ANTIBODY - DIRECTED DELIVERY
OF COPPER-67 TO TUMORS

by

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SUPERVISORY COMMITTEE APPROVAL

of a dissertation submitted by

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I have read the dissertation of Mahesh Kantilal Bhalgat in its final form and have found that (1) its format, citations and bibliographic style are consistent and acceptable; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the supervisory committee and is ready for submission to The Graduate School.

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ABSTRACT

The advent of monoclonal antibodies led to the reawakening of the "magic bullet" concept of Ehrlich, which suggests that antibodies against tumor-associated antigens could potentially be used for the delivery of cytotoxic agents specifically to tumors. The rationale for the use of such antibodies as selective carriers for the site-specific delivery of anticancer agents, and for increasing the therapeutic index of anticancer agents, is based on observations that antitumor antibodies or their immunologically reactive fragments do indeed selectively localize in vivo. Because in vivo localization of parenterally administered antitumor antibodies can be achieved, it has become feasible to investigate the efficiency and benefits of both diagnostic imaging as well as radiotherapy, using antibodies labeled with appropriate radioisotopes.

Whole antibody molecules and their fragments were used for the synthesis of radioimmunoconjugates. A radioisotope with both therapeutic and diagnostic utility, copper-67, was attached to a renal cell carcinoma antibody, A6H, or its fragments, using a synthetic porphyrin for chelation. A panel of conjugates was synthesized, either by direct attachment of the porphyrin to the protein or via the use of intermediate polymeric molecules.

Each of the synthesized conjugates was subjected to in vitro testing to determine the coupling yield, radiometalation yield, immunoreactivity, cell binding ability, and aggregation
properties. The copper-67-A6H conjugate with the highest level of immunoreactivity was injected in nude mice and its biodistribution was studied. Although a respectable tumor to blood ratio of 16 was obtained 45 hours after injection, the liver and spleen radioactivity levels were unusually high. Further investigation led to the possibility that aggregates present in the injection solution may be responsible for the high uptake of the conjugates by the reticuloendothelial system.

Dendrimer molecules used as polymeric intermediates in the immunoconjugates were subjected to preliminary biological evaluation to determine their toxicity, immunogenicity, carcinogenicity, and biodistribution pattern. Results from these experiments suggest that low doses of lower generation dendrimers are not associated with adverse effects that would preclude their use as intermediates in drug delivery.
To my family, especially my parents, for everything they have done for me,

and to all those wonderful people whose guidance I will never forget...
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CHAPTER 1

INTRODUCTION

Targeted Drug Delivery

One of the coveted goals of Western medicine is to develop therapeutic agents that can work like smart bombs or magic bullets, zeroing in on diseased tissue but leaving healthy cells unscathed (Amato, 1993). The reason this strategy is so appealing is because the single most serious problem associated with optimal drug action and systemic pharmacotherapy is the lack of selectivity of the drugs. Very often a drug has limited access to its intended site of action or is prematurely metabolized or excreted. Sometimes the drug travels freely throughout the body, exerting its effect on target and nontarget organs alike. As a consequence, drugs exhibit toxic side effects. A better understanding of the cellular and molecular biological processes in the human body has provided new and exciting opportunities for the design of site-specific therapeutic agents. The science aimed at improving the efficiency of a biologically active substance by increasing its localization at the desired site of action, decreasing its metabolism, and preventing unwanted uptake at other organs and tissues is referred to as drug targeting. It is a challenging concept that involves packaging drugs into complexes which can be addressed and delivered to specific cellular locations. This idea is particularly relevant for cancer chemotherapeutic agents since
present-day anticancer drugs possess little intrinsic ability to discriminate cancer cells from normal cells (Gupta, 1990). A wide variety of imaginative biochemical strategies has been employed to design site-specific drug delivery systems. Most of these are approaches that use physical properties or chemical recognition to facilitate selective localization and accomplish targeted drug delivery. These delivery systems are based on the presumption that molecular, genetic, or metabolic differences exist between the target and nontarget cells, such as a structural membrane protein, a cell-surface receptor, an intracellular enzyme, or an altered sequence in the genome (Pietersz et al., 1994).

**Rationale for Site-Specific Drug Delivery**

Site-specific drug delivery serves to improve the therapeutic index by localizing the drug at the desired site, and optimizing the interaction of the drug with its pharmacological receptor, as well as protecting the drug and the body from any deleterious disposition which could lead to undesirable degradation products.

The drug targeting concept is based on the use of carrier systems to deliver drugs to specific locations in the body. Generally speaking, the carriers used for this purpose are biological molecules with an inherent or acquired ability to interact selectively with specific receptor-bearing cells (Gregoriadis, 1981). Biological molecules such as antibodies or antibody fragments, hormones, and biological response modifiers have the inherent ability to function as carriers and are capable of targeting agents to specific locations in the body. In contrast, polymeric matrices, polymeric or proteinaceous microspheres or nanospheres, and lipid vesicles can serve as carriers but lack targeting ability until suitably modified with
a targeting moiety.

An area of targeted delivery systems that has attracted serious attention is the field of immunotargeting. This approach is based on the use of antibodies to deliver cytotoxic agents selectively to specific locations. It has been suggested that monoclonal antibodies against tumor-associated antigens may turn out to be the sword Excalibur in the fight against cancer (Larson, 1986). This approach uses preparations referred to as immunoconjugates, which are formed by attachment of lethal antitumor substances such as toxins, drugs, isotopes, etc., to tumor-specific antibodies. These preparations are macromolecular with relatively low molecular weights and dimensions, as compared to particle type carriers such as liposomes and microspheres.

Antibodies as Delivery Vehicles

Historical Perspective

The idea of using antibodies for selective delivery of drugs is usually credited to Paul Ehrlich and his motto, "We must learn to aim." At the turn of the century, Paul Ehrlich proposed the use of "bodies which possess a particular affinity for a certain organ" as a vehicle for delivering therapeutic agents, and he believed that these targeted agents could destroy malignant cells while sparing normal host cells (Ehrlich, 1906). More than 50 years after Ehrlich put forth the idea of drug targeting using antibodies, Mathe and coworkers (1958) reported some success in the treatment of mouse leukemia (L1210) using diazo-linked conjugates of the antitumor drug, methotrexate, and hamster antibodies raised against L1210 cells. In the early 1950s, Pressman pioneered the use of antitumor antibodies as
carriers of radioisotopes to cancer cells in animal models (Pressman & Korngold, 1953; Korngold & Pressman, 1954). However, the use of antibodies for drug targeting did not gain momentum until the early 1970s for several reasons: lack of relevant preclinical in vivo models, apprehension regarding the consequences of administering large quantities of foreign protein to patients, lack of specificity of the antibodies, and poorly defined targets (Dullens & De Weger, 1980; Ghose & Blair, 1978; Ghose et al., 1972). Some of these obstacles have been reduced if not fully eliminated (Ford & Casson, 1986). The discovery of antigenic sites specific for some tumor cells raised the hopes that antibodies against such antigens could serve as carriers to deliver cytotoxic drugs to the cells (Gold & Freedman, 1965; Bagshawe, 1983).

A landmark event that has had a major impact on this area of research was the development of hybridoma technology by Kohler and Milstein (1975). This method provides large amounts of pure antibodies with a predetermined specificity from clones of an immortalized cell line. The monoclonal antibodies produced by this technique have profoundly influenced biomedical research and medical diagnostics around the world (Waldmann, 1991). Monoclonal antibodies, by virtue of their unique specificity, the ability to select for the desired affinity, and ease of production, have surpassed polyclonal preparations as carriers for targeted delivery to tumors (Pietersz et al., 1994). It has now been demonstrated that not only can monoclonal antibodies be safely administered but they also can have a therapeutic effect on their own in xenograft models (Herlyn et al., 1985; Katano & Irie, 1984) and in patients with leukemias and lymphomas (Dillman et al., 1982; Miller & Levy, 1981). Modern immunotherapy uses antibodies alone (unconjugated or
"naked" antibodies) as passive therapy or as active immunogens (i.e., vaccines), or antibodies as carriers of toxic agents (drugs, toxins, isotopes, and enzymes) called immunoconjugates (Goldenberg, 1994).

In 1992, Eurocetus (The Netherlands) launched the first radiolabeled monoclonal antibody, OncoScint, approved for cancer diagnosis (OncoScint, 1992). Also, one monoclonal antibody (OKT3, Orthoclone, Ortho Pharmaceutical, Raritan, NJ) has already been fully approved for use as an immunosuppressive agent in cases of acute renal allograft rejection (Ortho multicenter transplant study group, 1985).

Antibody Structure

Antibodies are complex multichain biological macromolecules, proteinaceous in nature, with affinity for specific antigens and, therefore, are a natural choice as targeting moieties for drugs. Physiologically, they are molecules that powerfully bind to and help in the elimination of foreign material. Antibodies are found in the globulin fraction of the proteins that circulate in the blood and hence are also referred to as immunoglobulins. Specificity for the target antigen is the hallmark of antibody molecules. Thus, the use of antibodies to target "warheads" such as isotopes, drugs, or toxins has great theoretical appeal (Ford & Casson, 1986).

Although there are several different classes and subclasses of antibodies, they all show the presence of a basic structural unit. This basic unit of an immunoglobulin is comprised of the following:

1) two identical antigen binding regions, which are each formed from a pair of
disulfide-linked polypeptide chains, the heavy and light chains; and

2) one effector region, which is involved in biological effector functions such as complement-binding, skin fixation, placental permeability, etc., (Putnam, 1977) and is formed by conserved regions of the two heavy chains.

The two identical heavy (H) chains are approximately 53,000 Da each and the two identical light (L) chains are approximately 22,000 Da each. They are attached to each other through disulfide bonds. A more detailed description of the antibody structure has been provided for a specific class of immunoglobulin, Immunoglobulin G (IgG, see Figure 1.1).

Each chain is made up of a variable region (V) and a constant region (C), also known as domains. The N-terminal 110 amino acids in the H and L chains form the variable regions designated as \( V_H \) and \( V_L \), respectively. The greatest variability in antibody amino acid sequences occurs in three separate regions of each \( L \) and \( H \) chain, which are called the hypervariable regions or complementarity-determining regions (CDRs) (Kabat, 1983). The less variable spaces between these hypervariable regions are called framework regions. In a folded, intact antibody molecule, CDRs come together to constitute a combining site, which is complementary to an epitope, the region on the antigen that is capable of accommodating an antibody (Sikora & Smedley, 1984). The carboxy-terminal portions of each chain have a constant amino acid sequence for molecules of the same subclass and allotype and are referred to as \( C_H \) and \( C_L \) for the heavy and light chains, respectively. Heavy chains have four to five domains (one variable and three to four constant regions) depending on the class of antibody. Light chains have one variable and one constant region. A stretch
Figure 1.1. Schematic representation of an IgG molecule. Adapted from Gallagher, 1983.
of about 15 amino acid residues between $C_{H1}$ and $C_{H2}$ corresponds to the flexible junction between Fab (fragment antigen binding) fragment Fc (fragment crystallizable) portions of the molecule. This region is called the hinge region and contains the disulfide bonds that link the two heavy chains of each molecule. The abundance of proline and cysteine residues in the hinge region apparently confers rigidity and makes this site susceptible to proteolytic enzyme cleavage (Nisonoff et al., 1975; Anzel & Poljak, 1979). A dominant, but nonessential, homologous feature is the presence of an internal disulfide bridge between cysteine residues separated by 60 to 70 or 50 to 60 residues in the primary sequence of the V and C regions, respectively. Other conserved features of primary amino acid sequence include invariant tryptophan residues and conservative amino acid interchanges. These conserved sequences presumably reflect selective pressures that include a requirement to fold into a globular conformation. The result is a characteristic tertiary structure referred to as the immunoglobulin fold, which may be described as two surfaces of antiparallel $\beta$-pleated sheet between which the disulfide bridge is formed (Jefferis, 1990). Hydrophobic amino acid side chains are mostly orientated to the interior of the domain, thus stabilizing the globular protein structure. In addition, carbohydrates are present in the $C_{H2}$ domain below the hinge region. The oligosaccharide portions overlay the hydrophobic surface that would otherwise be exposed to solvent.

The entire family of immunoglobulins can be divided into classes and subclasses, each containing antibody molecules with distinct characteristics. The antibody classes - IgA, IgD, IgE, IgG and IgM - differ from each other in the composition of the heavy chain effector regions. IgG is the predominant antibody class in blood (in human serum, 85% of
total immunoglobulin is IgG), where it circulates as a monomeric, four chain unit. IgG is susceptible to proteolysis, particularly in the hinge region. Treatment with papain produces two Fab fragments, which retain the ability to bind antigen, and one Fc fragment, which does not bind the antigen but can be crystallized out of solution (see Figure 1.2). Treatment with pepsin yields a bivalent antigen-binding fragment, F(ab')₂. Antibodies are of the order of 100 Å long and 130 Å wide. IgA and IgM monomers are similar to IgG, but because of the presence of an extra cysteine residue in the C-terminus extension of their heavy chains, these monomers can polymerize. Polymerization is important for IgM, the antibody class produced on first exposure to the antigen. Although the antigen-binding sites are relatively low in affinity, the pentameric structure greatly increases the ability of IgM to bind antigen-bearing cells because avidity, a qualitative description of antigen-antibody interaction, increases markedly with valency. Two of the antibody classes, IgA and IgG, are further subdivided into subclasses, defined by antigenic uniqueness of their heavy chains. Some of the important antibody classes and subclasses and their characteristics are summarized in Table 1.1. Each of the nine classes has antigenically distinctive heavy chains (named with the corresponding Greek letter, α, γ, μ, δ and ε), but the light chains are similar in all cases (Eisen, 1980). Each of the four IgG subclass heavy chains may be expressed in combination with either kappa (κ) or lambda (λ) light chains to give an essentially symmetrical molecule composed of two identical heavy chains and two identical kappa or lambda light chains. The heavy chain of IgG is referred to as the gamma (γ) chain and thus the IgG subclass molecules have heavy chains γ₁ through γ₄.
Figure 1.2. Proteolytic fragmentation of an IgG molecule. Adapted from Gallagher, 1983.
Table 1.1

Characteristics of human antibody classes and subclasses.
Adapted from Saltzmann, 1993.

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<th>MW&lt;sup&gt;b&lt;/sup&gt; (kDa)</th>
<th>Concentration in fluid (mg/mL) and distribution between subclasses (%)</th>
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<td></td>
<td></td>
<td></td>
<td>Serum</td>
<td>CSF</td>
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<td>IgG</td>
<td>1</td>
<td>150</td>
<td>13.5</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>IgG1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>IgG2</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>IgG3</td>
<td></td>
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<tr>
<td></td>
<td>IgG4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td>1, 2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>150, 300</td>
<td>3.5</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>IgA1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgA2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>5</td>
<td>950</td>
<td>1.5</td>
<td>----</td>
</tr>
<tr>
<td>IgE</td>
<td>1</td>
<td>190</td>
<td>Trace</td>
<td>----</td>
</tr>
<tr>
<td>IgD</td>
<td>1</td>
<td>180</td>
<td>Trace</td>
<td>----</td>
</tr>
</tbody>
</table>

<sup>a</sup>DP = degree of polymerization of secreted form

<sup>b</sup>MW = molecular weight

<sup>c</sup>Levels vary throughout the cycle: low during midcycle; high during early proliferative phase.

<sup>d</sup>Also contains some molecules with DP = 3 and higher. Serum IgA contains 80% monomeric (DP = 1), whereas mucus secretions contain 90% polymeric (DP ≥ 2).
Antibodies are produced by B lymphocytes following stimulation by antigen. Antibodies found in the body are secreted by lymphoid cells in various organs, especially the bone marrow, spleen, and lymph nodes (Zola, 1987). Although bone marrow is the primary source of cells destined to make antibodies, it is rarely the locus of large-scale antibody production. Rather, the bone marrow is the site of intense lymphocyte proliferation that leads to the production of B lymphocytes that quickly escape the marrow and travel to the peripheral lymphoid tissues. There, the B lymphocytes may meet an appropriate antigen, become stimulated to divide and differentiate into numerous, identical antibody-secreting plasma cells, and actively manufacture antibodies. A given B cell can recognize and respond to only one antigenic determinant and produce antibody of only one immunoglobulin class. The antibody against the specific antigen is already present on the surface of a mature B cell and is responsible for the recognition process. Thus, a B cell, even before activation by antigen, has on its surface membrane a small sample of antibody that it can synthesize when it is properly activated by the appropriate antigen (Guttmann et al., 1987). Upon secretion by the B cells, antibodies distribute throughout the body and accumulate in blood plasma and in secretions (Table 1.1). B lymphocytes are plentiful in areas of antibody production such as the germinal centers of lymph nodes and diffuse lymphoid tissue of the gastrointestinal and respiratory tracts. Lower concentrations of antibodies are also found in the interstitial space of tissues. An organism produces millions of different B cells that are each genetically programmed to produce antibody of a certain class, specificity, and antigen affinity. Thus, when these pure products mix in the serum, the mixture obtained is
considered a polyclonal composition of antibodies. The rate of synthesis for IgG, IgA, IgM, IgD, and IgE is 34, 66, 7, <1, and <1 mg/kg/day, respectively (Saltzman, 1993). If a single antibody-producing cell is isolated and cloned, a population of pure (monoclonal) antibody is obtained, where each immunoglobulin binds to the same epitope. Frequently, antibodies for clinical use are obtained from the sera of patients known to have high concentrations, or titers, of antibodies specific for an antigen. In general, however, it is difficult to obtain sufficient quantities of a specific antibody by this method. To produce antibody of a predefined specificity, hybridoma technology is most commonly used (see Figure 1.3).

Briefly, the steps involved in the generation of a hybridoma cell line are as follows (Bankert, 1984): Animals (usually mice or rats) are immunized with an antigen preparation that contains macromolecules of interest, for example, tumor-specific determinants, until a significant response is detected. The spleen cells from the immunized animals are fused with myeloma cells using polyethylene glycol as a cell fusion promoter. The most commonly used myelomas are drug-resistant mutants that lack the enzymes, hypoxanthine guanine phosphoribosyl transferase (HPRT) or thymidine kinase (TK). These cells are unable to incorporate externally supplied hypoxanthine or thymidine. The blockage of endogenous synthesis of DNA precursors with aminopterin results in the death of the unfused myeloma cells, even when hypoxanthine and thymidine are supplied in the special hypoxanthine-aminopterin-thymidine (HAT) selection medium. However, the spleen cells that fuse with the myeloma cells supply the required salvage pathway enzymes, including HPRT and TK, thereby selectively permitting the growth of the resultant hybrids in the HAT medium. All
Figure 1.3. Schematic representation of hybridoma production. Adapted from Zola, 1987.
of the immune spleen cells that have not fused with a myeloma cell eventually die since they cannot propagate \textit{in vitro}. The hybridized cell line produces monoclonal antibody molecules that are identical in chemical structure, antigen specificity, and antigen binding affinity. To obtain large quantities of monoclonal antibody, hybridomas can be grown as ascitic tumors in animals or in culture.

