doses lower than 1 Gy cause more cell killing than observed for higher dose exposure (Joiner and Johns 1988). This indicates a high-dose-inducible repair process that requires doses of 0.5-1.0 Gy for induction. Additionally, continuous irradiation at very low dose-rates, < 0.05 Gy/h, during several days sensitize many cell lines to subsequent high-dose irradiation (Williams et al. 1992). Therefore, pre-treatment of low-dose rate RIT prior to conventional radiotherapy may be an advantage.

It has been suggested that at certain dose-rates, that a block in the more radiation sensitive G2 phase of the cell cycle may occur (Langmuir et al. 1993, Buchsbaum and Wessels 1995). Such a G2 block, may be produced by exponentially decreasing dose rate, which is the case at RIT, causes larger radiation-induced cell killing (Van Oostrum et al. 1990). Reoxygenation of hypoxic cells is crucial for the success of radiation induced cell killing. Cells lacking oxygen require 2.5-3 times as much radiation to be killed. A course of RIT may last for several weeks, which may be accompanied by an incomplete reoxygenation of hypoxic cells (Nylen et al. 1989), although rapid reoxygenation has been reported even during continuous irradiation (Kal 1972). As discussed by Langmuir et al. (Langmuir et al. 1993), hypoxic cell sensitizers should be useful with RIT, since the cells prone to be hypoxic are also those which receive a low radiation dose from RIT because of their distance from the blood vessels.

In summary, the tumours which are most likely to respond to RIT are those which are inherently radiosensitive, having a poor capacity to repair radiation damages or with long repair half-times. Such RIT-sensitive tumours also comprise those susceptible to blockade in sensitive phases of the cell cycle, express relevant antigen homogenously and reoxygenate rapidly (Langmuir et al. 1993).
1.6 Radionuclides

Labelling of antibodies with radioisotopes is crucial for antibody mediated imaging and treatment of tumours. The selection of the optimal radionuclide for cancer targeting may primarily be based on the required energy necessary for imaging or therapy. Moreover, a large number of factors involving the nuclide's availability, cost, labelling-chemistry, decay properties and relative biological effectiveness have also to be considered. Also, several biological parameters such as antibody kinetics, the tumour type, size, location as well as antigen properties, location, and expression should influence the choice of radionuclide.

Table 1 lists some radionuclides for imaging and therapy, including their physical properties (Volkert et al. 1991, Brady et al. 1988 In: Radiobiology in Radiotherapy.).

### Table 1. Examples of potential radionuclides for imaging and therapy with monoclonal antibodies.

I. For imaging

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Half-life (T1/2)</th>
<th>Energy (keV)</th>
<th>Decay Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>^{99m}Tc</td>
<td>6.0 h</td>
<td>141</td>
<td>γ</td>
</tr>
<tr>
<td>^{111}In</td>
<td>2.8 d</td>
<td>171</td>
<td>β, γ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>245</td>
<td></td>
</tr>
<tr>
<td>^{123}I</td>
<td>13.3 h</td>
<td>159</td>
<td>EC, γ</td>
</tr>
<tr>
<td>^{125}I</td>
<td>60.1 d</td>
<td>28</td>
<td>EC, γ</td>
</tr>
<tr>
<td>^{131}I</td>
<td>8.0 d</td>
<td>284</td>
<td>β, γ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>364</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>637</td>
<td></td>
</tr>
</tbody>
</table>
II. For therapy

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Half-life (T1/2)</th>
<th>Max.-Energy (MeV)</th>
<th>Decay Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{89}$Sr</td>
<td>53 d</td>
<td>1.46</td>
<td>$\beta$</td>
</tr>
<tr>
<td>$^{90}$Y</td>
<td>64.1 h</td>
<td>2.27</td>
<td>$\beta$</td>
</tr>
<tr>
<td>$^{131}$I</td>
<td>8.0 d</td>
<td>0.81</td>
<td>$\beta, \gamma$</td>
</tr>
<tr>
<td>$^{186}$Re</td>
<td>3.7 d</td>
<td>1.1</td>
<td>$\beta, \gamma$</td>
</tr>
<tr>
<td>$^{188}$Re</td>
<td>17 h</td>
<td>2.1</td>
<td>$\beta, \gamma$</td>
</tr>
<tr>
<td>$^{211}$At</td>
<td>7.2 h</td>
<td>6.8</td>
<td>$\alpha, EC$</td>
</tr>
<tr>
<td>$^{212}$Bi</td>
<td>1.0 h</td>
<td>7.8</td>
<td>$\alpha, \beta, \gamma$</td>
</tr>
</tbody>
</table>

The Max.-Energy refers to the $\beta$-component and not to the $\gamma$-rays energies, which not are listed here.

For the $\alpha$-emitters $^{212}$Bi and $^{211}$At, the mean $\alpha$-particle energy is listed.