Although this cell fusion technique works very well for production of mouse and rat monoclonal antibodies, obtaining stable fusions of human cells has been difficult (Saltzman, 1993). To get around this, recombinant DNA technology has been used to produce "humanized" antibodies, which are essentially chimeric molecules consisting of mouse variable regions and human constant regions (Morrison, 1985). Because of their prolonged plasma half-life, these chimeric molecules have improved biological activity, reduced immunogenicity, and improved pharmacokinetics.

**Monoclonal and Polyclonal Antibodies Compared (Zola, 1987)**

A solution of monoclonal antibodies contains a single antibody specificity and affinity and a single immunoglobulin isotype with identical amino acid sequence, whereas a polyclonal antibody preparation contains a variety of antibody molecules with no particular specificity.

**Different Antibodies Against the Same Antigen**

A polyclonal antibody preparation contains not only antibodies against "irrelevant" antigens, it contains multiple different antibodies against the antigen of interest. There will
be antibodies which react with the same antigen but with different affinity.

**Single or Multiple Antigenic Epitopes**

The monospecificity of monoclonal antibodies prevents them from exhibiting those antibody functions that require the presence of antibodies against several determinants on the antigen. Thus, monoclonal antibodies do not generally precipitate antigens unless the determinant detected is present in multiple copies on the molecule. This is because a sufficiently large antigen-antibody lattice is not formed.

**Sensitivity and Specificity**

A consequence of the specificity of monoclonal antibodies is that relatively few antibody molecules bind to the target cell, thus limiting sensitivity. This could be advantageous since the lower sensitivity of monoclonal antibodies compared to polyclonal mixtures leads to greater specificity and lower background staining. Another possible disadvantage is that there could be high concentrations of the polyclonal antibody binding to nontarget tissues.

**Labile Epitopes**

If the epitope detected by a monoclonal antibody is labile under certain conditions, the monoclonal antibody may not bind. A polyclonal preparation, on the other hand, contains antibodies to various different structures and has a higher possibility of binding since some epitopes may remain undamaged.
Labile Antibodies

Similarly, stability of the monoclonal antibody is also crucial. In a classical antiserum, if some antibodies are unstable, a drop in titer may be observed. However, if a monoclonal antibody happens to be an unstable protein, the loss of activity is complete.

Cross Reactivity

A major difficulty in the use of polyclonal antisera is cross reactivity - the reaction of the serum with an antigen differing from the antigen of interest. Monoclonal antibodies, on the other hand, are less likely to cross react.

Reproducibility

Above all, monoclonal antibodies provide the opportunity for standardization. Once a clone is established, it should be possible to produce identical antibodies in unlimited amounts. This can eliminate a major source of variability which is not possible when polyclonal preparations are used.

Handling and Manipulations (Bankert, 1984)

The homogeneous character of monoclonal antibodies presents several problems not encountered with conventional heterogeneous antibodies. For example, whereas conventional antibodies are, for the most part, tolerant to low pH, freezing, freeze-drying, iodination, and a variety of conjugation chemistries, monoclonal antibodies have demonstrated a variable susceptibility to these procedures. The apparent sturdy nature of
conventional antibodies is most likely a reflection of their heterogeneity, whereas monoclonal antibodies, being homogenous, show an all-or-nothing response.

In most applications, monoclonal antibodies are preferred because of reproducibility, ease and economy of production, and precision of epitope reactivity.

**Antibody Fragments**

Some emphasis has been placed on reducing the size of antibody preparations by using antibody fragments instead of whole antibodies. Antibody fragments such as F(ab')₂ (MW ~ 110 kDa) and Fab (MW ~ 55 kDa) are easily prepared and are smaller than the intact immunoglobulin (MW ~ 150 kDa) and, therefore, are likely to penetrate into tumors more easily. Fragments are also cleared more rapidly than intact IgG (Covell et al., 1986). Radioimmunolocalization studies have demonstrated that antibody fragments can give earlier and superior localization compared to intact monoclonal antibodies (Andrew et al., 1986); however, this is probably due to faster clearance of fragments from the blood, thereby reducing the background rather than giving absolutely higher levels of antibody in the tumor. In addition, cleaving the Fc portion from the antibody molecule should decrease nonspecific binding to nontumor cells possessing Fc receptors and reduce the immunogenicity associated with the monoclonal antibody. Single chain antigen binding proteins (SCA, sFv) have also been engineered. These proteins consist of antibody V₅ and V₇ regions linked via a peptide (Huston et al., 1988). Full antigen-binding capacity is retained by these fragments with some decrease in binding affinity (Glockshuber et al., 1990). In a study by Yokota et al., the penetration of various immunoglobulin forms, IgG, F(ab')₂, Fab, and sFv, were
compared *in vivo* using autoradiography (Yokota et al., 1992). It was observed that maximum penetration into tumors, for sFv was at 0.5 hour, whereas IgG reached a maximum at 48 to 96 hours postinjection. This study also showed that sFv penetrated more deeply (more distal from blood vessels) into the tumor than intact IgG.

The Antigen-Antibody Reaction (Sikora & Smedley, 1984)

Perhaps the most important function of antibody molecules is to combine with the corresponding antigen to form an antibody-antigen complex, which is then eliminated from the circulation by the cells of the mononuclear phagocytic system (Steward, 1984). Folding of the antibody chains creates clefts and hollows, which conform to the shape of the antigen, so that weak intermolecular forces can bind the antigen to the antibody (Guttmann et al., 1987). Each antibody combines with a particular antigenic determinant, or epitope. Although an antigen may have many different epitopes and react with a number of different antibodies, an individual antibody binds only one epitope, and this epitope (and the antibody's combining site for it) consists of five to seven amino acids. The locking of an antibody and its antigen is rather like the linking of two pieces in a jigsaw puzzle. The intermolecular forces which contribute to the stabilization of the antigen-antibody complex are the same as those involved in the stabilization of the configuration of proteins and other macromolecules. These include: hydrogen bonding, apolar or hydrophobic interaction, ionic or Coulombic interaction, van der Waal's forces, and steric factors.

The interaction of the antigen and its specific antibody sets in motion a series of events within the body which may result in the expulsion of the antigen from the body.
First, the complex forms a molecular lattice which grows in size, and eventually such complexes will be removed from the blood by phagocytic cells. Second, the combination of such molecules can cause the activation of complement, a complex cascade of proteins present in an inert form in blood which, when activated, may cause the lysis of a foreign cell. Alternatively the macrophages of the reticuloendothelial system may be directed to a specific site in the body and phagocytosis of foreign cells enhanced.

Antibody-Mediated Delivery

Over the past 20 years, considerable interest has been focused on targeting systems designed to permit selective delivery of drugs, radioisotopes, and toxins to tumors for both diagnosis and therapy. A great deal of research has been performed utilizing antibodies as carriers to deliver these agents. An important consideration for the use of antibodies as carriers has been the demonstration that they can reach tumor cells and bind to them - \textit{in vitro}, \textit{in vivo} with animal tumors and human tumors growing as xenografts in immune deprived mice, and in patients with cancer. The advantages of antibodies as carriers include (Bander, 1994; Pietersz et al., 1994): 1) the ability to specifically and precisely target cytotoxic agents to tumor sites while sparing normal tissues; 2) the ability to target individual tumor sites leading to the preferential uptake of the cytotoxic agent-carrier conjugate by tumor cells; 3) the ability to administer extremely cytotoxic agents that cannot be used alone because of toxicity; 4) the ability to bind cytotoxic agents to carriers, thus preventing the agent from enzymatic degradation and rapid excretion; and 5) the ability to target several different diagnostic or therapeutic agents.
In order to benefit from the above advantages associated with antibody-mediated drug delivery, it is essential that the antibody selected should meet certain criteria. Some of these criteria have been outlined here (Gallagher, 1983; Goldenberg et al., 1987): 1) abundant tumor-specific antigen targets; 2) homogeneous preparation; 3) high immunoreactivity and high affinity; 4) complete bioavailability of antigen targets; 5) high purity with minimal ascitic fluid impurities; 6) good stability during and after conjugation reactions (IgMs are unstable); 7) plenty of surface groups available for coupling a variety of agents; 8) nonantigenic in patients; 9) selective or no reactivity with nontarget tissues; 10) ready availability; 11) efficacious carrier of agents for imaging or therapy.

Although there are advantages to the use of antibodies as drug delivery vehicles, antibody-mediated tumor targeting also has certain problems associated with it. Some of these problems have been described (Burchell & Taylor-Papadimitriou, 1985; Larson et al., 1994).

**Antigenic Modulation**

Antigenic modulation, defined as the redistribution of surface antigen after binding of antibody (Poste & Kirsh, 1983), reduces the localization of the monoclonal antibody at the tumor site, and could be responsible for failure of antibody-mediated delivery. Antigenic modulation may involve internalization and degradation of the antigen or shedding of the antigen-antibody complex from the cell surface. Binding of monoclonal antibodies to circulating antigen (shed from the tumor) or to antigen present on normal tissue can divert monoclonal antibodies away from the target.
**Tumor Cell Heterogeneity**

Tumor cell heterogeneity is also an important cause of monoclonal antibody treatment failure and represents a formidable barrier to the development of effective regimes of antibody-targeted cytotoxic agents. Heterogeneity is particularly well-illustrated in colon carcinoma where the unstable phenotypic composition of tumors produces a mosaic of antigen expression (Durrant et al., 1986). Effective targeting of cytotoxic agents to tumors may, therefore, be improved by using a cocktail of antibodies which could be established by determining the antigenic repertoire of tumor cell populations. Goldenberg et al. (1987) have prepared mixtures referred to as "engineered polyclonal antibodies". These are mixtures of monoclonal antibodies, not only directed against different epitopes of the same antigen, but also reactive with distinctly different tumor-associated antigens.

**Antibody Specificity and Antibody Affinity**

One of the problems that exists in targeting drugs in the human subject is the very small absolute amount of antibody which reaches the target tumor. Increasing the amount of antibody administered may increase the concentration of modified antibody deposited in tumors, but this will be limited by a concomitant increase of deposition in normal tissues. One approach is to use bifunctional or bispecific antibodies, where there is linkage of an antibody or fragment against the tumor target with an antibody or fragment against a cytotoxic agent (Goodwin et al., 1988; Stickney et al., 1989). Upon injection, the bifunctional antibody localizes at the site of the tumor. After enough time has elapsed to clear the bifunctional antibody from normal tissues and blood, the cytotoxic agent is
introduced and, since this agent can react with the second antibody arm, it preferentially localizes at the tumor site. Still another, somewhat simpler, method to reduce nonspecific antibody localization is to inject a second antibody that binds the immunoconjugate after sufficient time has elapsed for optimal tumor targeting, thus clearing the primary antibody from the circulation and enhancing the tumor to blood ratio of the immunoconjugate preparation (Goodwin et al., 1984; Goldenberg et al., 1987).

Development of an Antimurine Immune Response

Often, an antibody response develops in humans after administration of mouse or rat monoclonal antibody to tumors (Carrasquillo et al., 1984), resulting in the presence of a human antimouse antibody (HAMA). These antibodies bind to the administered monoclonal antibody, forming a complex of a larger size, which in turn results in reduced circulation time of the administered antibody.

Adverse Effects of Monoclonal Antibody Therapy

Toxic reactions to the administration of monoclonal antibodies are primarily immune reactions, particularly if foreign antibodies are involved (Goldenberg, 1994). An immune reaction may be caused when monoclonal antibodies bind to their antigen target, particularly a circulating antigen, or when the administered antibody evokes host antibodies. The clinical manifestations of both mechanisms can be quite similar. In the first instance, fever, chills, urticaria, dypsnea, hypotension, and diarrhea can occur. In the second instance, reactions usually occur two to three weeks after the first or repeated injections and are
unrelated to the dose administered. The reactions can be manifested by anaphylaxis, serum
sickness, urticaria, fever, and hypotension. Despite these diverse reactions to conjugated
and unconjugated monoclonal antibodies, the clinical experiences to date have indicated that
immunoconjugates are relatively safe cancer therapeutics, and the adverse reactions are
generally easy to control, e.g., by changing antibody dose and/or infusion rate; administering
corticosteroids and antihistamines; or, possibly, treating with immunosuppressants
(Ledermann et al., 1991).

Pharmacokinetics of Antibody Delivery (Saltzman, 1993)

Following intravenous injection, the antibody preparation typically disappears from
the plasma in two phases: a rapid phase, α, due to disposition, and a slower phase, β, due
to metabolism and elimination. The half-life for an antibody in the plasma is usually
determined from the rate of decline in concentration during the β phase. Plasma half-life
depends on antibody class, with IgG having the longest half-life (see Table 1.2) (Saltzman,
1993).

The antibody molecules present in the plasma circulate throughout the body. By
slowly permeating through the capillary wall, the antibody can enter the interstitial space of
tissues. The rate of antibody entry into the interstitial space of any particular tissue depends
on the intrinsic permeability and the capillary surface area per volume of tissue. The
respective intrinsic permeableites of IgG, F(ab')2, and Fab are generally assumed to be 0.2-
0.7, 2, and 20 x 10^-7 cm/sec in normal tissue and approximately a factor of 10 higher (6, 20,
and 150 x 10^-7 cm/sec) in tumor tissue (Renkin, 1977; Baxter & Jain, 1989). Recently,
Table 1.2

Metabolism of human antibodies in humans.
Adapted from Saltzmann, 1993.

<table>
<thead>
<tr>
<th>Antibody class</th>
<th>t_{1/2} (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>21^a</td>
</tr>
<tr>
<td>IgA(^b)</td>
<td>6</td>
</tr>
<tr>
<td>IgM</td>
<td>6</td>
</tr>
<tr>
<td>IgD</td>
<td>3</td>
</tr>
<tr>
<td>IgE</td>
<td>2</td>
</tr>
</tbody>
</table>

^aDependent on IgG subclass: t_{1/2} (IgG1, IgG2, IgG4) = 21 days;
t_{1/2} (IgG3) = 7 days

\(^b\)Neglects differences between the various molecular forms of IgA
compartmental models have been used to describe nonspecific biodistribution and clearance in the mouse (Covell et al., 1986). These models are useful for relating plasma concentrations of antibody to tissue concentrations and degree of antibody localization. In general, antibody fragments are able to permeate through capillaries, and therefore enter the interstitial space, more rapidly than the intact antibody. Once a monoclonal antibody or an antibody conjugate leaves the circulatory system and reaches the interstitial space of a tissue or tumor, its ability to penetrate through the interstitial space determines its effectiveness.

The Tumor Target (Pietersz et al., 1994; Order, 1985)

To increase the selective targeting of cytotoxic agents to neoplastic cells, it is desirable to have clearly defined targets which ideally are expressed on the surface of tumor cells but not of normal cells. Although the search for specific antitumor monoclonal antibodies produced by murine or, optimally, by human hybridomas is in progress, such monoclonal antibodies of absolute specificity may not be necessary. For example, an antigen which has a higher expression on tumor than normal cells or is absent on vital normal cells (e.g., hematopoietic stem cells) may be a suitable target for the delivery of cytotoxic agents. Many potential antigens have been found to be highly tumor-associated; three of the best known examples are α-fetoprotein (AFP) (Abelev et al., 1963), carcinoembryonic antigen (CEA) (Gold & Freedman, 1965), and common acute lymphoblastic leukemia antigen (CALLA) (Greavel et al., 1975). Although more and more monoclonal antibodies against different antigens are being produced and characterized, to date, monoclonal antibodies against protooncogene products do not appear to be suitable vectors for targeting cytotoxic
agents such as methotrexate to tumor cells (Embleton et al., 1983), possibly due to their poor access to oncoproteins in tumor cells as most are expressed intracellularly and not on the surface (Heldin & Westermark, 1984).

Although abundantly expressed tumor antigens are desired for the specific targeting of cytotoxic agents with monoclonal antibodies, other important factors are the localization of monoclonal antibodies at tumor cells \textit{in vivo} and their uniform penetration into the tumor. Before localization, antibodies injected in the blood stream have to pass through a number of compartments, including vascular and extravascular spaces (organs, tissues, and body fluids), and have to cross several barriers before reaching the tumor target. The barriers encountered by the antibody are as follows.

\textbf{Physical Barriers}

\textbf{The Endothelial Barrier}

Endothelial cells lining the vasculature serve to demarcate the vascular and extravascular compartments and to regulate the flow of macromolecules between these compartments (Freudenberg et al., 1983; Anderson, 1981). Most of the exchange of solutes takes place at the level of the capillary endothelium, with a surface area of about 60 m$^2$ in humans (Simionescu & Simionescu, 1983). The three main types of capillary endothelium are continuous, fenestrated, and sinusoidal. These types of endothelial layers exhibit very different permeabilities to blood-borne macromolecules. Continuous endothelial layers show the lowest permeability characteristics, although sinusoidal capillaries are more or less permeable (Seymour, 1992). In the majority of continuous endothelial layers, the tight
occluding junctions in the capillary bed are generally impermeable to materials greater than 1.8 to 2.0 nm in diameter (Bundgaard, 1980). Materials are able to extravasate in normal endothelium by a process of transcapillary pinocytosis as well as by passage through interendothelial cell junctions. This vesicular pathway has a theoretical size limit for transport of about 70 nm (Seymour, 1992). The permeability of capillaries to proteins differs with tissue site and protein molecular weight (Renkin, 1977) and is lowest in the brain. This is because the endothelial layer of the brain microvasculature is the tightest in the body, containing no fenestrations at all and showing only very limited transcellular pinocytic activity. The bone marrow and spleen are two examples of tissues and organs possessing a sinusoidal vascular structure, with large interendothelial cell junctions (fenestrations up to 150 nm diameter) and only an irregular basement membrane. The liver also has a sinusoidal vasculature, with a complete absence of basement membrane. Access of macromolecules to the surface of hepatocytes and Kupffer cells is particularly easy.

The permeability of macromolecules in capillaries of tumor tissue is generally higher than that in normal tissue. The detailed vascular characteristics of tumors show considerable variability, even within a single tumor, and there are frequently differences from normal tissues in the cellular composition of the capillary wall, the form of the basement membrane, and sometimes in the size of interendothelial cell fenestrations. It has been suggested that deficiency of vascular pericytes may be responsible for leakiness of tumor vasculature (Blood & Zetter, 1990). The elevated interstitial pressure often associated with solid tumors may be another factor underpinning differences in the behavior of tumor capillaries from those serving normal tissues (Jain, 1989). Morphological indications of increased
permeability such as enlarged interendothelial cell junctions and incomplete basement membranes has also been observed (Heuser & Miller, 1986). Dvorak et al., (1988) however, have suggested that the increased rates of extravasation observed in tumors might be mediated by enhanced vesicular transport or via transendothelial channels, rather than by simple leakage between endothelial cells. Given the potential importance of this research area, it is surprising that greater effort has not been committed to its elucidation (Seymour, 1992).

Molecular size and charge are known to be important in determining rates of macromolecular extravasation (Taylor & Granger, 1984). The vascular endothelium appears to have a higher permeability for water soluble macromolecules ranging in size from the smaller plasma proteins like albumin (MW = 68 kDa) to large plasma-borne lipoprotein particles with molecular weights in excess of $2 \times 10^6$ kDa (Smith & Staples, 1982). The negative charge on the surface of endothelial cells results in differential rates of extravasation for charged species (Brenner & Beeuwkes, 1978). Negatively charged molecules move much more slowly, whereas material bearing a net positive charge passes through endothelial layers even faster than neutral molecules (Seymour, 1992).

The Basal Lamina Barrier

Except for capillaries in the liver, spleen, and bone marrow, the capillary endothelium is subtended by a layer of dense fibrillar material referred to as the basal lamina or basement membrane (Martinez-Herandez, 1981). The insoluble nature of this material has been attributed to the presence of type IV collagen. Apart from collagen, high molecular weight
adhesive proteins such as laminin and fibronectin may also be present. Thus the ability of macromolecular drug-carrier complexes to transit the basal lamina depends on the size, charge, and specific macromolecular binding characteristics of the carrier complex.

**Cellular Barriers**

After crossing the barriers discussed above, the drug-carrier complex must reach its ultimate site of action, which may or may not involve internalization into cells. Ultimately, if a drug has to interact with an extracellular surface receptor, then a carrier that brings the drug into proximity of the receptor has achieved its goal. However, if the target is intracellular, then the drug-carrier complex has to begin the process of intracellular migration. This migration may occur by any of the following uptake processes: phagocytosis, pinocytosis, or receptor-mediated endocytosis.

**Phagocytosis** is a discontinuous process undertaken by macrophages responding to the presence of particles. During phagocytosis, the cell internalizes a large particle (1000 Å or greater) by engulfing into a membrane-bound vesicle which is then internalized. The vesicle is then fused with lysosomes and its contents are digested by lysosomal hydrolases.

**Pinocytosis** is a nonspecific process and, because of its continuous nature and extent, may be useful as a general process for transporting macromolecular material through epithelia, some endothelia, and into various blood cells. In pinocytosis, cells pinch off and internalize small fluid-filled plasma membrane vesicles of about 250 Å in diameter. These vesicles, like phagocytic vacuoles, ultimately fuse with and deliver their contents to the lysosomes.
In some cases where high-affinity receptors exist, a process referred to as receptor-mediated endocytosis has been identified for a variety of physiological ligands including metabolites, hormones, and immunoglobulins (Steinman et al., 1983; Anderson & Kaplan, 1983; Hopkins & Trowbridge, 1983). Biological molecules such as polypeptide hormones, growth factors, and serum proteins undergo continuous recycling from the plasma membrane to the cell interior and back again by this process. Unlike phagocytosis or pinocytosis, receptor-mediated endocytosis is much more versatile, encompassing an ability of specific cells and tissue to recognize or select, and to transport into the cell (and sometimes through them), complex molecules ranging in size from less than 100 Da to more than one million Da (e.g., virus particles) (Tomlinson, 1986). This process is mediated by coated pits, bearing specific receptors, and the internalized molecules are delivered to the lysosomes for processing. This information regarding traffic across membrane barriers is important in designing an appropriate drug-carrier system, so that delivery of the drug into cells is achieved when necessary.

Physiological Barriers

The Reticuloendothelial Barrier

In order to reach target cells successfully, a drug-carrier complex must not only exit from the circulation and pass the endothelial and basal lamina barriers, but it must also be able to bypass the reticuloendothelial system (RES). The RES, which represents the body's disposal mechanism for foreign particles and macromolecules, is composed of a set of mononuclear phagocytic cells. The most important cells participating in this function are
the Kupffer cells of the liver and the splenic macrophages. In performing their function, macrophages not only engulf foreign particles, but also engulf certain proteins that interact with receptors on the macrophage surface. The receptor-protein complex is internalized into a vesicular structure which then fuses with lysosomes. The macrophage membrane contains a variety of receptors, including two types of receptors for the Fc domain of IgG (Ogmundsdotti & Weir, 1980; Silverstein et al., 1977). Antibody-drug conjugates, where the antibody is of the IgG1 or IgG3 subclasses, can bind directly to human macrophages via protease-sensitive Fc receptors. Antibody-drug conjugates involving IgG2 or IgG4 antibodies can bind to the trypsin-insensitive Fc receptor if they first bind to the antigen (Werb, 1983). Liposomes, microspheres, and other particulate drug delivery systems are rapidly cleared from the circulation due to uptake by liver and splenic macrophages in vivo (Juliano, 1981; Poznansky & Juliano, 1984). Manipulating the surface characteristics can help reduce this uptake but cannot totally eliminate it (Poste, 1983). A number of approaches has been adopted for reducing carrier interception by the RES. Recent examples are the deglycosylation of the A chains of ricin in immunotoxins, which enables them to avoid the relevant sugar receptors in the RES (Blakey et al., 1987) and the co-administration of ricin immunotoxin with yeast mannan which competes for uptake by the liver (Bourrie et al., 1986). Thus, the role of the RES in drug delivery is very important since it can lead to clearance and sequestration of a variety of particulate and macromolecular drug carriers. This not only reduces the total drug available for targeting but poses the threat of selective destruction of macrophages by drug action.

Considering all the barriers involved, it is not surprising that radioimmunolocalization
has revealed marked variation in the localization of antitumor antibodies in animal and human tumors (Foon et al., 1983; Baldwin & Pimm, 1983). Some monoclonal antibodies have shown fivefold greater localization in tumors than in normal tissues, whereas others have failed to selectively localize in tumors. Typically, between 0.0007 and 0.01% injected dose per gram (% ID/g) of the antibody actually reaches the tumor tissue (Bradwell et al., 1985).

**Agents Delivered Using Antibodies**

Despite the problems involved, several different types of agents have been delivered to tumors using antibodies. Broadly classified, these agents fall into four different categories: 1) toxins; 2) drugs; 3) enzymes; and 4) radioisotopes. Each of these agents possesses distinct advantages and disadvantages as seen in Table 1.3. For example, although it is relatively easy to synthesize an antibody-radioisotope conjugate, the handling of radioisotopes can be difficult. Also radiolabeled antibodies can cause damage to surrounding tissues. Whereas radiolabeled antibodies do not depend on internalization into cells for their cytotoxic effect, internalization is usually essential for a drug or toxin to be effective. Each of these agents, drugs, toxins, and radionuclides, can be either directly attached to the antibodies or can be attached through the use of intermediary linker/spacer molecules. The groups that are available on the monoclonal antibodies or intermediary carriers for crosslinking are the reactive groups such as ε-amino groups of lysine residues, N-terminal amines and other amino groups, phenolic hydroxyl groups of tyrosine residues, carboxylic acid groups, sulfhydryl groups of cysteine residues, cis-diols of carbohydrates,
Table 1.3

Comparison of immunoconjugates. Adapted from Pietersz et al., 1994.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Isotope</th>
<th>Drug</th>
<th>Toxin/Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemistry</td>
<td>Easy</td>
<td>Difficult</td>
<td>Easy</td>
</tr>
<tr>
<td>Yields</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Stability/half life</td>
<td>Low (depends on isotope used)</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>Handling</td>
<td>Difficult</td>
<td>No problem</td>
<td>No problem</td>
</tr>
<tr>
<td>Tumor heterogeneity</td>
<td>Good</td>
<td>Not good</td>
<td>Not good</td>
</tr>
<tr>
<td>Cocktail of monoclonal antibodies</td>
<td>Good</td>
<td>Good</td>
<td>Good</td>
</tr>
<tr>
<td>Damage to surrounding tissues</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Damage elsewhere</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Antibody to conjugate</td>
<td>No</td>
<td>Usually no</td>
<td>Yes (toxins), possibly with enzymes if non-human</td>
</tr>
<tr>
<td>Access to tumors</td>
<td>Good</td>
<td>Good</td>
<td>Not good due to large size</td>
</tr>
</tbody>
</table>
and imidazole groups of histidine residues (Pietersz et al., 1994).

Direct Attachment

Toxins

Several recent review articles have been published on the use of toxins conjugated to antibodies for use in cancer therapy (Brinkmann & Pastan, 1994; Gottstein et al., 1994; Pai & Pastan, 1994). A variety of potent plant, bacterial, and fungal toxins may be coupled with a carrier antibody to produce a potentially therapeutic molecule referred to as an immunotoxin. Most of the work has been done with either whole ricin or ricin A chain. Ricin is a heterodimeric glycoprotein of approximately 63 kDa, and is obtained from the seeds of the castor plant, *Ricinus communis*. Other toxins that have received a great deal of attention are diphtheria toxin and *Pseudomonas* exotoxin. Toxin entities used are generally polypeptides that consist of two chains: one which binds to carbohydrate residues present on virtually all cells (the B chain), and a second, the A chain. The ricin A chain mediates the toxic effect and is an enzyme that inactivates the elongation factor 2 binding site of the 60S ribosomal subunits in eukaryotic cells (Olsnes et al., 1975), leading to inhibition of protein synthesis. The ricin B chain, or carbohydrate binding chain, mediates binding of the toxin to galactose residues on cell surface glycoproteins and glycolipids (Olsnes & Pihl, 1973). Once bound to a cell surface, the receptor-toxin complex is endocytosed, and the A chain is translocated across the membrane of the endocytic vesicle into the cytosol where it inhibits protein synthesis. These toxic agents are usually large and are very potent indeed; one molecule of ricin is sufficient to interfere with the protein
synthesis machinery and lead to cell death. Since whole, intact molecules such as ricin bind to almost all cells through the B chain, isolated A chains have been conjugated to antibodies for therapy. Immunotoxins have also been prepared by blocking the galactose binding sites of the ricin B chain. Several attempts have been made to use affinity labels or protein modification to inactivate the galactose-binding sites of the ricin B chain. Houston (1983) has reported the successful inactivation of the galactose-binding sites of ricin using a photoactivated derivative of galactose. The resultant ricin molecules exhibited an approximately 300-fold reduction in nonspecific toxicity. It has now been shown that even when the native ricin A chains are conjugated to monoclonal antibodies, their carbohydrate residues bind to liver cells (Blakey et al., 1988). Thus, second generation immunotoxins have been prepared by using deglycosylated A chains.

In general, the chemical linkage of the toxins to antibodies is straightforward and often uses disulfide bonds. However, the efficiency of the procedure is low and the yields are accordingly poor (Pietersz & McKenzie, 1992). Genetic engineering approaches have also been used for preparing immunotoxins (FitzGerald & Pastan, 1992; Siegall, 1994).

The possible disadvantages of such potent compounds is their nonspecific toxicity to sites such as liver. Deglycosylated compounds, however, partly overcome this nonspecific binding problem. The large size of antibody-toxin conjugates may also be a disadvantage since it can lead to poor penetration into tissues. Also toxin molecules can be antigenic and, even before treatment, many humans have antibodies which cross react with ricin curtailing further treatment with ricin immunotoxins.
**Enzymes**

A novel concept that has emerged over the last few years has been to conjugate enzymes to antibodies. These immunoconjugates can be targeted to the tumor site, and a second injection is then given of a nontoxic prodrug from which a toxic drug is released by enzymatic cleavage at the site of the tumor. Enzymes such as carboxypeptidase G, cytidine deaminase, alkaline phosphatase, and β-lactamase have been targeted using this approach (Pietersz & McKenzie, 1992) and seem to give local therapeutic effects (Svensson et al., 1992, Senter et al., 1988). One clinical trial has been reported with an enzyme-antibody conjugate involving the delivery of carboxypeptidase G2 coupled to an anti-CEA antibody in colon cancer patients; 12 patients were treated and 5 patients showed a tumor growth delay of 5 months, 4 showed symptomatic relief, and in others no response was observed (Bagshawe et al., 1992). Unfortunately, all patients produced human antimouse antibody as well as an antienzyme antibody. The enzyme-antibody conjugates could theoretically suffer from some of the disadvantages listed for immunotoxins, such as size and antigenicity, but antigenicity could be overcome by using human enzymes.

**Drugs**

Cancer chemotherapeutic agents usually exhibit a narrow therapeutic index, and investigators have tried different approaches to increase the delivery of drug molecules to tumor cells. One such approach has been the attachment of anticancer agents to tumor-specific antibodies to prepare antibody-drug conjugates or chemoimmunoconjugates. An exhaustive list of the different classes of antineoplastic drugs used in antibody-drug
immunoconjugates has been published in a recent review article (Pietersz et al., 1994). A listing of the clinical studies performed with antibody-drug conjugates has also been published by Pietersz and McKenzie (1992).

The conjugation of drugs to antibodies can be chemically difficult. The portion of the anticancer drug that is responsible for its cytotoxic effect should not be modified, and, therefore, close attention needs to be given to the structure-activity relationship of these drugs when designing coupling protocols. Also it is important that the drug should be released from the immunoconjugate close to its site of action and not prematurely. Some of the coupling strategies used have been described below. Carboxylic acid-containing drugs are activated by forming the N-hydroxysuccinimide ester which can then be reacted with antibody amino groups to form conjugates with an amide linkage between drug and antibody (Kanellos et al., 1987). Hydroxyl-containing drugs are first reacted with succinic anhydride to introduce a carboxylic acid group and are then coupled to the antibody in a manner similar to other carboxyl-containing drugs (Goerlach et al., 1991). To facilitate coupling to periodate-oxidized sugar residues present on antibody molecules, carboxyl- or ester group-containing drugs have been converted to reactive amines or hydrazides (Apelgren et al., 1990). Sulphydryl groups on the drug and antibody have also been used for conjugation. These methods have been extensively reviewed (Pietersz, 1990) and result in usually 4 to 10 molecules of drug being conjugated to each molecule of antibody (Pietersz & McKenzie, 1992). During the coupling procedure, there is usually loss of antibody activity as well as up to 10-fold loss of drug activity. The resulting conjugates were generally inefficient because of drug crosslinking and altered immunoreactivity (Baldwin &
Byers, 1987; Koppel, 1990). An exception to these general observations has been observed in the case of alkylating agents such as chlorambucil or melphalan where an increase in drug activity has been observed after conjugation to antibody, and up to 30 molecules of drug can be bound to each molecule of antibody (Smyth et al., 1986a; Smyth et al., 1986b). To increase delivery of drug molecules to a tumor, methods have been developed that allow more molecules to be bound to an antibody without affecting its immunoreactivity and targeting capability (see below).

At present, clinical studies with drug conjugates of anthracyclines, vinca alkaloids, and folic acid antagonists are in progress. An advantage in the use of these cytotoxic agents is that considerable information has been gained regarding the utility of these drugs in cancer. As with other attempts at therapy with murine monoclonal antibodies, the human antimouse antibody response presents a serious limitation to delivering effective doses, necessitating the development of drug immunoconjugates using human or humanized monoclonal antibodies. Although most drugs are unlikely to be immunogenic by themselves, it is theoretically possible for these entities coupled to antibody to act as haptens; there is one report of the vinca alkaloid used in a vinca-antibody conjugate being immunogenic (Peterson et al., 1991). An important point, essential for the success of drug-antibody conjugates, is that in most cases the drug needs to be internalized by the cell for cytotoxicity.
Radioisotopes

Most drug and toxin immunoconjugates need to be internalized and transferred into the cytoplasm to be effective. With only a limited amount of antibody being delivered to the tumor, and even less being internalized by the tumor cells, the use of an agent that does not need internalization could be beneficial. For this reason, radioisotopes have been conjugated to antibodies to form radioimmunoconjugates. In contrast to drug and toxin immunoconjugates, radiolabeled antibodies can kill target cells without the monoclonal antibody conjugate being internalized. Therefore, radioimmunoconjugates can distribute their cytotoxic energy to antigen-negative cells in the neighborhood of antigen-positive cells (Goldenberg, 1994). In this way, tumor cells which do not bind the immunoconjugates (as they lack the antigen or have a low density of antigen expressed) can be irradiated and destroyed. A number of reviews have appeared on the use of radiolabeled antibodies for tumor imaging and therapy (Goldenberg, 1983; Fritzberg et al., 1988; Grossman & Rosebrough, 1988; Kosmas et al., 1993; Buchsbaum et al., 1993; Larson et al., 1994).

Radioisotopes have been widely used in immunoconjugate preparation for several reasons. In general, radionuclides are readily available, can be readily conjugated to antibodies and, being radioactive, it is convenient to study their in vivo biodistribution, half-life, and accumulation in tumors (Pietersz & McKenzie, 1992). These advantages have contributed to many more clinical studies being done with isotopes, particularly iodine-131.

Units of radiation (Roberts, 1992). The most basic characteristic of radiation, radioactivity, is defined as the number of nuclear transformations per unit time. Radioactivity is expressed in either Curies (Ci), Becquerels (Bq; SI unit), or disintegrations
per minute (dpm). One Curie is equal to $2.2 \times 10^{12}$ dpm and 1 Bq is equal to 1 dpm. For many years, the unit of dose commonly used was the rad (radiation absorbed dose), defined to be an energy absorption corresponding to 100 ergs per gram of irradiated material. This unit has been replaced by the Gray (Gy; SI unit), defined to be an energy absorption of 1 joule per kilogram. One Gray is equivalent to 100 rad.

**Choice of radionuclide.** A partial list of the radioisotopes that have been evaluated for antibody-mediated delivery is given in Tables 1.4 and 1.5 (Fritzberg et al., 1988). Each radionuclide decays at a particular rate involving the release of certain emissions. The most useful types of decay are $\gamma$-emissions, $\beta$-decay, $\alpha$-decay, and electron capture. The decay involves emission of particles or photons that differ in the distance they travel in tissue (Goldenberg, 1994). $\gamma$ Rays possess the capability of passing through cell layers and can irradiate organs and tissues that are away from the point of decay, enabling them to be detected by $\gamma$ cameras outside the body. $\beta$ Particles from low energy $\beta$-emitters such as iodine-131 travel less than 1 mm, whereas those from yttrium-90 have path lengths up to a few millimeters. The high energy emissions of $\alpha$-particle emitting radionuclides (6 to 9 MeV, which is ten times higher than the emissions of $\beta$- or $\gamma$-emitting radionuclides) travel even shorter distances (40 to 80 $\mu$m) (Waldmann, 1991). Radionuclides, such as iodine-125, that decay by electron capture (whereby low-energy electrons are emitted with energies in the range of a few keV) deposit this energy over very short distances (about 20 nm). Thus radioimmunoconjugates with short-distance radionuclides resemble drug and toxin immunoconjugates by needing to be targeted close to the tumor cell. As with other immunoconjugates, the radionuclide should be bound tightly to the monoclonal antibody
Table 1.4

Radionuclides available for radioimmunoimaging. Adapted from Fritzberg et al., 1988 and Mercer-Smith et al., 1988.