$d=$days, $h=$hours

It is important to obtain a high tumour to whole body dose which results in low toxicity to dose-limiting tissues such as the bone marrow (Buchsbaum and Wessels 1995). It has been demonstrated both experimentally and clinically that the tumour content and retention time of radiometal-labelled antibodies is higher than for radioiodinated antibodies (Rainsbury and Westwood 1982, Buchsbaum and Lawrence 1990, Buchsbaum and Lawrence 1991). $\alpha$-emitters such as $^{211}$At possess a high linear energy transfer (LET), which cause a larger radiobiological effectiveness compared to low LET photon- and $\beta$-emitters (Hall 1993). Furthermore, the $\alpha$-emitters are short-ranged and the effects are mostly direct, which limits the repair capacity to the DNA of the cells (Langmuir et al. 1993, Buchsbaum and Wessels 1995). Rapidly accessible cancer cells such as leukaemia cells in the circulation and micrometastases elsewhere, which may be targeted homogenously, would therefore preferably be treated with $\alpha$-emitters. $\beta$-emitters on the other hand can, with their long-range emission, be useful to treat solid tumours with a heterogenous target-antigen expression (Buchsbaum and Wessels 1995).
2. AIMS OF THIS STUDY

* Can repetitive in vivo injections of a mixture of $^{131}$I-labelled mAbs against the tumour associated antigens cytokeratin and PLAP cause a significant tumour growth inhibition?

* Can high affinity antiidiotypic mAbs be generated and used as tools to regulate serum levels of antibodies? Which are the characteristics of these idiotypic-antiidiotypic interactions in vitro and in vivo?

* Which are the in vivo effects of the antiidiotypic mAb $\alpha$TS1 when used as a secondary clearing antibody at experimental radioimmunolocalisation?

* Which tumour dose and tumour to non-tumour dose ratio can be obtained using the $\alpha$TS1 mAb as clearing antibody combined with pre-injection of unlabelled TS1 at experimental radioimmunolocalisation with $^{125}$I-labelled TS1?

* Are single-, oligo-, or poly-injections of the $^{125}$I-labelled TS1 mAb using the same total amount of antibody to be preferred, with respect to tumour doses and tumour to non-tumour dose ratios at experimental radioimmunolocalisation?
3. RESULTS

**Paper I**: Radioimmunotherapy of HeLa cell tumors in nude mice by a combination of iodine-131-labelled monoclonal antibodies against placental alkaline phosphatase and cytokeratin

*Tumor Targeting, 1, 239-244, 1995.*

In this paper we have evaluated one strategy with the potential to improve radioimmunotherapy, i.e. simultaneous targeting of two different antigens using multiple injections of two $^{131}$I-labelled monoclonal antibodies. The two target antigens, placental alkaline phosphatase (PLAP) and cytokeratin 8, represent two conceptually different antigens. PLAP is a membrane bound antigen on the cell surface, while cytokeratin 8 constitutes part of the intracellular cytoskeleton and is immunochemically available only in damaged or necrotic cells.

During the entire observation period (3-months), untreated control animals demonstrated a 20 fold increase in tumour volume. In one experimental group, which received a single injection of an unlabelled mixture of these two antibodies, a slightly higher growth rate was observed during the initial 35 days. Following the first injection of the $^{131}$I-labelled mAb mixture (13 MBq), there was a small reduction in growth rate. However, this reduction, or delay in growth rate, lasted only for approximately 7 days. In the group receiving totally 4 injections of the labelled mAb mixture, the same pattern was seen after the second injection of 13 MBq at day 10. Following the third injection of 18.5 MBq of the mAb mixture at day 22, an even more pronounced growth delay was seen compared to the other groups. As a result of the fourth and last injection of 18.5 MBq at day 42, the tumour volumes in this group decreased during the rest of the experimental period.

The dissected tumours presented a morphology which was virtually the same in the different experimental groups. The necrotic volume densities were considerable in all groups irrespective of treatment. Although the relative amounts of viable cells and connective tissue were slightly higher in the group which received the multiple injections of the labelled mAb mixture, no differences were statistically significant. This indicates that the low dose rate radiation following the multiple injections of the $^{131}$I-labelled mAb mixtures do not necessarily cause changes in the relative amounts of viable tumour cells, connective tissue or necrotic areas. The morphology of the tumours and the high relative proportion of necrotic regions may be a result of the experimental model itself and may imply involvement of the angiogenesis within the tumour, whereas the radiation caused a deceleration of growth. The
paper demonstrates that significant tumour growth retardation can be achieved by repetitive
injections of a mixture of $^{131}$I-labelled anti-PLAP and anti-cytokeratin antibodies in a nude
mice experimental model.
Paper II: In vivo and in vitro interactions between idiotypic and antiidiotypic monoclonal antibodies against placental alkaline phosphatase


This study was designed to generate a high affinity mAb against an anti-PLAP mAb H7 and to characterise this idiotypic-antiidiotypic interaction in vitro and in vivo.