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Half-life</th>
<th>Particle energies (%) keV</th>
<th>Decay mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorine-18</td>
<td>0.08 days</td>
<td>511 (97%)</td>
<td>Positron</td>
</tr>
<tr>
<td>Gallium-67</td>
<td>3.62 days</td>
<td>185 (24%), 296 (22%)</td>
<td>Electron capture</td>
</tr>
<tr>
<td>Copper-67</td>
<td>2.58 days</td>
<td>91 (8%), 93 (32%), 184 (48%)</td>
<td>Beta</td>
</tr>
<tr>
<td>Bromine-76</td>
<td>0.67 days</td>
<td>511 (38%)</td>
<td>Positron</td>
</tr>
<tr>
<td>Bromine-77</td>
<td>2.38 days</td>
<td>240 (30%), 520 (24%)</td>
<td>Electron capture</td>
</tr>
<tr>
<td>Zirconium-89</td>
<td>3.26 days</td>
<td>511 (22%)</td>
<td>Positron</td>
</tr>
<tr>
<td>Ruthenium-97</td>
<td>2.88 days</td>
<td>215 (91%), 324 (8%)</td>
<td>Electron capture</td>
</tr>
<tr>
<td>Technetium-99m</td>
<td>0.25 days</td>
<td>140 (90%)</td>
<td>Isomeric transition</td>
</tr>
<tr>
<td>Indium-111</td>
<td>2.83 days</td>
<td>172 (89%), 247 (94%)</td>
<td>Electron capture</td>
</tr>
<tr>
<td>Iodine-123</td>
<td>0.54 days</td>
<td>159 (83%)</td>
<td>Electron capture</td>
</tr>
<tr>
<td>Iodine-131</td>
<td>8.05 days</td>
<td>364 (82%), 637 (7%)</td>
<td>Beta</td>
</tr>
<tr>
<td>Lead-203</td>
<td>2.20 days</td>
<td>279 (81%)</td>
<td>Electron capture</td>
</tr>
</tbody>
</table>
Table 1.5

Potentially useful radionuclides for radioimmunotherapy.
Adapted from Fritzberg et al., 1988.

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Half-life</th>
<th>$E_{\text{max}}$ (MeV)</th>
<th>Particle energies (%) (keV)</th>
<th>Decay mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorus-32</td>
<td>14.30 days</td>
<td>1.7</td>
<td>-</td>
<td>Beta</td>
</tr>
<tr>
<td>Scandium-47</td>
<td>3.43 days</td>
<td>0.6</td>
<td>163 (73%)</td>
<td>Beta</td>
</tr>
<tr>
<td>Copper-67</td>
<td>2.58 days</td>
<td>0.575</td>
<td>184 (48%)</td>
<td>Beta</td>
</tr>
<tr>
<td>Yttrium-90</td>
<td>2.66 days</td>
<td>2.27</td>
<td>-</td>
<td>Beta</td>
</tr>
<tr>
<td>Rhodium-105</td>
<td>1.44 days</td>
<td>0.568</td>
<td>319 (20%)</td>
<td>Beta</td>
</tr>
<tr>
<td>Palladium-109</td>
<td>0.56 days</td>
<td>1.028</td>
<td>88 (5%)</td>
<td>Beta</td>
</tr>
<tr>
<td>Iodine-131</td>
<td>8.05 days</td>
<td>0.606</td>
<td>364 (82%)</td>
<td>Beta</td>
</tr>
<tr>
<td>Samarium-153</td>
<td>1.95 days</td>
<td>0.80</td>
<td>103 (28%)</td>
<td>Beta</td>
</tr>
<tr>
<td>Rhenium-186</td>
<td>3.70 days</td>
<td>1.02</td>
<td>137 (7%)</td>
<td>Beta</td>
</tr>
<tr>
<td>Rhenium-188</td>
<td>0.71 days</td>
<td>2.12</td>
<td>155 (15%)</td>
<td>Beta</td>
</tr>
<tr>
<td>Astatine-211</td>
<td>0.30 days</td>
<td>5.87</td>
<td>-</td>
<td>Alpha</td>
</tr>
<tr>
<td>Bismuth-212</td>
<td>0.04 days</td>
<td>6.09</td>
<td>-</td>
<td>Alpha</td>
</tr>
</tbody>
</table>
without affecting its targeting potential. Considerable progress has been made in the chemistry of antibody radiolabeling. Some nuclides, such as isotopes of iodine, can be conjugated directly to the antibodies, whereas others are linked by an intermediary group, such as chelating agents for radiometals.

Selecting the ideal radionuclide for radiolabeling depends on certain criteria (Wolf & Shani, 1986): 1) It is necessary to consider the purpose for radiolabeling the monoclonal antibody (diagnosis, prognosis, follow-up, therapy). For a diagnostic or prognostic procedure, such as radioimmunoimaging (an in vivo diagnostic technique in which radiolabeled antibody binds to target antigen, so that the antigen-containing tissue can be seen on γ camera images), it is desirable that the radionuclide deliver sufficient photon density for achieving the resolution needed for the imaging studies, when contributing as small a radiation dose as possible. For therapeutic purposes, radioimmunotherapy, the aim is to deposit as much energy as possible at the target site, so as to obtain the desired tumor dose of approximately 6000 rad (Fawwaz et al., 1986). Radioisotopes that emit particles such as β particles are ideal for this purpose. 2) The physical characteristics of the radionuclide, such as half-life and photon and/or particle energy, are also important. This is because the selected radionuclide must retain a sufficient activity (e.g., deliver the desired number of photons for imaging) at the time an image is to be taken. 3) Chemical reactions used for attaching the radionuclide to the monoclonal antibody also need to be considered in selecting the appropriate radionuclide. The chemical changes required to attach the radiolabel to the monoclonal antibody should have no effect on biological properties such as immunointegrity and immunoreactivity of the antibody. Also, chemical stability of the
radiolabeled monoclonal antibody is very important. 4) Choice of the radioisotope is also dictated by the radiation dose expected (desired) to be delivered to the tumor, as well as to other organs of the patient.

Iodine-131 has been the most widely used nuclide for imaging of tumors using monoclonal antibodies in humans. However, it has the following disadvantages: 1) iodine-131 is associated with high radiation exposure, particularly from β energy; 2) iodine-131 emits a 364 keV photon, which is not optimal for use with the current imaging equipment; 3) iodination involves exposure of the antibody to varying levels of oxidizing reagents during the labeling process, which could affect antibody integrity; and 4) iodine-labeled antibodies undergo extensive deiodination in vivo, and there is high accumulation of iodine in the thyroid gland (Torchilin & Klibanov, 1991) and small amounts in the stomach (Fritzberg et al., 1988).

In order to obtain good images in radioimmunoimaging, high localization of the antibody in the tumor tissue, as opposed to other organs, is required. This high contrast is not easily achieved because after the radioimmunoconjugate traverses across the different barriers, it has to find its tumor-specific antigen which is present in the picogram to nanogram range (Grossman & Rosebrough, 1988). Thus, in general, radioimmunoimaging results have been somewhat disappointing, because of poor antibody localization in the tumor. Despite these difficulties, reports indicate that tumors as small as 0.5 cm, which are sometimes missed by other radiological methods, can be imaged with antibodies or antibody fragments labeled with suitable radionuclides (Waldmann, 1991). Some of the potential uses of radioimmunoimaging are outlined in Table 1.6.
Table 1.6

Potential uses of radioimmunoimaging.
Adapted from Goldenberg et al., 1987.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Contribute to preoperative clinical staging.</td>
</tr>
<tr>
<td>2.</td>
<td>Evaluate recurrence or dissemination.</td>
</tr>
<tr>
<td>3.</td>
<td>Confirm other detection measures that are less tumor-specific.</td>
</tr>
<tr>
<td>4.</td>
<td>Evaluate tumor response to therapy.</td>
</tr>
<tr>
<td>5.</td>
<td>Serve as a basis for antibody-mediated therapy.</td>
</tr>
</tbody>
</table>
Unlike radioimmunoimaging, which only requires a high target/nontarget ratio of counts for optimal results, therapy with radiolabeled antibodies depends on a high concentration of radioactivity delivered to tissue for a long duration (Order, 1990). The amount of radioactive antibody delivered to a tumor can be measured in vivo noninvasively by serially acquiring data from tomographic scanning of the patient and recording tissue radioactivity counts. This information can be analyzed to determine the dose delivered to various organs, including the tumor (Siegel et al., 1991). The attractive feature of radioimmunotherapy is the prospect that most normal tissues distant from the tumor are spared intensive radiation.

Small amounts of nonspecific radiation of normal tissues during the distribution and elimination of the radionuclide, however, are inevitable. Nonspecific radiation problems may also arise based on the duration of binding of radiolabeled monoclonal antibody to tumor cell surface antigen. The antigen-antibody complex which is initially located on the cell surface may, after a delay, become unstable; the antibody may then disassociate from the cell surface. When that occurs, the monoclonal antibody may re-enter the circulation and can irradiate other areas of the body. Metabolism of the antibody and its fragments can cause organs such as the kidneys and liver to be subjected to unnecessary radiation. Other problems associated with manufacturing and handling high amounts of radioactivity have inhibited progress to date (Order, 1985).

**Dosimetry of radiolabeled antibodies** (Order, 1985). In diagnostic applications, the distribution of the radioisotope is often monitored by calculating its partition between tumor and normal tissue. The tumor to normal tissue ratio must be especially high with
respect to radiosensitive organs such as bone marrow, intestinal mucosa, liver, and kidneys (Fawwaz et al., 1986). The maximum radiation dose that these organs can tolerate and still continue to function adequately to support life are listed in Table 1.7. With any effective antibody, the tumor to normal tissue ratio should increase as tumor-bound antibody is retained and background diminishes. Fab fragments are particularly useful in this regard. However, it is the concentration of radiolabeled antibody in mCi per gram over time that determines tumor dose. The regulation of radioactivity at the target, therefore, depends on the biologic half-life and physical half-life of the isotope, the composite result being the tumor-effective half-life. Radiolabeled antibodies are compared using parameters such as the dosimetry in concentration (mCi per gram) and tumor-effective half-life. These parameters help in the development of better therapeutic and imaging agents. It is estimated that the administration of a safe therapeutic dose of 150 mCi of iodine-131 delivers approximately 30,000 rad to a well-differentiated thyroid tumor approximately 1 cc in size (Koral et al., 1982).

**Chelating agents** (Torchilin & Klibanov, 1991; Waldmann, 1991; Roberts et al., 1987). The preparation of a monoclonal antibody-chelate complex suitable for in vivo use should meet some general criteria, in addition to those that are true for all monoclonal antibodies: 1) The binding of the chelating agent and subsequent metal binding should not compromise antibody specificity and affinity for target antigen. 2) The chelation and radiolabeling procedure should not alter the distribution and metabolism of the monoclonal antibody; the complex should accumulate at the tumor site with minimal nonspecific binding. 3) The bifunctional chelate should provide tight binding of the metal label to the protein.
Table 1.7

Sublethal radiation doses in humans.
Adapted from Fawwaz et al., 1986.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Dose (rad)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>&lt; 2500</td>
</tr>
<tr>
<td>Kidney</td>
<td>&lt; 1500</td>
</tr>
<tr>
<td>Intestinal Mucosa</td>
<td>&lt; 700</td>
</tr>
<tr>
<td>Bone Marrow</td>
<td>&lt; 200</td>
</tr>
</tbody>
</table>
It should not detach and there should be no premature release of the radiometal in vivo. 4) The complex formed must have high specific activity of labeling. 5) The chelating agent and the complex should be nontoxic. 6) The antibody-chelating agent complex should allow for easy metal incorporation. 7) The covalent bond between the metal and the chelating agent must be stable in vivo.

Chelation chemistry has developed considerably over the years; current methodology permits efficient and easy attachment of indium-111 to antibodies, with relatively low nonspecific binding to liver, and considerably diminished in vivo transchelation (Larson et al., 1994). Gansow et al. (1990) and the Meares group have developed several methods for attachment of indium-111 and yttrium-90 to antibodies (Deshpande et al., 1989). Several reports have described the use of chelating agents suitable for use with copper (Moi et al., 1985; Fujiwara et al., 1985; Meares, 1986; Roberts et al., 1987; Deshpande et al., 1988; Mercer-Smith et al., 1988; Green et al., 1988; Morphy et al., 1989; Smith-Jones et al., 1991; DeNardo et al., 1991b; John et al., 1994; Kukis et al., 1995).

Use of Intermediary Carriers/Linkers

In order to couple multiple molecules of the cytotoxic agent to a single antibody molecule, the use of intermediate linkers or polymer chains was proposed (Torchilin et al., 1986). The use of intermediary carriers involves coupling cytotoxic molecules to a carrier molecule, which has multiple reactive sites, and then linking this complex to a monoclonal antibody. A variety of carriers has been used for conjugation, including human serum albumin, dextran (polyaldehyde and amino), poly-L-glutamic acid, poly-L-lysine, and poly-
L-aspartic acid (Pietersz et al., 1994). Human serum albumin has been widely used because it has a defined and acceptable molecular weight (~ 67,000 Da), a large number of both free carboxyl and amino groups for easy chemical substitution, and good biological activity in aqueous solutions and in several organic solvents. Dextran as a carrier has several advantages in that there are no limitations on its chemical derivatization, and it is a nondigestable polymer suitable for human use (Hurwitz et al., 1980). An interesting polymeric system that has been developed by Jindrich Kopecek and Ruth Duncan uses copolymers of N-(2-hydroxypropyl)methacrylamide (HPMA) as targetable vehicles for drug delivery (Duncan et al., 1984; Kopecek et al., 1985). These methacrylamide-based copolymers have extremely versatile chemistry, and various drugs can be covalently linked to the polymer backbone using a range of linkages. During the design and testing of these conjugates, a linker had been identified that would permit rapid liberation of active drug by intracellular enzymes, but would not show significant degradation in the circulation. Eventually, a tetrapeptide sequence (glycine-phenylalanine-leucine-glycine), designed as a substrate for the cathepsins B, H, and L, was shown to fulfill all of the necessary criteria (Duncan et al., 1984; Kopecek et al., 1985). The delivery of doxorubicin and daunorubicin, both anthracycline antibiotics with a broad spectrum of anticancer activity, has been extensively studied using the HPMA system (Figure 1.4) (Seymour et al., 1987; Duncan et al., 1988; Rihova et al., 1988; Duncan et al., 1989). To date, over 250 different HPMA conjugates have been produced and tested, and some are in clinical trials in the United Kingdom. Intermediary carriers have been used for several purposes including the following.
Figure 1.4. Chemical structure of a doxorubicin-N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugate. Adapted from Seymour, 1992.
To Increase the Number of Cytotoxic Molecules That Can Be Delivered

For example, in the preparation of radiolabeled antibodies, multiple chelator residues need to be attached to a single antibody molecule. This can be effectively done using intermediate linkers molecules.

To Effect a Concomitant Decrease in Host Toxicity

(Shih et al., 1990; Shih et al., 1991)

Marshall and coworkers (Marshall et al., 1977; Marshall, 1978) and Vegarud and Christensen (1975) have demonstrated that linkage of enzyme analogs to dextran results in reduced immunogenicity.

To Prevent Overmodification of the Antibody

Direct attachment of several cytotoxic agents to the protein molecule may result in the alteration of the native protein conformation and may be associated with the decrease of the antibody affinity (Paik et al., 1983) or even complete denaturation.

To Provide Increased Modification Potential

It is possible to attach various other groups to the polymer chain, providing a range of possibilities for the additional modification of antibody-carrier complex. One such opportunity is that of varying the net charge or hydrophobicity of the complex, which can influence modification potential. Charge and hydrophobicity may also influence the antibody
To Modify the Pharmacokinetics of the Conjugate

Coupling of certain ligands results in the binding of the modified antibody conjugate to the corresponding receptors in vivo. This opens up the possibility of the specific clearance of the preparation, or its rapid binding to the receptor preliminary to the target site. Also, Marshall and coworkers (Marshall et al., 1977; Marshall, 1978) and Vegarud and Christensen (1975) have demonstrated that linkage of enzyme analogs to dextran results in a longer in vivo half-life.

Despite difficulties in consistently preparing and purifying drug-carrier-antibody conjugates, many have displayed highly specific and cytotoxic properties in vitro. To date, however, few have demonstrated enhanced antitumor activities in vivo against human tumor xenografts in nude mice (Pietersz et al., 1994). Indeed, in vivo problems such as poor tumor localization, rapid clearance from the circulation, and nonspecific toxicity to the RES may result, depending on the size, biological nature, and stability of the intermediate carrier used.

Components of the Copper-67 Delivery System

This section will serve to introduce the various pieces that have been used in the design of an antibody-mediated drug delivery system for the site-specific delivery of copper-67 to renal cell carcinoma.
The Tumor Model: Renal Cell Carcinoma

Incidence

Renal cell carcinoma, also called Grawitz tumor (Grawitz, 1883), is the most common malignant disease arising from the cells of the proximal tubule of the kidney (Vessella et al., 1988). Renal cell carcinoma accounts for approximately 80% to 90% of all primary renal neoplasms (Garnick & Richie, 1991) and 2% to 3% of all cancers in adults (Millan, 1989). In the United States the incidence of the disease is 7.5 cases per 100,000 (Kantor, 1977). The median age of patients with renal cell carcinoma is between 50 and 60 years (Garnick & Richie, 1991); however, the incidence increases steadily between the ages of 40 and 90 years, with a male to female ratio of approximately 1.6:1 (Boring et al., 1993). The 1993 incidence and mortality estimates for cancer of the kidney was 27,200 (Boring et al., 1993). Factors that may be of etiologic relevance in the development of kidney cancer include smoking, chewing tobacco, eating a high-fat diet, and exposure to petrochemical products (Davis, 1993).

A great deal of information has become available with respect to the genetics of renal cell carcinoma. The best known example of inherited renal cell carcinoma is associated with the von Hippel-Lindau patients disease (Dijk, 1991). However, in the majority of cases, renal cell carcinoma is sporadic and involves alterations in the 3p chromosome (Kovacs et al., 1987). In one study, 15 out of 22 patients (68%) showed terminal 3p deletions (Bergerheim et al., 1989). Kovacs et al. (1988) observed that in 18 out of 21 patients (86%) chromosome 3p was deleted. These data suggest that rearrangements or loss of alleles of loci involving chromosome 3p are involved in the pathogenesis of familial and
nonfamilial cases of renal cell carcinoma, and argues for the presence of a tumor suppressor gene in this region. Furthermore, activation of H-ras and myc oncogenes has also been suggested in renal cell carcinoma (Dijk, 1991).

**Diagnosis**

Sadly, renal cell carcinoma is often diagnosed rather late because of the myriad of ways it may present clinically. The most common symptoms at diagnosis are pain and hematuria, either gross or microscopic. Sometimes, hypertension, hypercalcaemia, liver dysfunction, or polycythaemia may each be the only symptom of the disease (Parks & Kellett, 1994). Because renal cell carcinoma may remain clinically occult for most of its course, patients often enter the clinic with a late stage of the disease. Metastatic disease is documented at presentation in approximately 25% of patients (Davis, 1993). Also, one-third of those patients initially diagnosed with localized renal cell carcinoma will develop metastatic disease (Wilbur et al., 1993).