By use of the hybridoma technology, we obtained one clone which in the inhibition-ELISA test system caused an inhibition similar to that caused by the polyclonal control serum taken from the mouse providing the spleen for the fusion. This clone produced, after recloning and ascites production, the mAb αH7 of the isotype IgG1× and with an average pl of 8.0.

The binding patterns of H7, αH7 and PLAP could be displayed by gel-electrophoresis. H7 was found to generate stable complexes with PLAP as well as with αH7, but as expected, αH7 and PLAP did not interact at all. A mixture of H7, αH7 and PLAP appeared to give a PLAP/H7 complex, indicating a higher affinity between H7 and PLAP, than between H7 and αH7. These interactions were also followed in real-time using biosensor technology (BIAcore, Pharmacia) and the association-rate (kₐ), dissociation-rate (kₐ) and affinity constants (Kₐ) were calculated. The affinity between H7 and PLAP (6.7 × 10⁹) was approximately 2.1 times higher than between H7 and αH7 (3.2 × 10⁹), confirming the electrophoretic results. This was due to a slightly faster kₐ and a somewhat slower kₐ.

In vivo, αH7 promoted a rapid clearance of the labelled H7. A dose relationship was documented; injection of only the labelled ¹²⁵I-H7 resulted in T¹/₂ of approximately 10 days. Injection of αH7 in molar ratio of 1:10 to H7 caused an initial small rapid decrease of H7, and in a ratio of 1:2 a pronounced rapid decrease was observed. Approximately 48 hours after the αH7 injection the curves were parallel to the curve of the non αH7 treated group. In a similar experiment, serum and urine analyses using the αH7 in a ratio 1:2 to H7 revealed that 32 hours after the αH7 injection serum radioactivity was decreased to on average 28 %, whereas mice receiving only ¹²⁵I-H7 retained 86 % of their serum radioactivity. This decrease in serum radioactivity was followed by a concomitant rapid increase in low-molecular weight bound-¹²⁵I (not possible to retain on gels), excreted into the urine.

It was concluded that high affinity antiidiotypic mAbs would be useful tools to regulate the titre of idiotypic antibodies in vivo.
Paper III: Use of anticytokeratin monoclonal anti-idiotypic antibodies to improve tumor:nontumor ratio in experimental radioimmunolocalization


A high affinity antiidiotypic mAb was raised against the anticytokeratin mAb TS1 and used as a secondary clearing antibody at experimental radioimmunolocalisation.

The hybridoma technology was used to generate this antiidiotypic. One single clone was obtained, which caused significant inhibition compared to the positive control serum in the developed ELISA screening system. After recloning, ascites production and purification on Protein A-Sepharose, an IgG1,κ mAb called αTS1 was obtained. This antiidotypic mAb, with an average pI of 7.4 was further characterised using the sensitive biosensor-based technology BIAcore™. The TS1-αTS1 interaction, was found to be of high-affinity. The affinity constant, as high as 8.6 x 10^{10} M^{-1}, was a result of a very slow dissociation-rate constant of 2.6 x 10^{-6} s^{-1} and an association-rate constant of 2.2 x 10^{5} s^{-1} x M^{-1}. In non-denaturing polyacrylamide gel-electrophoresis, high molecular weight complexes were formed, with consumption of both TS1 and αTS1.

In vivo, the injection of αTS1 in Balb/C mice (without tumours) in molar ratio of 0.75:1 (αTS1/TS1) promoted a rapid decrease in total body radioactivity, resulting in a retained activity of approximately 20%, compared to controls not receiving αTS1. A similar experiment was set up to analyse the changes of blood and urine radioactivity after αTS1 treatment. Using a ratio of 0.5:1 (αTS1/TS1), autoradiographic comparisons of serum samples between mice receiving αTS1 and mice receiving only 125I-labelled TS1 revealed that mice treated with the antiidiotypic cleared 125I-labelled TS1. Urine samples from such mice demonstrated that the clearance of 125I-labelled TS1 was followed by a concomitant rapid increase in 125I (or 125I-Tyrosine) excreted into the urine. This high urine content of 125I was absent in mice receiving only the 125I-labelled TS1. In a final in vivo experiment, the use of αTS1 as secondary clearing antibody was evaluated at experimental radioimmunolocalisation in nude mice xenografted with HeLa Hep2 cell tumours. Following injection of αTS1 to TS1 (0.5:1), a clear demarcation of tumour to non tumour tissues was observed upon a scintigraphy 48 hours after the αTS1 injection. Relative 125I levels/g tissue of ten different organs were measured after the scintigraphies. Approximately 60-65% of the activity in the tumours and 12-45% of the activity in other organs remained, as compared to the group receiving the 125I-labelled TS1 only, increasing the tumour to non tumour ratio at least 2-3 fold.
It is concluded that a rapid and controllable decrease of non-tumour targeting $^{125}$I-labelled anti-tumour mAbs can be achieved using syngeneic a high affinity secondary antiidiotypic mAb. This strategy increases the diagnostic window at radioimmunolocalisation and reduces toxicity to radiosensitive normal tissues at radioimmunotherapy, and furthermore leaves the host with an intact endogenous antibody repertoire.
Paper IV: Experimental radioimmunolocalization combining non-labeled, $^{125}$I-labeled and antiidiotypic anticytokeratin monoclonal antibodies - a dosimetric evaluation

Submitted to Cancer

A dosimetric evaluation of a combination of two strategies to render radioimmunotargeting more efficient i.e., the use of a preinjection of unlabelled TS1 mAbs prior to the $^{125}$I-labelled TS1 and the use of an antiidiotypic mAb to clear the circulation from non-targeted $^{125}$I-labelled TS1, was performed in this paper.

All experimental groups presented progressively increasing tumour to non-tumour values, reaching their highest values in the tenth week of observation. The increase appeared simultaneously with non-tumour tissue levels of $^{125}$I-labelled TS1 approaching zero. In the experimental group receiving $^{125}$I-TS1 (80 MBq/mg), a high accumulation of approximately 5% injected dose (% I.D.)/g persisted during the three initial weeks. This yielded a total absorbed dose to the tumour of 3.9 Gy, most of which was delivered during the first month, whereas the whole body dose was calculated to 1.3 Gy. Using an additional injection of αTS1 an expected rapid decrease in the accumulation in non-tumour tissues, i.e. from 4 to 2% I.D./g was observed. During the entire experimental period, the total dose absorbed by the tumour was calculated to be 2.0 Gy and by non-tumour tissues to be 0.9 Gy, for this group. A preinjection of unlabelled TS1, but without αTS1, resulted in absorbed doses by normal tissues and by the tumour of 1.7 and 5.2 Gy respectively. By combining the preinjection of unlabelled TS1 with the αTS1, the highest absorbed dose ratio between tumour: non tumour tissues was observed, i.e. 3.3.

The total tumour uptake was, as expected, highest in the groups not receiving the secondary clearing αTS1 mAb reaching 2% of the I.D.

It can be concluded that the concentration and total amount of labelled anti-tumour antibody administered had a significant impact on the total tumour uptake. By combining a clearance strategy of non-tumour targeting mAbs and a strategy to increase the relative accumulating proportion of administrated anti-tumour mAb, it is possible to reach higher accumulated dose ratios between tumour and normal tissues than using either of these strategies alone.
This paper is a review of the "secondary antibody clearing concept" to improve tumour to non-tumour ratio at radioimmunotargeting. A model describing the kinetics of the involved components (i.e., the antigen, the primary and secondary antibodies) is presented. It is concluded that the use of secondary antibodies as tools to clear non-tumour targeting primary antibodies over the past years have developed in the direction of using more specific, less immunogenic antibodies of higher affinity. Such antibodies should typically be syngeneic and monoclonal. Well characterised high affinity idiotypic-antiidiotypic antibody interactions as primary and secondary antibodies enable smaller doses of antibody to be used and give a more controlled clearance. The use of new technologies, such as the BIAcore™, to analyse kinetic constants in vitro, i.e. association-, dissociation-rate and affinity constants of primary and secondary antibody interactions has the potential to significantly improve the "secondary antibody clearing concept".
In this paper we have evaluated the dosimetry at experimental radioimmunolocalisation in HeLa Hep 2 cell xenografted nude mice, using different administration schedules, but the same total protein dose of $^{125}$I-labelled TS1. Three experimental groups were analysed, i.e., one group receiving a single bolus dose of 100 µg TS1 (22.2 MBq), a second receiving three (nine days apart) consecutive injections of 33 µg TS1 (7.4 MBq) and the third ten consecutive injections (three days apart) of 10 µg TS1 (2.22 MBq). The interfraction interval for the latter group, was based on previously determined pharmacokinetic data (T1/2) for this mAb such that a comparatively low, but persisting serum antibody level could be obtained. It could be demonstrated that the observed pattern of the whole body retention curves were in agreement with theoretically calculated predictions and a new T1/2 (8.6 days) for this mAb was determined. A significantly higher tumour growth rate was seen in the group receiving a single bolus injection, compared to the group receiving 10 injections as long as additional injections were administrated. After the last injection in the latter group, the tumour growth-rates were similar in all groups. The tumour uptake increased faster and reached a higher peak value (1.65 % I.D.) in the group receiving a single bolus injection compared to the other groups, in particular the group receiving 10 injections, in which a slower tumour uptake was seen with a 10-fold lower peak value (0.17 % I.D.). When the tumour uptake was related to the tumour weight (% I.D./g), the differences between the groups were smaller. Doses were calculated according to the MIRD-formalism and based on approximately 700 repetitive quantitative radioimmunoscintigraphies. Following a single bolus dose of 100 µg TS1 (22.2 MBq) the highest tumour dose was delivered i.e. as high as 17 Gy, whereas the tumour doses to the other groups were 6 Gy (three injections) and 3 Gy (ten injections). The high tumour dose following the single bolus injection was accompanied by higher tumour to non tumour dose ratios compared to the other groups during the entire observation period, reaching approximately 5, at the end of the experiment. Interestingly, this high bolus furthermore caused a significantly faster tumour growth compared to the group receiving 10 injections. Unambiguously, the present report demonstrates, within this target antigen system, that the protein amount per fraction affects the tumour uptake, tumour dose, and tumour to non-tumour dose ratios. In summary, this study demonstrates that both the protein amount and the radioactivity dose per fraction should be considered for optimal fractionated radioimmunotherapy.
4. GENERAL DISCUSSION