For the diagnosis of renal cell carcinoma, many techniques are available such as intravenous pyelography, computed tomography, arteriography, ultrasound, and magnetic resonance imaging (Garnick & Richie, 1991). Usually, multiple imaging modalities are used to obtain complete information. However, these methods remain nonspecific; detection is dependent on different physical characteristics between normal and tumorous tissue. Furthermore, and most important for prognosis, metastatic lesions are often not visualized by these methods, due to the small size of most metastatic lesions (Dijk, 1991). Considering all these factors, it is obvious that renal cell carcinoma requires better diagnostic methods.
for early detection.

**Therapy**

**Surgery.** The standard procedure for treatment of localized renal cell carcinoma is radical nephrectomy (deKernion & Mukamel, 1987). Radical nephrectomy includes complete removal of Gerota's fascia and its contents, including the kidney and the adrenal gland. Apart from this technique, patients with tumor in a solitary kidney may be treated with partial nephrectomy, which helps preserve normal kidney function (Morgan & Zincke, 1990). About 30% of all patients show recurrence of disease within 5 years after nephrectomy (deKernion et al., 1978).

**Chemotherapy.** Investigational and commercially available cytotoxic agents with different mechanisms of action have been widely investigated alone and in combination in renal cell carcinoma. To date, a striking lack of activity has been demonstrated by all known agents (Davis, 1993). Vinblastine, either by intravenous bolus or continuous infusion, remains the most active single chemotherapeutic agent, with response rates ranging from 1% to 16% (Dorr, 1990).

**Hormonal therapy.** Progestational agents, androgens, and antiestrogens have been studied as single agents or in combination with response rates being reported ranging from 2% to 17%, the higher percentage perhaps reflecting subjective responses (Hrushesky & Murphy, 1977).

**Immunotherapy.** With better understanding of the immune system and the availability of various immunomodulatory reagents, such as interleukins, interferons, and
tumor necrosis factor, the field of immunotherapy has been greatly stimulated. Of the 
various types of interferons investigated in renal cancer for their cell growth inhibition and 
immunostimulatory properties, the alpha interferons appear to exhibit more activity than 
beta or gamma interferons (Linehan et al., 1989). Interleukin-2 is also capable of inducing 
tumor regression with an overall response rate paralleling that achieved by alpha interferon. 
In fact, the Food and Drug Administration approved the use of interleukin-2 for metastatic 
lymphocytes, demonstrated to be more active than lymphokine-activated killer cells in 
inducing tumor regression in animal models, are also being studied (Bellido et al., 1991).

Radiotherapy. Theoretically, preoperative radiation may sterilize tumor cells, thus 
rendering them incapable of growth in the event of dissemination by surgical manipulation 
and thereby improving survival (Forman, 1989). This theory has not been confirmed in 
clinical trials. Reduction in tumor size, sclerosis of small blood vessels, and fibrosis with 
subsequent ease of surgical resection are postulated as benefits of preoperative radiotherapy 
(Davis, 1993). Palliative external beam radiation therapy has been demonstrated to provide 
symptomatic benefit (Forman, 1989).

Thus, renal cell carcinoma not only needs a good diagnostic system but also needs 
an efficient therapeutic system. Successful targeting of renal cell carcinoma sites in patients 
has been accomplished by the use of radioiodinated monoclonal antibodies (Vessella et al., 
1988; Vessella, 1991). Although these studies are continuing, it seems prudent to evaluate 
the use of monoclonal antibodies coupled with other nuclides as well. For reasons cited 
above, renal cell carcinoma forms an attractive human tumor model system against which
The copper-67 delivery system can be used.

The Monoclonal Antibody: A6H

Vessella and coworkers have generated a panel of tumor-preferential monoclonal antibodies reactive against renal cell carcinoma (Vessella et al., 1985; Vessella et al., 1988). From the dozens of monoclonal antibodies generated, one particular monoclonal antibody, A6H, has performed better than others under in vivo test conditions (Vessella, 1991). This monoclonal antibody has been chosen for modification and preparation of the antibody-mediated delivery system for specific delivery of copper-67 to renal cell carcinoma.

A6H belongs to the IgG1 subclass and expresses the κ light chain. The monoclonal antibody, A6H, resulted from a tandem immunization schedule using five different human fetal kidney homogenates (Vessella et al., 1985). A6H is relatively restrictive in that it is reactive against all of the 14 different renal cell carcinoma cell lines tested; however, it has also shown reaction against a few other cancer lines tested (five of 22): one of six transitional cell carcinoma of the bladder cell lines; one of two breast carcinoma cell lines; two of three colon and bowel cell lines; and one of three testicular carcinomas. A6H reacted with > 90% of the cells in each of the 14 renal cell carcinoma cell lines tested. Antigen recognition by A6H is found to be reduced when cells or membrane preparations containing the antigen are treated with methanol or 100°C for 5 minutes. Despite extensive efforts to immunoprecipitate the renal cell carcinoma-associated antigens recognized by A6H, no success has yet been obtained (Vessella et al., 1985).

Biodistribution studies have shown that A6H targeted three renal cell carcinoma
xenografts (TK-82, TK-39, and TK-177C) unusually well (Vessella et al., 1988). At an antibody dose of 10 μg and a xenograft weight of 100 mg, it is reasonable to expect more than 50% ID/g. Results of biodistribution studies performed using iodine-131- or indium-111-labeled A6H and its F(ab')2 are shown in Tables 1.8 through 1.10.

In preliminary nude mouse radioimmunotherapeutic studies, one 100 μCi dose of iodine-131-labeled A6H significantly retarded the growth of established renal cell carcinoma xenografts, whereas two doses over a period of 3 weeks arrested progression and initiated a long course (< 120 days) of tumor regression (Vessella et al., 1985). Based on successes in the renal cell carcinoma xenograft model, an Investigational New Drug application was acquired for a phase I-II radioimmunoscintigraphy and radioimmunotherapy clinical trial of monoclonal antibody A6H in patients with renal cell carcinoma (Vessella, 1991). These studies were carried out using iodine-131-labeled A6H antibody. In the radioimmunoscintigraphy study, six of eight patients were found to be image positive. In all of the radioimmunotherapy study patients, the estimated absorbed dose to the bone marrow was < 25 cGy. All of the patients tolerated a dose of 50 mCi without signs of significant side effects.

The Warhead to be Delivered: Copper-67

Before a radioisotope is selected for use in diagnostics or therapy, its half-life, type of radioactive decay, and chemical nature must be evaluated. Copper-67 is a radioisotope that has attractive nuclear decay characteristics (Table 1.11) (Raman & Pinajian, 1969) for use in both γ camera imaging of tumors and internal radiation therapy. It decays by β-
Table 1.8

Biodistribution results obtained with $^{131}$I-labeled A6H.
Adapted from Palme et al., 1991.

<table>
<thead>
<tr>
<th>RCC Xenograft</th>
<th>Days after injection</th>
<th>% ID/g</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tumor</td>
<td>Blood</td>
</tr>
<tr>
<td>TK-39</td>
<td>1</td>
<td>26 ± 7</td>
<td>10 ± 3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>46 ± 20</td>
<td>8 ± 2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>44 ± 20</td>
<td>4 ± 2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>17 ± 12</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>TK-82</td>
<td>1</td>
<td>86 ± 21</td>
<td>10 ± 2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>75 ± 17</td>
<td>6 ± 2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>55 ± 32</td>
<td>4 ± 3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>18 ± 14</td>
<td>1 ± 0.4</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SD
### Table 1.9

Biodistribution of A6H and its F(ab')<sub>2</sub> in normal mouse tissues.
Adapted from Chiou, 1989.

<table>
<thead>
<tr>
<th>Days after injection</th>
<th>Tissue: Blood ratio of radiolabel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>131I-labeled</td>
<td></td>
</tr>
<tr>
<td>2-3</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>111In-labeled</td>
<td></td>
</tr>
<tr>
<td>2-3</td>
<td>2.9 ± 1.8</td>
</tr>
<tr>
<td>131I-labeled</td>
<td></td>
</tr>
<tr>
<td>F(ab')&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SD
Table 1.10

*In vivo* localization of $^{111}$In-labeled A6H to TK-82 and a melanoma xenograft. Adapted from Chiou, 1989.

<table>
<thead>
<tr>
<th>Xenograft</th>
<th>Mouse number</th>
<th>Days after injection</th>
<th>Tumor : Blood ratio</th>
<th>% ID/g of tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK-82</td>
<td>3</td>
<td>3</td>
<td>31</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>23</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4</td>
<td>71</td>
<td>209</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>2</td>
<td>94</td>
<td>338</td>
</tr>
<tr>
<td>Melanoma</td>
<td>7</td>
<td>3</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>17</td>
</tr>
</tbody>
</table>
Table 1.11
Copper-67 nuclear decay properties.
Adapted from Mercer-Smith et al., 1988.

<table>
<thead>
<tr>
<th>$T_{1/2}$</th>
<th>Decay mode (%)</th>
<th>Particle energies (%) in keV</th>
<th>Gamma transitions (%) in keV</th>
</tr>
</thead>
<tbody>
<tr>
<td>62 hours</td>
<td>beta (100)</td>
<td>182 (1)</td>
<td>91 (7.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>392 (56)</td>
<td>93 (31.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>484 (23)</td>
<td>184 (48.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>576 (20)</td>
<td>209 (0.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>300 (0.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>309 (0.2)</td>
</tr>
</tbody>
</table>
emission with particle energies sufficiently high to be considered useful for treatment (395 to 577 keV) (Wessels & Rogus, 1984). Copper-67 also decays by γ-emission, with an abundant 184 keV photon, and therefore has the potential for use as an imaging agent. Its half-life of 61.7 hours is also appropriate for use with antibodies (DeNardo et al., 1983). The daughter of copper-67 is the stable zinc-67, which presents no possibility of undesired effects from radioactive decay products. Unlike other radiometals, copper-67 has no known biological pathways for deposition in the bone (DeNardo et al., 1991a). Due to its superior properties, copper-67 is being actively investigated by several groups (Meares, 1986; Smith-Jones et al., 1991; Morphy et al., 1989; Roberts et al., 1990; Zamora et al., 1992; Kukis et al., 1995).

Major producers of copper-67 are Los Alamos National Laboratory, Brookhaven National Laboratory, and the University of Missouri Research Reactor. The procedure for its preparation has been reported (Mercer-Smith et al., 1988). At the Los Alamos National Laboratory, copper-67 is produced by a (p,x) spallation reaction on a 70 g zinc oxide target; the primary nuclear reaction is probably 68Zn(p, 2p)67Cu. Irradiation of the target in the primary proton beam of 600 to 800 MeV protons at a current of 1 mA produces 2 to 4 Ci of copper-67 after 5 to 7 days of bombardment. The copper-67 is produced in high specific activity, typically 15,000 Ci/g. The spallation reaction produces not only copper-67 but also a variety of isotopes of lighter mass than zinc. The large amount of the target material and the presence of isotopes of other elements complicate the purification of copper-67 from the target. In addition, the level of radioactivity is so high that remote handling hot cells must be used to provide adequate shielding during processing of the irradiated targets.
The target material is dissolved in a 30:1 mixture of 3 M H$_2$SO$_4$ : 16 M HNO$_3$. The copper-67 is purified by a series of electrochemical platings that reduce Cu(II) to metallic copper on a platinum working electrode at -0.35 V vs. a Ag/AgCl electrode. This potential reduces only Cu$^{2+}$ to metallic Cu, leaving other metal ions in solution. After electrochemical plating of Cu, the platinum electrode is moved to a different electrochemical cell with fresh electrolyte (0.1 M H$_2$SO$_4$). A potential of +0.35 V is used for stripping the copper into solution by oxidation to the +2 state. The electrochemical stripping and plating process is repeated two times. This sequence of electrochemical reductions and oxidations serves to isolate copper-67 from zinc in the target material and the other isotopes produced by spallation reactions. The copper is stripped, plated onto the electrode, then stripped into a 0.1 M HNO$_3$ solution. The copper solution is applied to a Dowex AG1-X8 anion exchange column and eluted with 2 M HCl, which helps further reduce the concentration of entrained zinc. Zinc forms a negatively charged chloride complex, which is retained by the column material, but copper passes through the column and is collected as $^{67}$CuCl$_2$ in 2 M HCl. Analysis of the purified copper-67 solution by plasma emission spectroscopy indicates that the final concentration of zinc is typically 2 ppm, while the final concentration of all copper isotopes (stable and radioactive) is about 20 ppm. Other metals are below the limit of detection of the emission spectrometer ($<1$ ppm).

The Chelating Molecule: Porphyrin

The remarkable ability of porphyrins to retain bound copper ion and the great stability of synthetic porphyrin ring systems against degradation make them desirable metal chelators.
Naturally-occurring porphyrins complex a variety of metals. Iron porphyrins are present in hemoglobin, myoglobin, and many of the cytochromes. Magnesium porphyrin derivatives are present in the chlorophylls and bacteriochlorophylls. Copper porphyrins are found in the feather pigments of some birds. The nonmetalated porphyrins have found use in tumor phototherapy in much larger quantities (Dougherty, 1984) than would be used for imaging or for radiotherapy. Porphyrins have been selected as copper-67 chelating agents since they fulfill several general criteria for use in radiolabeling antibodies: 1) Metalloporphyrins, which are ubiquitous in nature, present little problem of toxicity at clinically useful concentrations. 2) They can be synthesized with a variety of functional groups suitable for conjugation with proteins or linkers. 3) Porphyrins can be modified to incorporate metals easily. 4) Some porphyrins are resistant to metabolism \textit{in vivo}. 5) Copper porphyrins are very stable to the loss of copper ions. Copper porphyrins are reported to be extremely stable to the loss of the copper dication from the chelate; the stability constant for copper-porphyrin complexes is so high that it cannot be determined (Buchler, 1975). For example, radiocopper does not exchange with copper porphyrin derivatives during two days in 80% acetone (Ruben et al., 1942), and 100% sulfuric acid is required to remove copper from copper porphyrins (Buchler, 1975).

A major drawback to the use of porphyrins has been the slow rates at which the relatively inflexible, planar porphyrins incorporate metal ions. Although Cu(II) is one of the most rapidly incorporated metal ions, the metalation rate in aqueous solutions, in the pH and temperature range that antibodies are stable, is too slow to make the reactions of copper-67 with a protein-linked porphyrin feasible. The only route available for typical porphyrins is
the synthesis of the copper-67 chelate in refluxing dimethylformamide, followed by coupling of the radiolabeled porphyrin complex to the antibody.

It is possible to achieve rapid metal complexation under mild conditions that are necessary for a procedure in which a prepurified porphyrin-antibody conjugate could be labeled with copper-67 immediately before use ("cold kit" concept) using suitably modified synthetic porphyrins. N-alkylation produces greater structural changes within a pyrrolenine ring of the porphyrin than does protonation (Lavallee, 1987). It has been shown that N-substituted porphyrins with aryl substituents at all the meso positions have greatly increased metal complexation rates and the rate is especially favorable for Cu(II) (Bain-Ackerman & Lavallee, 1979). The N-alkyl substitution distorts the plane formed by the four pyrrole nitrogens, allowing for easy metal incorporation (Lavallee et al., 1986). However, after the Cu(II) ion is bound, the N-substituent must be removed to give a highly stable porphyrin complex because metal ions are readily removed from N-substituted metalloporphyrins (Lavallee & Gebala, 1974). When the substituent used is the N-benzyl group, its removal is easy and can be accomplished by mild nucleophiles (Lavallee & Kuila, 1984). This process of metalation and removal of the N-benzyl group causes the internal porphyrin ring to revert back to planarity, thus locking the metal ion in the central cavity. The rate for the overall reaction of metal complexation and N-benzyl group removal in buffered aqueous solution is rapid and independent of Cu(II) concentration (Lavallee et al., 1986). The metalation reaction mechanism for the N-benzyl porphyrins is shown in Figure 1.5.

The N-benzyl substituted synthetic porphyrin of choice for the copper-67 delivery system was N-4-nitrobenzyl-5-(4-carboxyphenyl)-10,15,20-tris(4-sulfophenyl)porphine (N-
Figure 1.5. Mechanism of the metalation reaction of N-benzyl porphyrins. Adapted from Mercer-Smith et al., 1988.
bzHCS$_3$P (Figure 1.6). The carboxyl group serves as a handle for carrying out chemical modifications and for linking to amino groups of antibody/linker molecules via peptide bonds. The presence of the sulfonyl groups leads to increased water solubility of the porphyrin allowing for its use under conditions compatible with antibody integrity. The porphyrin is activated for metalation due to presence of the N-4-nitrobenzyl group.

The reactions to synthesize N-bzHCS$_3$P are shown in Figure 1.7. The first step consists of the formation of 5-(4-methylcarboxyphenyl)-10,15,20-triphenylporphine by condensation of 4-formylmethylbenzoate, benzaldehyde, and pyrrole in a 1:3:4 ratio. Because the condensation method forms porphyrins with various combinations of unsubstituted phenyl and 4-methylcarboxyphenyl groups, the desired porphyrin (the monoester) is isolated and purified by flash chromatography prior to the remaining synthetic steps. The N-benzylation of the porphyrin by diphenyl-4-nitrobenzylsulfonium tetrafluoroborate can form two different porphyrins, N-4-nitrobenzyl-5-(4-carboxyphenyl)-10,15,20-triphenylporphine and N-4-nitrobenzyl-5-[4-(4-nitrobenzyl)carboxyphenyl]-10,15,20-triphenylporphine, depending upon whether or not the porphyrin monoester from step 1 is hydrolyzed before the initiation of step 2. When 5-(4-methylcarboxyphenyl)-10,15,20-triphenylporphine, is the starting material, N-4-nitrobenzyl-5-(4-methylcarboxyphenyl)-10,15,20-triphenylporphine, is prepared by substitution of one of the pyrrole protons. Alternatively the porphyrin free acid, 5-(4-carboxyphenyl)-10,15,20-triphenylporphine (formed by basic hydrolysis of the monoester prepared in step 1), is employed as the starting material in step 2. In this case, two equivalents of diphenyl-4-nitrobenzylsulfonium tetrafluoroborate are used because the free acid is converted to the 4-
Figure 1.6. Structure of N-4-nitrobenzyl-5-(4-carboxyphenyl)-10,15,20-tris(4-sulfophenyl)porphine, N-bzHCS$_3$P.
Figure 1.7. Synthesis of N-bzHCS₃P. Adapted from Mercer-Smith et al., 1988.
nitrophenyl ester under conditions where N-substitution on the pyrrole occurs. These reactions form N-4-nitrobenzyl-5-[4-(4-nitrobenzyl)carboxyphenyl]-10,15,20-triphenylporphine. In step 3, concentrated sulfuric acid treatment of either of the porphyrins formed in step 2 causes: 1) addition of sulfonate groups to the 4-phenyl positions to give water solubility; and 2) hydrolysis of the ester to give the free carboxylate group.

The other porphyrin that has also been used in some of the studies is N-benzyl-5,10,15,20-tetrakis(4-carboxyphenyl)porphine, N-bzHTCPP. The synthesis of this porphyrin has been shown in Figure 1.8. The stages involved in the synthesis are: 1) synthesis of benzylidiphenylsulfonium tetrafluoroborate from benzyl alcohol and phenyl sulfide in methanesulfonic acid, followed by reaction with tetrafluoroboric acid; 2) condensation of 4-formylmethylbenzoate and pyrrole to form 5,10,15,20-tetrakis(4-methylcarboxyphenyl)porphine; 3) reaction with benzylidiphenylsulfonium tetrafluoroborate to form the N-benzyl derivative; and 4) hydrolysis of the ester in base to liberate the free acid groups.

One of the useful physical properties of a porphyrin is its intensive UV-Visible absorption spectrum, since it typically allows for characterization of solutions with concentrations in the micromolar range. The spectral characteristics of N-substituted porphyrins are similar to unsubstituted porphyrins, with molar absorptivities in the Soret region (400-500 nm) of $10^4$ and $10^5$ in the 500-700 nm range. The absorption bands of N-substituted porphyrins are generally red-shifted on the order of 10 nm from bands of corresponding non-N-substituted porphyrins (Lavallee, 1987).
Figure 1.8. Synthesis of N-benzyl-5,10,15,20-tetrakis(4-carboxyphenyl)porphine. Adapted from Mercer-Smith et al., 1988.
Intermediary Linkers Used

The two polymeric molecules used as linkers in the copper-67 delivery system are the Starburst™ dendrimer and poly(lys,ala) 2:1. Dendrimers are a class of three-dimensional, highly ordered macromolecules that can be synthesized with a variety of structural components and molecular weights. These polymers are based upon the application of mathematical progressions to organic synthesis, and thus possess a well-defined molecular topology (Newkome et al., 1992). The dendrimers resemble polymers in certain respects but differ from classical polymers by their extraordinary symmetry, high branching, and high terminal density. Polymerization processes are random in nature and produce a complex mixture of species within a molecular weight range. Starburst™ dendrimers, on the other hand, are prepared using a unique branching strategy that results in polymeric products of specified size, shape, and molecular weight depending on the "generation" of the dendrimer (Tomalia et al., 1985).

Dendrimers are synthesized in a step-wise process, which results in molecules with three distinguishing features: 1) an initiator core; 2) layers of repeating units radially attached to the core, called generations; and 3) an outer surface of terminal functional groups. These reactive end groups allow: 1) controlled molecular weight building, 2) controlled branching, and 3) versatility in design and modification of the terminal end groups. Figure 1.9 outlines the synthesis (Tomalia et al., 1990) of ammonia core amine-terminating polyamidoamine (PAMAM) dendrimers which have been used as linkers in the copper-67 delivery system.

1Starburst is a Trade Mark of Dendritech, Inc., Midland, MI.
Figure 1.9. Steps involved in dendrimer synthesis. Adapted from Tomalia, 1990.
The procedure for the preparation of polyamidoamines uses reiterative cycles of methylacrylate additions followed by ethylenediamine additions (Figure 1.9). Step A of the synthesis begins with an ammonia core and involves exhaustive Michael addition to methyl acrylate. This addition occurs very rapidly and in high yield with essentially complete selectivity and no amidation at room temperature. Step B involves addition of this triester intermediate to a large excess of ethylenediamine at room temperature to produce the terminal triamine core cell. Repeating the sequence of steps A and B leads, via a hexaester (generation=0.5), to a hexamine (generation=1.0). Continuing the sequence of steps A and B produces increasingly higher generations. Dendrimer growth beyond generation 3 simply introduces more concentric tiers of interior cells which provide scaffolding for surface groups whose number follows a geometric progression (Figure 1.10). The synthesis of up to generation 10 has now been accomplished (Tomalia, 1991). Ideal branching growth would produce dimensionally precise surfaces with a defined number of surface groups. The systematic change in the features of these dendrimers is shown in Table 1.12. The tridendron polyamidoamine dendrimer series increases its diameter by approximately 10 Å per generation, evolving from a disk-like shape (generations 0 to 2) to an oblate spheroid (generations 3, 4) to a nearly symmetrical spheroid at generations 5 and higher (Naylor et al., 1989).

Dendrimers have been prepared with different building blocks and have found utility in several different applications. Dendrimers have been synthesized with a wide variety of interior (ruthenium, silicon, ethers, thioethers, etc.) as well as exterior functionalities (Tomalia, 1993; Newkome et al., 1990; Serroni et al., 1992; Kim, 1992; Frechet, 1994; Jin
Figure 1.10. Tethering successive branch and repeat units around a core produces a series of generations. Adapted from Tomalia, 1991.
Table 1.12

Characteristics of PAMAM Starburst™ dendrimers.
Adapted from Tomalia et al., 1990.

<table>
<thead>
<tr>
<th>Generation</th>
<th>Molecular weight</th>
<th>Monomer units</th>
<th>Terminal groups</th>
<th>Diameter* (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>359</td>
<td>3</td>
<td>3</td>
<td>10.8</td>
</tr>
<tr>
<td>1</td>
<td>1043</td>
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<td>6</td>
<td>15.8</td>
</tr>
<tr>
<td>2</td>
<td>2411</td>
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<td>12</td>
<td>22.0</td>
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<td>3</td>
<td>5147</td>
<td>45</td>
<td>24</td>
<td>31.0</td>
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<tr>
<td>4</td>
<td>10619</td>
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*The diameter was determined by size exclusion chromatography
et al., 1993). It is possible to synthesize dendrimers with hydrophobic surfaces and hydrophilic interiors (Worthy, 1988). It is also possible to incorporate into the initiator core a metal atom or a chemical group that absorbs light at visible wavelengths, giving highly colored materials. By starting with an initiator core that is a linear polymer, such as polyethylenimine, it is possible to produce rod-like structures (Tomalia, 1991). One of the largest all-hydrocarbon molecules ever made, C_{1134}H_{1146}, has topology similar to that of a dendrimer constructed from phenylacetylene building blocks (Dagani, 1993). Damha and his colleagues have reported the success in training RNA to form an intricate branching molecule referred to as a biodendrimer (Hudson & Damha, 1993). Carboxylated Starburst™ dendrimers have been used as calibration standards for aqueous size exclusion chromatography (Dubin et al., 1992). A peptide dendrimer with a record high molecular weight of 24,205 Da has been synthesized by Rao and Tam (1994).

Since Starburst™ dendrimers seem to behave as micelles, yet consist of a single molecule, they form attractive drug delivery agents (Tomalia, 1991). Dendrimers are currently being evaluated for use in selective adsorption and catalysis, catalytic detoxification reagents against chemical weapons, for separation of optical isomers, and as artificial cells, filters, membranes, adhesives, and chromatographic materials (Tomalia, 1991). Polyamidoamine dendrimers conjugated to the chelator, 2-(4-isothiocyanatobenzyl)-6-methyl-diethylenetriaminepentaacetic acid, and complexed with gadolinium, form good magnetic resonance imaging agents. These conjugates provide better results than those obtained with the use of serum albumins, polylysine, or dextran by a factor of two (Wiener et al., 1994). Polyamidoamine cascade polymers have also been shown to be effective in
transfection of cells in culture (Haensler & Szoka, 1993). Constructing antibody immunoconjugates using dendrimers as intermediate linker molecules has been reported for the site-specific delivery of boron for boron neutron capture therapy (Barth et al., 1994).

Dendrimers have been used as linkers in the copper-67 delivery system because of their uniformity and hence their ease of use as chemical reagents. Their availability in a range of generations gives the opportunity to fine-tune and design the optimum delivery system. These reagents obviate problems of size distribution and side chain flexibility because of the symmetry, uniform surface chemistry, size, shape, and the rigidity of the interior topology (Tomalia et al., 1985; Tomalia et al., 1990; Wiener et al., 1994). Also dendrimers are virtually monodisperse, much more so than linear polymers of comparable molecular weight, such as polylysines (Tomalia et al., 1990).

A linear polymer, poly(lys,ala)2:1 (MW ~ 50,000 Da) was also used in the copper-67 delivery system in order to compare a linear polymer, such as poly(lys,ala) to a spherical dendrimer. This poly(lys,ala) is a random polymer formed by polymerization of lysine and alanine in a ratio of 2 to 1.

Poly-L-lysine has been used as a linker in several different studies (Ryan et al., 1992; Clegg et al., 1990; Slinkin et al., 1990). It has been shown that polylysine interacts with many cells in both specific and unusual ways (Arnold, 1985). Early studies indicated that polylysine possessed antiviral and antibacterial activities (Shahtin & Katchalski, 1962; Bichowski-Slomnicji et al., 1956). Some studies have indicated that polylysine has an affinity for cancerous tissue (Anghileri et al., 1976) and is capable of enhancing the uptake of macromolecules such as albumin and peroxidase into cells (Arnold, 1985).
In conclusion, pharmacologically active synthetic compounds and also natural substances can function as powerful therapeutic agents if the relevant tissues, cells, or subcellular targets can be reached. The beneficial effects fully depend on this prerequisite, especially since the therapeutic potential in certain cells and tissues coincides in most cases with toxicity of the same agents for other cells and tissues. Delivering drugs to a specific site by packaging them with appropriate carrier systems can improve the utility of pharmaceutical agents. Some of the most widely studied systems include those involving the use of antibodies, polymers, liposomes, erythrocytes, microspheres, nanoparticles, and implantable infusion pumps. Antibody-mediated drug delivery appears to be an approach with potential utility for the delivery of cytotoxic drugs. Of the various agents that can be delivered, radionuclides seem to provide more advantages than others. Furthermore, copper-67 provides the additional advantage of being both a $\beta$- and a $\gamma$-emitter, making it useful for therapeutic as well as imaging applications. Renal cell carcinoma is a good tumor model for evaluation of the antibody-mediated delivery approach and may benefit from an agent that can help in the early diagnosis and therapy of this disease.

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CHAPTER 2

SYNTHESIS, BIODISTRIBUTION, AND AGGREGATION STUDIES ON ANTIBODY-PORPHYRIN CONJUGATES

Introduction

Antibody and antibody fragments are attractive vehicles for targeted drug delivery to tumors because of the inherent specificity of the antibody-antigen interaction (Baldwin, 1982). The first study using this particular approach for tumor targeting was documented in 1953 by Pressman and Korngold (1953). Certain biological molecules, such as monoclonal antibodies directed against tumor-associated antigens, possess the ability of directing cytotoxic drugs, toxins, and radionuclides specifically to the tumor site. This method of delivery is under intense investigation because of the advantages offered over conventional methods currently available to clinicians. First, there is an increase in the tumor concentration of the agent being delivered. Second, localization of the agent at the specific site in the body leads to decreased side effects. In addition to these advantages, radioimmunoconjugates enjoy an additional benefit, in that internalization of the radionuclide into cells is not essential for activity.

Copper-67 is being studied as a potential nuclear medicine agent because of its favorable decay characteristics (Raman & Pinajian, 1969). Also, its half-life of 62 hours is
considered optimal for use with antibodies (DeNardo et al., 1983). The daughter of copper-67 is the stable isotope, zinc-67, which presents no possibility of undesired effects from radioactive decay products.

The approach described here involves the use of synthetic porphyrins as chelating agents for the radiometal that can then be covalently coupled to antibodies or antibody fragments for targeted delivery purposes. Previous work has shown the utility of N-4-nitrobenzyl-5-(4-carboxyphenyl)-10,15,20-tris(4-sulfophenyl)porphine (N-bzHCS$_2$P, 1) and N-benzyl-5,10,15,20-tetrakis(4-carboxyphenyl)porphine (N-bzHTCPP, 2) as powerful copper chelating agents for this application (Roberts et al., 1987; Mercer-Smith et al., 1988; Roberts et al., 1989; Roberts et al., 1990; Mercer-Smith et al., 1991). The key structural feature of these porphyrins (Figure 2.1) is the presence of the N-benzyl substituent which distorts the normally planar macrocycle and allows rapid metal ion incorporation from the opposite face (Lavallee et al., 1986). This allows for metalation of the porphyrin under mild conditions and in the presence of the antibody, a desirable synthetic sequence for radiopharmaceutical preparation. The N-benzyl group is lost as the alcohol, and the macrocycle reverts to planarity. The resultant metal chelate is very stable to loss of the copper ion under simulated physiological conditions (Roberts et al., 1987), confirming previous reports on the stability of copper porphyrins in general (Ruben et al., 1942; Buchler, 1975).

Several reports in the past have demonstrated the ability to conjugate the porphyrins to polyclonal antibodies (Roberts et al., 1987; Mercer-Smith et al., 1988; Roberts et al., 1989; Roberts et al., 1990), monoclonal anti-Thy 1.2 antibody (Mercer-Smith et al., 1990),
<table>
<thead>
<tr>
<th>R</th>
<th>R1</th>
<th>R2</th>
<th>Name</th>
</tr>
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<td>CO$_2^-$</td>
<td>SO$_3^-$</td>
<td>N-4-nitrobenzyl-5-(4-carboxyphenyl)-10,15,20-tris(4-sulfophenyl)porphine N-bzHCS$_3$P, 1</td>
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<td>CO$_2^-$</td>
<td>N-benzyl-5,10,15,20-tetrakis(4-carboxyphenyl)porphine N-bzTCP, 2</td>
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<td>Br</td>
<td>N-methyl-5,10,15,20-tetrakis(p-bromophenyl)tetraphenylporphine N-CH$_3$TPPBr$_4$, 3</td>
</tr>
</tbody>
</table>

Figure 2.1. Structures of synthetic porphyrin chelators.
and small autoantigenic peptides (Mercer-Smith et al., 1991). This coupling strategy has been extended to include the modification of monoclonal A6H, an antibody raised against human renal cell carcinoma (RCC) (Moon et al., 1985; Vessella et al., 1988).

RCC is a clinically relevant tumor model, constituting approximately 85% of all primary kidney tumors and 2 to 3% of all cancers in adults (Millan, 1989). La Vecchia has reported a periodic increase in mortality resulting from cancer of the kidney from 1955 to 1990 in most European countries (La Vecchia et al., 1992). In most cases, RCC is sporadic, but there are also familial forms, sometimes associated with von Hippel-Lindau syndrome (Millan, 1989). There are nearly 24,000 new cases of RCC annually, of which about 40% present with known metastatic disease, frequently because early disease is without symptoms (Patel & Lavengood, 1978). The remaining 60% of patients have apparently localized disease and undergo a nephrectomy, which is the treatment of choice and curative for truly kidney-confined tumors. Surgical tumor resection, most appropriately with the affected organ, is the only curative therapy available for RCC at this time (Wirth, 1993). Reports suggest that 13 to 21% of patients show distant metastases and 12 to 22% show regional lymph node metastases when diagnosed (Bassil et al., 1985; Hermanek & Schrott, 1990; Robson, 1963). RCC patients are among those with the highest spontaneous remission rates, 7%, of all human tumors. Unfortunately, one-third of these patients develop subsequent metastatic disease. Chemotherapy has been largely unsuccessful, with vinblastine being the most active agent, but response rates rarely exceed 16% (deKernion, 1983). Immunotherapy and hormonal therapy have also been largely unsuccessful; several studies are still in progress (Wirth, 1993). However, detection and earlier diagnosis of renal
tumors has improved recently due to imaging techniques such as sonography and computed
tomography (Smith et al., 1989). Unfortunately, these techniques lead to detection of both
benign and malignant renal masses, some of which do not require therapy. Thus, new
systemic therapies and specific diagnostic tools for RCC are critically needed.

The biodistribution of the two copper-67-labeled porphyrins, as well as of $^{67}$CuCS$_3$P-A6H conjugate, has been performed in human RCC xenograft-bearing nude mice.
Radiolabeled porphyrins were included in these studies to outline their site(s) of localization
should they become detached from the antibody. The goal was to determine the ability of
the modified antibody to localize at its antigenic determinant \textit{in vivo}. From the results
obtained, it was obvious that the biodistribution was primarily dictated by factors other than
immunoreactivity of the antibody. Thus, quantitative aggregation studies have been
performed to investigate the factors that might be responsible for aggregation and some
possible measures that can be taken to eliminate these aggregates.

\textbf{Experimental}

Materials and Methods

\textit{BALB/c} nude (nu/nu) male mice were purchased from Simonson Laboratories
(Gilroy, CA). Water and laboratory chow were available \textit{ad libitum} throughout the studies.
All animal studies were performed in compliance with guidelines established in the "Guide
for the Care and Use of Laboratory Animals," published by the U.S. Department of Health
and Human Services, NIH Publication 85-23, revised in 1985. Animals were housed in
facilities accredited by the American Association for Accreditation of Laboratory Animal
Care (AAALAC). Human RCC xenograft TK-82 used in this study has been established and characterized earlier (Clayman et al., 1985) and was maintained by serial passages in nude mice. ACHN cells, originally obtained from American Type Culture Collection (Rockville, MD), were maintained in minimum essential medium, α-modification (α-MEM) culture medium supplemented with 10% serum (5% fetal calf serum and 5% α-calf serum) and antibiotics (10 mL of antibiotic-antimycotic solution per 1 L of medium) by passage every 2 to 3 days. A6H was produced as previously described (Moon et al., 1985; Vessella et al., 1985). Distilled-in-glass grade N,N-dimethylformamide (DMF) from EM Science (Cherry Hill, NJ) was used. CuCl₂ was obtained from Aldrich Chemical Co. (Milwaukee, WI). Electrophoresis supplies were purchased from BioRad Laboratories (Richmond, CA). All other reagents, chemicals, the polyclonal antibody, rabbit immunoglobulin G (RIgG), and chromatography and cell culture supplies were purchased from Sigma Chemical Co. (St. Louis, MO). Water purified by a Barnstead Nanopure II system with > 17 mΩ resistivity was used for all applications. Counting of radiolabeled samples was done using a Micromedic® 4/200plus Automatic Gamma Counter with the spectral window that extended from 73-171 keV or with a LKB 1282 Gamma Counter.

Copper-67

Carrier-free copper-67 was obtained as ⁶⁷CuCl₂ in 2 M HCl from either Los Alamos National Laboratory (Los Alamos, NM) or the University of Missouri Research Reactor (Columbia, MO). The acid was evaporated to dryness under a flow of air or argon with gentle heating, and the radionuclide was taken up into DMF for metalation.
Synthetic Procedures

Preparation of $^{67}$CuTPCP and $^{67}$CuCS$_3$P

N-bzHTCPP and N-bzHCS$_3$P were synthesized and characterized as published elsewhere (Mercer-Smith et al., 1988). The metalation mechanics and kinetics to form copper porphyrins from N-benzyl porphyrins have been previously described (Lavallee et al., 1986; Mercer-Smith et al., 1988; Schulte et al., 1991). All metalation reactions were performed in dim light using acid-washed glassware to prevent inadvertent porphyrin metalation or photooxidation.