At radioimmunoscintigraphy (RIS), there is a need to rapidly achieve a high tumour to background ratio, whereas at RIT, an additional high persisting total tumour uptake of antibody is crucial. Several suggestions have been made to improve the efficacy of RIS and RIT. The results of this thesis will be discussed in relation to similar strategies under the following subheadings- "How to reduce background radioactivity and associated toxicity at radioimmunotargeting" and "How to improve tumour localisation".

5.1 How to Reduce Background Radioactivity and Associated Toxicity at Radioimmunotargeting

One of the major obstacles of radioimmunotargeting is the relatively low proportion of antibodies localising to the tumour. Considering passive diffusion of antibodies through the endothelium of the vessels to TAAs in the tumour as the mechanism for specific localisation, this may not be surprising (Begent 1985). In experimental xenograft systems, as much as 10% of the injected antibody can be tumour targeted whereas in man, two orders of magnitude lower can be obtained. (Mach et al. 1980, Begent 1985). This high background activity, caused by circulating non tumour-targeted antibodies reduces the imaging window and causes detrimental effects on radiosensitive organs such as the intestine and the bone marrow during therapy.

For imaging purposes, a subtraction procedure was developed in which human serum albumin was labelled with $^{99m}$Tc and the specific antibody with $^{131}$I (Goldenberg et al. 1978). By subtracting the $^{99m}$Tc image from that of $^{131}$I, the specific tumour localisation could be demonstrated as residual activity (Goldenberg et al. 1978). Although several tumours were detected when conventional radiology was negative, the subtraction method suffered from a number of disadvantages related to the different energies of radionuclides used. Subsequently, methods to reduce background radioactivity were developed. The use of secondary antibodies (SA) as clearing agents for circulating non-tumour targeted anti-tumour antibodies was initially proposed by Spar et al. (Spar et al. 1964). The SA bind to the anti-tumour antibodies forming immune complexes, which are rapidly taken up by the reticuloendothelial system (RES) and degraded, followed by rapid excretion of the free isotope into the urine as depicted in Fig. 1 in Paper V.

In the beginning of the 1980s Barratt et al., used liposome-entrapped SA (LESA), leaving the variable domains of the antibody available at the surface to bind the primary anti-tumour antibodies (PA) (Barratt et al. 1983). Using such a technique, tumour to background ratios were improved several fold experimentally and imaging was achieved with
LESA directed against goat antibody to CEA in patients with gastrointestinal cancer (Begent et al. 1982, Barratt et al. 1983). Subsequently, several investigators using both polyclonal xenogeneic PA and polyclonal xenogeneic SA, demonstrated that free SA without liposomal entrapment were equally efficient for clearance, and that smaller doses could be used (Bradwell et al. 1983, Sharkey et al. 1984, Goodwin et al. 1984). The approach of using free secondary antibodies as clearing agents has developed in the direction of using more specific and less immunogenic antibodies, enabling smaller doses of antibody to be used (Paper II, III, IV and V) (Begent et al. 1987, Goldenberg et al. 1987, Pedley et al. 1989, Sharkey et al. 1992, Pimm and Gribben 1992, Marshall et al. 1994). In Paper III, we accomplish improved tumour to non-tumour ratios by a factor of 2-3, i.e. similar to Sharkey et al. (Sharkey et al 1992). In both these investigations, completely syngeneic monoclonal primary and secondary antibodies were used, but the target antigens and PA/SA ratios were different. Whereas Sharkey et al. used a ratio of 1:100 (PA/SA), we used 1:0.5 which have implications for multiple cycle treatment. The smaller the antibody doses administered, the smaller the endogenous anti-antibody responses that should appear. It may be possible to reach a higher tumour to non-tumour ratio in our system, as no systematic evaluation of the optimal PA/SA ratio was performed in this investigation. By the use of extracorporeal clearance utilising the same concept with anti-antibody clearance in antibody columns, tumour to non-tumour ratios have been improved several fold (Lear et al. 1991, Norrgren et al. 1993, Norrgren et al. 1994, Garkavij et al. 1995). This technique, however, is more complicated and needs invasive surgery.

Another method to improve clearance is the use of biotinylated anti-tumour antibodies which can be cleared via administration of avidin or streptavidin. This approach takes advantage of the very high affinity in the avidin-biotin interaction \((10^{15} \text{ M}^{-1})\) and the rapid clearance of the avidin-biotin complexes and is therefore attractive. The avidin/biotin concept has been used in two- and three-step treatment regimes (Paganelli et al. 1991, Paganelli et al. 1992). In the latter, biotinylated non-labelled anti-tumour antibodies are administered, followed by non-labelled avidin which bind to the tumour targeted biotinylated antibody and complexes, circulating non-targeting antibodies which are cleared. Finally, labelled biotin which can localise to the avidin on the tumour is administrated. In order to find a general extracorporeal clearance strategy, biotinylated antibodies have also been cleared via adsorption columns containing avidin (Norrgren et al. 1993). A comparison of the clearance efficiency of primary anti-tumour antibodies using either streptavidin or second antibodies have been performed by Marshall et al. (Marshall et al. 1994). The second antibody was superior to the streptavidin as the clearance of blood pool activity was faster and the tumour to blood ratio was higher (Marshall et al. 1994). A rapid and fast clearance of primary anti-tumour antibody has been reported using galactosylated avidin (Marshall et al. 1995). By such a technique, the clearance of complexes did not involve the reticuloendothelial system, but was altered to the liver-asialoglycoprotein receptor (Marshall et al. 1995).
A more direct way of decreasing toxicity associated with non-targeting antibodies is the use of antibody fragments. Generally, antibody fragments (F(ab’), and F(ab’2)) display a more rapid blood clearance, greater kidney uptake, less tumour uptake and shorter biological half-life in tumours and lower affinity in vitro, compared to intact IgG (Sharkey et al. 1990, Buchsbaum et al. 1993, Ullén 1996 Unpubl. Results). The two reasons suggested for the faster blood clearance are the lack of Fc-portion of the molecule, which reduces the Fc-mediated binding within RES and the smaller size, which facilitates diffusion and results in a higher permeability coefficient (Blumenthal et al. 1995a). Better tumour to non-tumour ratios and less toxicity using F(ab’)2 fragments compared to intact antibodies have been reported both experimentally and clinically (Buchegger et al. 1990, Sharkey et al. 1991, Pedley et al. 1993, Lane et al. 1994). Also, longer tumour remissions have been reported using 131I-labelled F(ab’)2 fragments compared to their intact antibodies (Buchegger et al. 1990). However, equal efficiency and superiority of intact antibodies over F(ab’)2 fragments have been reported as well (Sutherland et al. 1987, Blumenthal et al. 1992a, Blumenthal et al. 1995a). Whether or not fragmented antibodies or intact antibodies are preferable for radioimmunotherapy is still not completely clear. The smaller size allows a more rapid penetration and even distribution within the tumour compared to intact antibodies. The lack of the Fc-part, and the smaller size of the fragments result in a rapid but non-controllable clearance of non-targeting antibodies. This causes increased tumour to non tumour ratios, but generally at the expense of lower tumour accumulation. It appears that the tumour type, size and perhaps most importantly, the individual antibody will influence the therapeutic outcome (Blumenthal et al. 1995a). If the two conceptually different techniques for clearance of non-targeting antibodies are compared i.e., SA and antibody fragments, it can be stated that the use of SA may provide a time-controllable and dose dependent (Paper II) clearance compared to fragments, but at the expense of more protein.

Multiple injections of radiolabelled antibody, i.e. fractionated radioimmunotherapy, have been tested as an approach to increase the therapeutic gain by reducing toxic side effects in normal tissues compared to adjacent tumours (Meredith et al. 1992). Increased survival with persisting anti-tumour effects, by dividing a single injection into three with the same or higher total radiation dose have been demonstrated by Schlom et al. (Schlom et al. 1990). We can demonstrate, however, that three or ten fractions (nine and three days apart, respectively) using the same total amounts of antibody (100 µg) and radioactivity (22.2 MBq) cause significantly lower tumour uptake (% I.D.), tumour dose and tumour to non-tumour ratio than a single bolus injection targeting cytokeratins with the TS1 mAb (Paper VI). Thus, an optimal fractionated RIT-schedule requires in our opinion, that the administered radiation dose per fraction, taken together with the total number of fractions, is adjusted to the maximally tolerated whole body dose, but also, and most importantly, that the
amount of antibody per fraction is adjusted to yield maximal tumour uptake and tumour to non-tumour ratios.