$^{67}$CuTPCP was prepared from N-bzHTCPP by a modification of the methods previously described (Mercer-Smith et al., 1988). A 10 μL portion of N-bzHTCPP (5.9 x $10^{-2}$ mg, 6.7 x $10^{-5}$ mmol) in DMF was placed in a conical vial equipped with a magnetic stirrer. To this dark green liquid was added 350 μL of borate-buffered saline (BBS, 0.1 M borate, 0.08 M NaCl, pH 8.5), and 360 μL of $^{67}$CuCl$_2$ (15.2 mCi) in DMF. The solution was magnetically stirred for 1.25 hours. Then 16.6 μL of CuCl$_2$ (1.1 x $10^{-2}$ mg, 6.6 x $10^{-5}$ mmol) was added to the solution. The mixture was magnetically stirred on a 40°C heating block for an additional 30 minutes. The solution took on the deep orange color of CuTPCP. The entire volume was applied to a 3.9 x 2.5 cm CM Sepharose CL-6B column that had been equilibrated with phosphate-buffered saline (PBS, 10 mM phosphate buffer, 120 mM NaCl, pH 7.4). Fraction 4 (1.5 mL) from the column contained $^{67}$CuTPCP and was orange in color. The solution was sterilized by filtration through a sterile 0.2 μ Acrodisc 13 filter into a sterile crimp seal vial.

$^{67}$CuCS$_3$P was synthesized using N-bzHCS$_3$P (8.7 x $10^{-2}$ mg, 6.7 x $10^{-5}$ mmol) and
$^{67}$CuCl$_2$ (15 mCi) by a method identical to that used to prepare $^{67}$CuTCPP. The orange-colored $^{67}$CuCS$_3$P was eluted in fraction 4 (2.0 mL) from the ion exchange column. Finally the solution was filtered through the 0.2 μ filter into a sterile crimp seal vial.

**Preparation of Porphyrin-Antibody Conjugates**

The synthesis of N-bzHCS$_3$P-antibody conjugates followed previous preparation schemes (Roberts et al., 1989) as follows (Figure 2.2): A 20 μL portion of a solution of N-bzHCS$_3$P (1.8 mg, $1.3 \times 10^{-3}$ mmol) in DMF (200 μL) was placed in an acid-washed, conical vial equipped with a magnetic stirrer. To this dark green liquid was added 10 μL each of a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (2.6 mg, $1.3 \times 10^{-2}$ mmol) and a solution of N-hydroxysuccinimide (NHS) (1.5 mg, $1.3 \times 10^{-2}$ mmol), both in 12.5 mM sodium dihydrogen phosphate, pH 6 (100 μL). The activation step was allowed to proceed at room temperature in the dark (to prevent porphyrin photooxidation) for 1 hour. For preparation of N-bzHCS$_3$P-A6H, 410 μL of an A6H solution (4.87 μg/μL in PBS, pH 7.4) was added to the activated porphyrin mixture. For preparation of porphyrin-polyclonal antibody conjugates, 200 μL of a 10 mg/mL solution of RIgG in BBS was used. In both cases, the coupling step was allowed to proceed for 4 hours in the dark at room temperature.

The porphyrin-antibody conjugates were purified from the excess free porphyrin in the reaction mixture using Sephadex G25 and eluting with BBS. (Phosphate buffers interfere with the later metalation step by producing insoluble salts.) The bright green eluent was stored on ice until further use.
Figure 2.2. Synthesis of N-bzHCS$_3$P-A6H and its metalation to CuCS$_3$P-A6H.
The amount of porphyrin attached to protein was determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions (Laemmli, 1970). A 5 μL aliquot of the reaction mixture was mixed with 5 μL of sample buffer (0.6 mM Tris, 10% glycerol, 2% SDS, pH 6.8), and the samples were loaded on a tube gel (6.5 x 0.2 cm) using a running gel of 7% polyacrylamide content at 2 mA per gel for the first 15 minutes and later increased to 8 mA per gel. The gels were scanned at 435 nm for porphyrin absorbance. The area under the peaks was integrated, and the areas were used to calculate coupling yields.

Metalation of the Porphyrin-Antibody Conjugates

All glassware was scrupulously acid washed to eliminate any metal ions that might metalate the porphyrin prematurely. For biodistribution studies, the purified N-bzHCS₃P-A6H conjugate was metalated with $^{67}$CuCl₂ at a 1:1 copper:porphyrin ratio at 40°C for 60 minutes. For aggregation studies, A6H and RIGG conjugates were metalated with either $^{67}$CuCl₂ or CuCl₂. Metalation with $^{67}$CuCl₂ was carried out at 40°C for 60 minutes. Metalation with stable copper (1 mM CuCl₂) at 40°C was conducted under various conditions as indicated. Following metalation, the conjugates were subjected to vacuum dialysis into PBS, pH 7.4, containing 0.05 M sodium citrate to complex any free copper. Unmetalated preparations were vacuum dialyzed under similar conditions with the exclusion of sodium citrate from the dialysis buffer. Metalation yield was calculated for preparations metalated with copper-67 by counting the sample before and after vacuum dialysis.
In Vitro and in Vivo Testing

Thin Layer Chromatography

The A6H conjugate was analyzed by thin layer chromatography (TLC) to estimate the level of protein-bound activity (Yamazaki et al., 1988). The radiolabeled preparation was spotted on aluminum backed silica gel plates that were developed in either acetone or 80% methanol. These were then cut into the top two-thirds and bottom one-third portions and were counted in the gamma counter.

Cell Binding Assay

A cell binding assay was performed to determine the immunoreactivity of the radiolabeled antibody (Vessella et al., 1988). Briefly, approximately 10,000 counts per minute (cpm) of the radioimmunoconjugate (contained in 0.1 mL PBS) were added to 2 x 10^6 RCC cells suspended in 900 μL of 1% bovine serum albumin (BSA) in PBS. Cells from a renal cell carcinoma cell line, ACHN, were obtained from a confluent culture. These cells were used due to their excellent reactivity against A6H (Vessella et al., 1985). Freshly trypsinized cells were washed three times with 1% BSA in PBS before use. Each tube was counted in a gamma counter to obtain Total Counts. The mixture was incubated at room temperature for 1 hour with rotary inversion mixing. The cells were pelleted by centrifugation, the supernatant decanted, and the cells resuspended in 1 mL PBS. The counts remaining associated with the cells, Bound Counts, were quantitated in the gamma counter. The percent immunoreactivity was calculated as Bound Counts/Total Counts x 100.
RCC Xenografts

BALB/c nude (nu/nu) male mice bearing the TK-82 RCC xenograft were used for the biodistribution studies as previously described (Vessella et al., 1988). The TK-82 xenograft does not grow in tissue culture but has been widely used in A6H biodistribution studies (Chiou et al., 1988; Chiou, 1989) due to A6H's preferential binding (Vessella et al., 1988). For biodistribution studies, a 25-mg piece of TK-82 xenograft tissue was implanted subcutaneously behind the right shoulder; the incision was closed with surgical staples. Tumor growth rate was moderately fast with tumors reaching about 100 mg within 4 weeks. Ideally, biodistribution studies are performed on subcutaneous tumors ranging in size from 50 to 250 mg.

Biodistribution Protocol

Nude mice bearing TK-82 tumors received a tail vein injection (1 to 2 μCi) of one of the following three preparations: 1) $^{67}$CuCS$_3$P-A6H immunoconjugate; 2) $^{67}$CuTCPP; or 3) $^{67}$CuCS$_3$P. Biodistribution studies were performed at either two (20 and 45 hours) or three (4, 24, and 48 hours) time points postinjection of the radiopharmaceuticals. Each time point consisted of five animals. For biodistribution, various samples (blood, tumor, muscle, lung, kidney, spleen, liver, intestine, and stomach) were recovered and weighed at the time of sacrifice, and the copper-67 activity was quantitated in a gamma counter. Whenever possible, urine was also collected from the bladder and was handled in a similar manner. The percent injected dose per gram of tissue (% ID/g) was calculated for each specimen.
Determination of Aggregates

**Size exclusion HPLC.** Gel permeation chromatographic (GPC) analysis of all conjugates and unmodified antibodies was carried out using a Hitachi HPLC system equipped with a variable wavelength UV-Vis detector. A Bio-Silect SEC 250-5 column (300 x 7.8 mm) along with a Bio-Select SEC 250 guard column (50 x 7.8 mm) was used. The following preparations were analyzed for their aggregation pattern: 1) unmodified A6H; 2) A6H subjected to the coupling protocol in the absence of porphyrin and coupling reagents; 3) A6H subjected to the coupling protocol in the absence of only the coupling reagents; 4) N-bzHCS$_3$P-A6H; 5) CuCS$_3$P-A6H; 6) $^{67}$CuCS$_3$P-A6H; 7) unmodified RIgG; 8) N-bzHCS$_3$P-RIgG; 9) CuCS$_3$P-RIgG; and 10) $^{67}$CuCS$_3$P-RIgG. All conjugates were analyzed immediately after vacuum dialysis and several times over a 1-week period. Samples were centrifuged (12,500 x g, 4°C, 2 to 3 minutes) to eliminate any particulate material before injecting on the HPLC column. Analysis was carried out with phosphate buffer (0.1 M sodium phosphate, 1 mM EDTA, 2 mM sodium azide, pH 6.8) as the mobile phase with a flow rate of 1 mL/min. The percent aggregation was calculated using the area under the monomer and aggregate peaks. To determine the error associated with replicate aggregation measurements, 20 µL of a solution of CuCS$_3$P-RIgG was injected 10 times.

**Attempts to decrease aggregates.** Ultracentrifugation to separate the aggregates from monomeric antibody was carried out in a Beckman Ultracentrifuge XL-90 using a 50 Ti rotor at 4°C at 145,000 x g. Following this process, a sample of the supernatent was withdrawn and was injected on the HPLC.

Ultrasonication of the conjugates was carried out in a Branson 32 ultrasonicator.
at room temperature for 20 minutes. A nonionic surfactant, 3-[(3-Cholamidopropyl)-
dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), at a final concentration of
3\%, was mixed with a solution of N-bzHCS\_3P-R\_IgG to study its ability to disassociate
aggregates. Also, 2 M NaCl and glycerol were tested for this ability by mixing with the
same conjugate in a 1:1 ratio (v/v), and all these solutions were injected on the HPLC both
before and after the treatment.

Molecular Modeling

The molecular modeling studies were performed on N-benzyl porphyrins and non-N-
benzyl porphyrins on Silicon Graphics Iris or Indigo work stations. The molecules were
constructed and visualized using the builder module of the Insight II software obtained from
Biosym Technologies, Inc. (San Diego, CA). Minimizations and dynamics were performed
using Discover software which was also obtained from Biosym Technologies, Inc.
Porphyrin structures were first minimized by using the steepest gradient for 1250 iterations.
To obtain a global minimum, molecular dynamics simulations were used. Dynamics were
initialized at 3000 K with gradual reduction in temperature to 300 K over a period of 140
picoseconds. The lowest energy conformer obtained in the molecular dynamics simulation
was further minimized by 3750 iterations. The lowest energy conformers obtained for the
N-benzyl and non-N-benzyl porphyrins were visually compared to analyze their
conformational differences.
Results

During the radiolabeling of the free porphyrins, column recovery of $^{67}$CuTCPP was 10.1 mCi with 1.68 mCi of colorless copper-67 activity retained on the column. After the final filtration step, 7.67 mCi of $^{67}$CuTCPP was obtained. For N-bzHCS$_3$P, the column recovery of $^{67}$CuCS$_3$P was 12.77 mCi with 1.14 mCi of unincorporated copper-67 retained on the column. The final yield of $^{67}$CuCS$_3$P, following filtration, was 10.8 mCi. Thus, the incorporation of copper-67 was about 86% for N-bzHTCPP and about 92% for N-bzHCS$_3$P. Porphyrin-antibody conjugates were prepared with a coupling efficiency of 25 to 30% as determined by SDS-PAGE, which is the equivalent of 2 to 3 porphyrins/antibody. Radiometalation yield was 41% for $^{67}$CuCS$_3$P-A6H and 27% for the $^{67}$CuCS$_3$P-RiG conjugate. The specific activity of the $^{67}$CuCS$_3$P-A6H conjugate was 0.88 mCi/mg of protein.

TLC analysis of radiolabeled A6H showed that the majority of radioactivity remained at the origin. Strips developed in 80% methanol resulted in an average of 98% activity at the origin, whereas those developed in acetone resulted in an average of 91% activity, confirming that most of the radioactivity was bound to the protein and very little free copper-67 or $^{67}$CuCS$_3$P was present.

In the cell binding assay, 75% of the total radioactivity remained associated with the cell pellet. This is about 25% above the minimal immunoreactivity (50%) typically desired for injection (Vessella et al., 1988).

Tables 2.1 and 2.2 outline the biodistribution of $^{67}$CuTCPP and $^{67}$CuCS$_3$P, respectively. As seen there and in Figures 2.3 and 2.4, both porphyrins show very similar
Table 2.1

Biodistribution of $^{67}$CuCS$_3$P in nude mice

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<th>Tissue</th>
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<tr>
<td>Blood</td>
<td>19.6 ± 1.9</td>
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<td>Tumor</td>
<td>10.9 ± 1.8</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.6 ± 0.4</td>
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<tr>
<td>Lung</td>
<td>13.8 ± 2.4</td>
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<tr>
<td>Kidney</td>
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<tr>
<td>Spleen</td>
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<tr>
<td>Liver</td>
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<tr>
<td>Intestine</td>
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<tr>
<td>Stomach</td>
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<tr>
<td>Urine</td>
<td>3.0 ± 1.3</td>
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Values are expressed as the mean ± standard deviation.
Table 2.2

Biodistribution of $^{67}$CuTCPP in nude mice

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<th>Percent Injected Dose per Gram ± SD</th>
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</tr>
<tr>
<td>Muscle</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Lung</td>
<td>9.3 ± 1.1</td>
</tr>
<tr>
<td>Kidney</td>
<td>31.4 ± 7.1</td>
</tr>
<tr>
<td>Spleen</td>
<td>15.2 ± 1.7</td>
</tr>
<tr>
<td>Liver</td>
<td>43.8 ± 5.8</td>
</tr>
<tr>
<td>Intestine</td>
<td>7.4 ± 0.8</td>
</tr>
<tr>
<td>Stomach</td>
<td>3.3 ± 2.1</td>
</tr>
<tr>
<td>Urine</td>
<td>3.3 ± 1.2</td>
</tr>
<tr>
<td>Tumor:Blood</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± standard deviation
Figure 2.3. Graphical representation of biodistribution of $^{67}$CuCS$_2$P in selected organs.
Figure 2.4. Graphical representation of biodistribution of $^{67}$CuTCPP in selected organs.
behavior with respect to their localization in various organs over a 48-hour period. Liver and kidney showed the highest levels of radioactivity, which peaked at or around 24 hours in the case of the kidney but decreased with time in the liver. For both porphyrins, the highest uptake after 4 hours was seen in the liver. At 4 hours postinjection, the tumor localization was less than that seen in the blood, but the tumor localization was greater than the blood localization after 24 hours postinjection. This resulted in a tumor:blood ratio which increased with time to 3.4 for $^{67}$CuCS$_3$P and 4.1 for $^{67}$CuTCPP. Spleen, intestine, and lung also showed significant localization of copper-67 activity. Levels in stomach and urine were quite low.

A comparison of the localization of the copper-67-labeled porphyrins reveals some differences in the uptakes of the two porphyrins. In the case of the $^{67}$CuTCPP, tumor localization increases slightly from 24 to 48 hours postinjection. However, the $^{67}$CuCS$_3$P uptake in the tumor remains almost constant from 24 to 48 hours postinjection. $^{67}$CuTCPP shows much greater uptake in the kidneys than does $^{67}$CuCS$_3$P at all time points studied. The tumor:blood ratios for $^{67}$CuCS$_3$P and $^{67}$CuTCPP are similar for the two porphyrins at the 4 hour and 24 hour time points; however, the $^{67}$CuTCPP tumor:blood ratio at 48 hours is greater than the $^{67}$CuCS$_3$P at that time point. After 4 hours, the $^{67}$CuCS$_3$P activity in the liver was higher than that of the blood, whereas both liver and kidney had higher activity than blood when $^{67}$CuTCPP was administered. However, for either porphyrin, the blood activity cleared rapidly such that levels in the tumor, liver, spleen, lung and kidney were all lower than that associated with the blood after 24 and 48 hours.

Significant differences in the biodistribution patterns for $^{67}$CuCS$_3$P (48 hours after
administration) and $^{67}$CuCS$_3$P-A6H (45 hours postinjection) are seen in Figure 2.5. Unlike $^{67}$CuCS$_3$P, the A6H conjugate showed high uptake in liver and spleen at 20 hours postinjection (57% and 23% ID/g, respectively). This high localization was found to persist through the 45-hour time point (Table 2.3). Both these organs showed activity levels much higher than those seen after administration of the free porphyrins. On the other hand, activity in the kidney was minimal after administration of the antibody conjugate. Other organs tested contained very low activity. The tumor tissue accumulated up to 12% ID/g, which translates to a tumor:blood ratio at 20 and 45 hours of 9.5 and 15.8, respectively.