Other methods to reduce normal tissue toxicity, i.e. the myelosuppressive toxicity, and to enable higher potential radiation doses to be delivered, includes the use of colony stimulating factors and bone marrow transplantation (Morton et al. 1990, Blumenthal et al. 1992b, Blumenthal et al. 1994, Blumenthal et al. 1995b). Morton et al. reported increased survival giving up to twice as high doses (250 μCi ⁹⁰Y-labelled anti-CEA antibody) when bone marrow transplantation was undertaken (Morton et al. 1990). By using the lymphokines IL-1 and GM-CSF, Blumenthal et al. have demonstrated that ¹³¹I-labelled antibody-induced decline in circulating white blood cells could be prevented in a hamster experimental model (Blumenthal et al. 1992b). By combining these myeloprotective strategies, i.e. syngeneic bone marrow transplantation and administration of IL-1 and GM-CSF, the same group have recently demonstrated greater effect on the number of peripheral white blood cells, than either strategy could reach alone (Blumenthal 1995b).

5.2 How to Improve Tumour Localisation

Antibodies do not penetrate uniformly in a solid tumour mass, nor do they bind to all cells (Fujimori et al. 1989, Jain 1991). Administration of mixtures of antibodies specific for different antigens may be one way to target a greater number of cells and to achieve a more homogeneous antibody microdistribution (Buchsbaum et al. 1993). By using two- or three mAbs targeting different antigens, Andrew et al. showed a 2- to 3 fold-higher % I.D./g in human colon xenograft system (Andrew et al. 1990a). Blumenthal et al. demonstrated a better tumour growth inhibition by simultaneous targeting of CEA and colon-specific antigen-p using two different ¹³¹I-labelled antibodies, compared to the inhibition produced by either antibody alone at equal radionuclide dose (Blumenthal et al. 1991). Along the same lines, we report that simultaneous targeting of two different antigens (PLAP and cytokeratin 8) using multiple injections of two ¹³¹I-labelled mAbs caused significant growth retardation in nude mice, although no comparison to targeting these antigens separately was performed (Paper I).

Another approach to achieve better targeting in terms of total tumour uptake and retention time may be the use of antibodies with higher affinity or avidity. A significant therapeutic advantage of high affinity antibodies compared to ones with lower affinity, was demonstrated by Schlom et al. (Schlom et al. 1992) in LS174T colon xenografts. Using the same model system, a direct correlation between the affinities of six different mAbs and their tumour uptake was demonstrated by Muraro et al (Muraro et al. 1988). Andrew et al., classified four murine anti- Ly-2 and Ly-3 antibodies in terms of affinity, and found correlations to their localisation in tumours (Andrew et al. 1990b), in contrast to McCready
et al., who evaluated two antibodies against melanoma xenografts finding no such correlation (McCready et al. 1989). It has been suggested that if high amounts of circulating shedded antigen is present, antibodies of lower affinity may be superior to high affinity antibodies (Buchsbaum 1995). Moreover, low affinity antibodies have been demonstrated to result in a more homogeneous distribution within the tumour compared to antibodies of higher affinity (Langmuir et al. 1992). In our opinion however, affinity is directly related to specific binding i.e., higher the affinity, higher the tumour localisation. Specific targeting of TAA's form the basis for the concept radioimmunotargeting and high affinity antibodies should always be preferred. How to overcome shedded antigens is discussed below, whereas a homogenous antibody distribution within the tumours should be possible to achieve using mixtures of high affinity antibodies targeting different parts of a tumour. In this respect, simply performed kinetic characterisations and comparisons between antibodies using the biosensor technology BIAcore™, should be advantageous (Paper V).

Injection of the anti-tumour antibody unlabelled, prior to the therapeutic labelled antibody has been suggested as a technique to increase the localisation to the tumour (Buchsbaum et al. 1992). Improved localisation has been demonstrated both experimentally (Buchsbaum et al. 1992) and clinically (Kaminski et al. 1993) and the suggested mechanism of action has been that Fc-binding sites in normal tissues are saturated (Buchsbaum 1995). In our opinion, clearance of shedded antigen contributes partially or entirely to improved localisation following such preinjections of unlabelled antibody (Paper IV).

The use of fractionated antibody administration is one way to reduce normal tissue toxicity, as described above. Sublethal damage from an initial injection may also cause release of more target antigen, which may be targeted by subsequent injections. The obstacle however is the tendency of the host to produce anti-antibody responses during such a procedure. Such endogenous anti-antibody responses could decrease the localisation efficiency, particularly for the latter injections. To overcome such phenomena, chimeric (Lo Buglio et al. 1989, Baker et al. 1991, Chester et al. 1994), humanised (Isaacs et al. 1992, Kashmiri et al. 1995, Stephens et al. 1995) and recently, human mAbs with reduced immunogenicity, have been used in patients (Erdi 1994). Indeed, human antibodies have been demonstrated to evoke less anti-antibody responses in patients than all other suggested antibody types (Klein et al. 1995). Anti-idiotypic antibodies may, however, still be produced and the degree related to the immunogenicity of the variable region of each individual mAb. The use of immunosuppressive agents may be an approach to minimise such residual responses (Ledermann et al. 1988).

Furthermore, interferons have been used to enhance the mAb localisation to tumours by stimulating the tumour-target antigen expression (Kuhn et al. 1991, Greiner et al. 1992, Greiner et al. 1994, Nieroda et al. 1995). Kuhn et al. have demonstrated that interferon treatment could increase the CEA expression in tumours by a factor of 6.9 which caused twice
as high tumour dose after 120 $\mu$Ci $^{90}$Y-labelled mAb (Kuhn et al. 1991). Increased localisation of both anti-TAG-72 and anti-CEA mAbs have been demonstrated in nude mice xenografted with human colon cancer after interferon treatment (Kuhn et al. 1991, Greiner et al. 1992, Greiner et al. 1994, Nieroda et al. 1995). Local hyperthermia, the use of vasoactive drugs and external radiation have all been suggested as approaches to increase the tumour vascular permeability, thereby increasing the degree of mAb uptake in tumours (Misrikale et al. 1987, Wilder et al. 1993, Hennigan et al. 1993, Pedley et al. 1994, Pedley et al. 1996). By the use of the drugs flavone-8-acetic acid and its analogue, 5,6-dimethylxanthenone-4-acetic acid (DMXAA), which both reduce tumour blood flow and induce immunomodulation, Pedley et al. could significantly enhance experimental RIT (Pedley et al. 1994). Recently, the same group could demonstrate ablation of colorectal xenografts using DMXAA and an additional vasoactive agent, serotonin, to potentiate RIT (Pedley et al. 1996). Retention of higher doses of the $^{131}$I-labelled mAb and drug-induced tumour necrosis were two mechanisms suggested for the improved RIT efficacy (Pedley et al. 1994, Pedley et al. 1996). Wilder et al. have demonstrated that local hyperthermia in combination with the hypoxic cytotoxin SR 4233, is another way to enhance radiolabelled antibody anti-tumour efficiency (Wilder et al. 1993). Delgado et al. demonstrated a promising new approach to increase the localisation of an anti-CEA F(ab') fragment, i.e. using a poly(ethylene glycol) (PEG) modification (Delgado et al. 1996). The maximal tumour to non-tumour ratios were not statistically improved after the PEG modification, but persisted over a longer time, yielding at least double the total tumour levels as compared to the un-modified fragment (Delgado et al. 1996). The mechanisms contributing to the improved localisation were, however, unclear (Delgado et al. 1996).

Finally, some techniques which do not typically enhance tumour localisation, but nevertheless have been suggested to render RIT more efficient will be addressed. These include new techniques to make more stable radioiodinated antibody products with less dehalogenation in vivo (Shuster et al. 1991). In cases of radiometal-labelling, efforts to find new chelates with improved stability have been performed (Kozak et al 1989).

Moreover, radiosensitizers, normally used to enhance the therapeutic efficacy with external beam radiation have been evaluated also at RIT (Pedley et al. 1991). Using the hypoxic radiosensitizer misonidazole, combined with anti-CEA $^{131}$I-labelled antibodies, significantly prolonged tumour growth inhibition could be demonstrated compared to using the radiolabelled antibodies alone in xenografted nude mice (Pedley et al. 1991).
5. FUTURE PERSPECTIVES

Since the advent of hybridomas for production of mAbs, antibody targeting of tumours has been advancing, but RIT is still limited by three basic problems i.e., inadequate antibody accretion in the tumour, dose-limiting myelotoxicity and murine antibody immunogenicity. As discussed above, a large number of technologies have been presented lately to overcome these drawbacks.

Some trends within targeting research, emerge from last years efforts to improve the efficacy in targeting by various means. One of the most fashionable new tendencies is to use different modes of multistep procedures i.e., combinations of predosing, enhanced specific targeting and removal of redundant antibodies, as substitutes for the simple "magic bullets".

Another significant trend, especially for diagnostic purposes, is the use of small single-chain recombinant antibodies with low-molecular-weight, which enabling good penetration and rapid clearance. Such antibodies may approach the same affinities of those of intact antibodies. Large combinatorial phage libraries might furthermore enable findings of antibody specificities not yet identified, targeting structures typically conserved between species and thus not within reach with conventional hybridoma technologies.

Within the next years, RIT may constitute an important complement to other established strategies such as conventional radiotherapy, surgery and chemotherapy. The use of radiotherapy and RIT simultaneously, to enhance the therapeutic effect, represents a novel technique not yet explored, attracting significant attention. Furthermore, intralesional therapy may considerably contribute to generate high initial tumour doses, if the antibody remains within the tumour.

A clear awareness of the importance of antibody kinetics can be traced, as manifested by ambitions to document time-dose relationships. Dose calculations at RIT have been incorporated as irrevocable procedures to document and predict efficacy of different strategies. It seems necessary in future evaluations of different RIT-modalities to present obtained tumour and non-tumour tissue doses. With respect to the large number of different target antigens, antibodies and tumours, such calculations should be of fundamental importance if accurate comparisons should be possible to make.
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