Results from aggregation studies have been shown in Tables 2.4 to 2.8. A representative chromatogram obtained after injection of $^{67}$CuCS$_3$P-R1gG is shown in Figure 2.6. The peak at 9.37 minutes represents monomeric antibody, and the peaks at 7.45 and 8.04 minutes represent aggregated protein. Multiple injections of a solution of CuCS$_3$P-R1gG showed that the retention times are within ± 0.05 minutes of each other. The error in aggregation values for multiple injections of the same preparation of CuCS$_3$P-R1gG was found to be ± 2%. As seen in Table 2.4, a frozen solution of A6H, when thawed and analyzed by HPLC, did not contain any aggregates. In direct contrast, freshly reconstituted solutions of R1gG always showed 18 to 22% aggregation. A6H stirred without porphyrin or coupling reagents showed no aggregation. Mixing the porphyrin with A6H in the absence of coupling reagents still did not cause any aggregation. Covalent attachment of the porphyrin, however, had a considerable influence on the aggregation tendencies of both antibodies. With the attachment of 1.6 porphyrins/A6H, 29% aggregation was observed. This number increased to 38% after metalation with stable copper. Metalation with
Figure 2.5. Comparison of the uptake of $^{67}\text{CuCS}_3\text{P}$ (48 hours) and $^{67}\text{CuCS}_3\text{P}$-A6H (45 hours postinjection) in selected organs.
Table 2.3

Biodistribution of $^{67}$CuCS$_3$P-A6H in nude mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Percent Injected Dose per Gram ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 h</td>
</tr>
<tr>
<td>Blood</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Tumor</td>
<td>11.9 ± 1.6</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Lung</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.9 ± 0.7</td>
</tr>
<tr>
<td>Spleen</td>
<td>23.4 ± 6.6</td>
</tr>
<tr>
<td>Liver</td>
<td>56.6 ± 15.1</td>
</tr>
<tr>
<td>Intestine</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>Urine</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Tumor:Blood</td>
<td>9.5</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± standard deviation
Table 2.4

Aggregation levels for A6H\(^a\) and R\(\text{IgG}\)\(^b\) conjugates immediately after preparation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Aggregates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6H</td>
<td>0</td>
</tr>
<tr>
<td>Stirred A6H(^c)</td>
<td>0</td>
</tr>
<tr>
<td>Uncoupled A6H + N-bzHCS(_3)P</td>
<td>0</td>
</tr>
<tr>
<td>N-bzHCS(_3)P-A6H</td>
<td>29</td>
</tr>
<tr>
<td>CuCS(_3)P-A6H</td>
<td>38</td>
</tr>
<tr>
<td>(^{67})CuCS(_3)P-A6H</td>
<td>40</td>
</tr>
<tr>
<td>R(\text{IgG})</td>
<td>20</td>
</tr>
<tr>
<td>N-bzCS(_3)P-R(\text{IgG})</td>
<td>38</td>
</tr>
<tr>
<td>CuCS(_3)P-R(\text{IgG})</td>
<td>47</td>
</tr>
<tr>
<td>(^{67})CuCS(_3)P-R(\text{IgG})</td>
<td>47</td>
</tr>
</tbody>
</table>

\(^a\)1.6 Porphyrins/Antibody
\(^b\)2.4 Porphyrins/Antibody
\(^c\)A solution of A6H that was stirred in the absence of coupling reagents and porphyrin.
Table 2.5

Correlation between the number of porphyrins/antibody and aggregation using CuCS₃P-RiG

<table>
<thead>
<tr>
<th>Porphyrins/Antibody</th>
<th>Aggregates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2</td>
<td>50</td>
</tr>
<tr>
<td>8.7</td>
<td>60</td>
</tr>
<tr>
<td>18.3</td>
<td>68</td>
</tr>
</tbody>
</table>
Table 2.6

Effect of the porphyrin:copper ratio used in metalation on aggregate formation using CuCS$_3$P-RlG$^a$

<table>
<thead>
<tr>
<th>Porphyrin:Copper</th>
<th>Aggregates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>52</td>
</tr>
<tr>
<td>1:2</td>
<td>53</td>
</tr>
<tr>
<td>1:10</td>
<td>50</td>
</tr>
</tbody>
</table>

$^a$5.2 Porphyrins/Antibody

Table 2.7

Effect of heating time used in metalation on aggregate formation using CuCS$_3$P-RlG$' as sample

<table>
<thead>
<tr>
<th>Heating Time (minutes)</th>
<th>Aggregates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>54</td>
</tr>
<tr>
<td>45</td>
<td>54</td>
</tr>
<tr>
<td>75</td>
<td>55</td>
</tr>
</tbody>
</table>

$^a$4.4 Porphyrins/Antibody
Table 2.8
Attempts to Decrease Aggregates

A. Ultracentrifugation

<table>
<thead>
<tr>
<th>Ultracentrifugation time (minutes)</th>
<th>Aggregates (%) Before Treatment</th>
<th>Aggregates (%) After Treatment</th>
<th>Loss of monomeric RlgG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>33</td>
<td>29</td>
<td>Not detectable</td>
</tr>
<tr>
<td>18</td>
<td>33</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>25</td>
<td>33</td>
<td>19</td>
<td>29</td>
</tr>
<tr>
<td>30</td>
<td>33</td>
<td>14</td>
<td>33</td>
</tr>
</tbody>
</table>

B. Other Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Aggregates (%) Before Treatment</th>
<th>Aggregates (%) After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 M NaCl</td>
<td>28</td>
<td>27</td>
</tr>
<tr>
<td>Glycerol</td>
<td>28</td>
<td>26</td>
</tr>
<tr>
<td>Ultrasonication</td>
<td>32</td>
<td>31</td>
</tr>
</tbody>
</table>
radiocopper gave aggregation levels of 40%, suggesting that metalation by either stable or radiocopper has a similar influence on aggregation tendencies. Aggregation levels remained unchanged over a 7-day period when conjugates were stored at 4°C (data not shown). Aggregation levels for an RIgG conjugate with 2.4 porphyrins/RIgG were 38%, which is 10% higher than the corresponding levels for an A6H conjugate. As was seen with the A6H conjugates, metalation of the RIgG conjugate with either stable or radiocopper increased aggregation levels by 10%. Once again this suggests that metalation leads to an increase in aggregation levels, and this increase is independent of the copper isotope used for metalation.

Table 2.5 shows the aggregation levels as a function of the number of porphyrins attached to each antibody. Whereas a preparation with 4.2 porphyrins/RIgG showed about 50% aggregation, the preparation with 8.7 porphyrins/RIgG resulted in 60% aggregation. When a preparation with 18.3 porphyrins/RIgG was prepared, the aggregation levels increased to 68%.

As seen in Table 2.6, changes in the copper:porphyrin ratio used in metalation has no effect on aggregation levels. Metalation carried out with even a 10-fold molar excess of copper showed about the same aggregate content, 50%, as that obtained when a 1:1 copper:porphyrin ratio was used. A preparation with an intermediate copper:porphyrin ratio of 1.2 was also aggregated to the same extent.

Table 2.7 shows the aggregation levels after heating the conjugate at 40°C for varying periods of time. Heating for 15 minutes led to the same level of aggregation, 54 to 55%, as observed when the conjugates were heated for 45 or 75 minutes, indicating that the
extent of aggregation was independent of the heating time for the time periods studied.

As seen in Table 2.8, ultracentrifugation for 10 minutes led to minimal reduction in aggregate content, 4%, with almost no loss of monomeric antibody. When the ultracentrifugation time was increased, the efficiency of aggregate removal was improved. However, the increased removal of aggregates was accompanied by higher losses of monomeric protein. For example, ultracentrifugation for 30 minutes led to a 43% decrease in aggregates, but was accompanied by a 33% loss of monomeric antibody.

The attempts to reduce noncovalent interaction within aggregates were only partially successful (Table 2.8). Addition of CHAPSO in a final concentration of 3% decreased aggregation levels from 29% to 17%. Mixing with 2 M NaCl or with glycerol in a ratio of 1:1 did not result in a reduction in aggregation levels. Similarly, ultrasonication under the conditions described did not reduce aggregation levels. Inclusion of 150 mM NaCl in the mobile phase also had no effect on aggregation levels (data not shown).

Discussion

Due to the lack of suitable diagnostic or therapeutic agents, the overall survival rate in RCC patients has not improved in the past 10 years (Couillard & deVere White, 1993). The continuing need for therapeutic and diagnostic agents has led to an extensive evaluation of radiolabeled antibodies that might be useful in RCC patients (Wilbur et al., 1993; Vessella et al., 1988). Isotopes that decay by β- and γ-emission are good candidates for radiolabeling antibodies since they can be used for both imaging (γ-emission) and therapy (β-emission). For example, 131I-labeled anti-RCC monoclonal antibodies have been used in radioimaging
and radiotherapy studies since they possess decay properties suitable for both (Vessella et al., 1988). Similarly, copper-67 offers the potential for both uses and, in particular, has an excellent gamma photon (185 keV) which could result in valuable images for diagnostic purposes using conventional γ cameras, because of their operation in this energy range.

The synthetic porphyrins offer an exceptional chelating system for radiocopper because of the ease of metalation. Other attractive features of these porphyrins include their nontoxic nature at clinically useful concentrations and the range of functional groups possible for conjugation with proteins. Also, metalloporphyrins are very resistant to loss of the metal ion (Roberts et al., 1987) and their stability is comparable to that of other copper chelating systems currently under study (Fujiwara et al., 1985; Meares, 1986). Direct attachment of copper-67 to antibodies is also receiving renewed attention (Zamora et al., 1992).

As reported by Lavallee et al. (1986), metalation of the porphyrins is almost quantitative under mild conditions. However, radiometalation of the A6H conjugate proceeded in a 41% yield, which is somewhat lower that the 55% average metalation yield observed in the past (Mercer-Smith et al., 1988). This difference may be attributed to the difference in the nature of the antibody used in each study. The reduction in metalation yield of a conjugate as opposed to that of the free porphyrin may be due to the steric bulk of the attached antibody and/or porphyrin orientation. Orientation of the porphyrin is of added importance in the case of N-substituted porphyrins because the metal ion may only complex on one side of the porphyrin to form a "sitting atop" complex, which is an important step in porphyrin metalation (Bain-Ackerman & Lavallee, 1979; Lavallee et al., 1986). The
"sitting atop" complex with copper has been observed spectrally for N-substituted porphyrins and antibody-porphyrin conjugates (Mercer-Smith et al., 1991).

The \textit{in vitro} cell binding analysis of the A6H radioimmunoconjugate suggests that damage to the antigen binding site has occurred. This may be explained by the 'random' nature of the chemistry used to attach the porphyrins to the antibody. Amino groups from the antigen binding site, along with those from other parts of the immunoglobulin, can potentially participate in coupling with the porphyrin and cause a reduction in its immunoreactivity. Also conformational changes or general damage to the antibody during the manipulations may also be responsible for the reduction in immunoreactivity. Aggregation tendencies observed in these conjugates could also lead to reduction in antigen recognition capabilities.

The biodistribution pattern for the free porphyrins, as well as for porphyrin-conjugated A6H, was investigated in RCC-bearing nude mice. The two porphyrins showed similar localization tendencies, with the highest activity residing in the kidney. This may actually be due to quicker clearance from the blood than from the kidney and not due to increased kidney uptake past 4 hours. This behavior is expected because molecules smaller than about 15 Å undergo glomerular filtration readily and are found in almost the same concentration in glomerular filtrate as in the plasma (Duling, 1988). By 24 hours the kidneys and liver localize almost 7 times more $^{67}$CuCS$_2$P than the blood. For $^{67}$CuTCPP the localization in kidneys is 18 times whereas that in the liver is almost 11 times that of the blood after 24 hours. Thus kidneys and liver are the primary organs responsible for quick removal of porphyrins from the blood. High activity was also found to be associated with
the spleen. The work of Kaelin and Zanelli has shown similar high liver and spleen localization when biodistribution studies were performed with sulfonated and nonsulfonated $^{125}$I-labeled hydroxynaphthyl porphyrins (Kaelin & Zanelli, 1990); high liver and spleen uptake was also observed in rats after $^{67}$CuTCPF administration (Mercer-Smith et al., 1990). The activity associated with the intestines, muscle, and stomach was comparable to the activity associated with blood suggesting that localization in these organs was only limited to the level of porphyrins circulating in the blood. The slow and steady increase in the tumor:blood ratio shown by both the porphyrins was expected based on previous reports demonstrating the ability of porphyrins to localize in tumor tissue (Kessel, 1984; Cole et al., 1991). Investigation by Megnin et al. (1987) suggests that a selective transport mechanism, which differs between normal and cancer cells, may be responsible for the retention of free porphyrins by tumor tissue. This conclusion was arrived at after elimination of other mechanisms, such as enzymatic alteration of porphyrins in cancer cells or physical trapping by compartmentalization.

In the case of the antibody conjugate, recognition exists between the antibody and tumor cells, causing an increase in the tumor:blood ratio. This increase is also partly caused by the elimination of activity from other organs where such specific interaction is absent. However, substantial levels of activity continue to remain associated with liver and spleen after 45 hours suggesting the presence of aggregates or macromolecular species in the injection solution. Antibody molecules being on the order of 100 Å long are incapable of glomerular filtration. The lower radioactivity levels associated with the kidney indicated that little, if any, free radiolabeled porphyrin was present either in the injection solution or
*in vivo* after approximately 2 days of circulation. The *in vivo* release of free porphyrins from these conjugates was not expected since previous results (Roberts et al., 1989) indicated that 70% of the antibody-porphyrin conjugates remained intact upon incubation in serum for a period of 7 days at 37°C. In addition, copper porphyrins are stable to the loss of copper under identical experimental conditions in serum for at least 12 days (Mercer-Smith et al., 1988). As seen in Figure 2.5, kidneys show highest uptake of ^67^CuCS₃P, whereas uptake of the conjugate was predominant in the liver. Also the elimination of ^67^CuCS₃P was found to be more rapid than that of ^67^CuCS₃P-A6H. This may be due to the fact that antibodies are normally present in the circulatory system and are expected to clear more slowly than a much smaller molecule such as ^67^CuCS₃P. This difference in localization and elimination patterns suggests that conjugate biodistribution seems to be dictated by the larger antibody molecule as compared to the smaller porphyrin. Since antibodies are known to be metabolized by the liver and spleen (Keenan et al., 1985), a higher concentration of the conjugate is expected in the liver and spleen than in the kidneys.

Aggregation of antibody molecules has been reported in the past. Kummer et al. (1993) have found that certain anti-Thy-1 monoclonal antibodies of the IgG2c isotype have the inherent tendency to self-associate, probably through homophilic Fc-Fc contacts, which renders them mitogenic. Aggregation has also been observed in single-chain antibody variable fragment (sFv) proteins (Whitlow et al., 1993; Kortt et al., 1994). In fact, Whitlow et al. (1993) have found that the use of a longer linker (proteolytically stable 18 residue peptide) between the V\(_H\) and V\(_L\) domains of the anti-fluorescein sFv 4-4-20 led to an increased affinity for fluorescein and a reduction in its aggregation tendency. Kortt et al.
1994) report that the sFv of monoclonal antibody, NC10, constructed with a 15-residue linker, is predominantly in the monomeric form when expressed in E. coli. However, at concentrations of approximately 5 mg/mL, dimers and higher molecular mass aggregates are formed. A detailed study of aggregation kinetics of immunoglobulins was performed by Feder and Joessang (1986). Their investigation suggests that aggregation of IgG molecules is an irreversible process, confirming the unsuccessful attempts to reverse aggregation using NaCl, glycerol, or CHAPSO. Although GPC is the most common technique for determination of aggregate content (Caldwell, 1988), Litzen et al. (1993) have demonstrated the utility of asymmetrical field-flow fractionation in the separation and quantitation of monoclonal antibody aggregates. This latter method suffers from problems such as precision and the possibility of overloading the channel in a field-flow fractionation instrument.

A representative chromatogram obtained during the analysis of a CuCS₃P-RiG conjugate is shown in Figure 2.6. Since aggregation was not observed after stirring A6H with porphyrin in the absence of coupling reagents, it follows that covalent linking of the porphyrin to the antibody is essential for aggregate formation. Confirmation of this behavior comes from the correlation observed between the extent of aggregation and number of porphyrins attached to the antibody. The kinetics of the aggregation process are such that aggregates are formed soon after conjugate formation; aggregation levels remain largely unchanged over a 7-day period when the preparation is stored at 4°C. The 10% increase in aggregation levels following metalation may be explained on the basis of conformational
Figure 2.6. Chromatogram obtained after HPLC analysis of CuCS$_3$P-R1gG. Peaks at 7.45 and 8.04 minutes represent aggregated antibody. The peak with retention time 9.37 minutes represents monomeric antibody.
changes accompanying porphyrin metalation. The metalation mechanism and kinetics to form copper porphyrins from N-benzyl porphyrins have been previously described (Lavallee et al., 1986; Mercer-Smith et al., 1988; Mercer-Smith et al., 1991; Schulte et al., 1991). The metalation reaction is a two-step process. The first step involves formation of the N-benzyl metalloporphyrin, which then undergoes attack by water to release the benzyl alcohol as the second step. Thus, metalation of N-bzHCS₃P leads to the loss of p-nitrobenzyl alcohol, and this causes the otherwise puckered porphyrin to revert back to its planar conformation (Lavallee, 1987). This planar, highly conjugated molecule then has a better opportunity to stack and, consequently, has higher aggregation tendencies. Crystallographic studies have shown that the angle between the alkyl substituent and the plane of the alkylated pyrrole ring is 120° for N-methyl-5,10,15,20-tetrakis(p-bromophenyl)tetr phenylporphine (N-CH₃TPPBr₄, 3), whereas the corresponding angle for the protonated pyrrole is 180° (Lavallee, 1987) (see Figure 2.1). Conformational differences observed between the molecular modeling images generated for the N-benzyl and non-N-benzyl porphyrins can be seen in Figure 2.7. These images serve to clarify the conformational changes accompanying metalation. As seen in Figure 2.7, the N-benzyl porphyrin has a cup shaped conformation whereas the non-N-benzyl porphyrin is planar. The stacking tendency is not as high for N-substituted porphyrins because the nitrogen bearing the substituent lies in a plane different from that formed by the three nonsubstituted nitrogens. Structures of N-methylporphyrin complexes demonstrate the nonplanar nature of the ligand (Lavallee et al., 1978; Anderson & Lavallee, 1977). In addition, reports from Pasternack’s laboratory suggest that porphyrins with negative charges at the periphery tend to localize the electron
Figure 2.7. Molecular modeling images generated for N-benzyl porphyrin and porphyrin without N-substitution formed after metalation.
density near the center. This leads to stronger van der Waals interactions for a stacking type
dimer (Pasternack et al., 1973). N-bzHCS₃P, with its negatively charged peripheral
substituents, would possess aggregation tendencies which may continue to play a role even
after attachment of the porphyrin to the antibody molecule.

The zwitterionic detergent, 3-[(3-cholamidopropyl)dimethylammonio]-1-
propanesulfonate (CHAPS), is a good membrane solubilizing agent (Dickey et al., 1987).
It has been proven to be better than bile salts in reducing nonspecific interactions between
the different protein components of adenylate cyclase (Bitonti et al., 1982). The
hydroxylated derivative of CHAPS, CHAPSO, was used because of its improved water
solubility. Due to its zwitterionic nature, CHAPSO was partially effective in reducing
noncovalent interactions between antibody molecules and thus reducing aggregation. None
of the attempts successfully produced a preparation free of aggregated species. The only
efficient method then to obtain a nonaggregated preparation would be by preventing the
formation of these aggregates.

A6H has been labeled with ¹³¹I, ¹²¹I, ¹²³I, ¹³¹I, ¹⁸⁵Re, and ⁹⁹ᵐTc (Chiou et al., 1988;
Chiou, 1989; Palme et al., 1991; Wessels et al., 1989) in studies that have shown
exceptional tumor targeting abilities. A6H has also undergone preclinical (Vessella et al.,
1985; Vessella et al., 1987; Vessella et al., 1988) and clinical (Vessella, 1991) evaluations
for therapy of RCC. ¹³¹I-labeled A6H gave a tumor:blood ratio of 9:1 24 hours after
injection (Palme et al., 1991). Radioimmunoscintigraphy using ¹³¹I-labeled A6H has led to
the detection of tumors as small as 7 mg (Vessella et al., 1987).

The characteristics of copper-67 continue to make it an attractive label for future in
vivo antibody studies. From these results, it has been demonstrated that the coupling strategy used yields immunologically reactive, copper-67-labeled A6H with good tumor localization capabilities. Biodistribution results suggest that A6H controls the localization of the $^{67}$CuCS$_3$P-A6H conjugate. However, direct attachment of the metalloporphyrin to the antibody seems to be responsible for aggregate formation leading to unacceptable liver and spleen localization. Since the nature of the porphyrin could also be responsible for aggregation, a change in the porphyrin ring substituents may provide conjugates with reduced aggregation. Refinements in the chemical modification strategy to place the porphyrin farther away from the antibody (by the use of intermediate linker molecules) could also lead to preparations with good immunoreactivity and reduced aggregation.

References


Buchler, J. W. (1975) Static coordination chemistry of metalloporphyrins. Porphyrins and


CHAPTER 3

ANTIBODY-PORPHYRIN CONJUGATES PREPARED USING A6H FRAGMENTS FOR DELIVERY OF COPPER-67

Introduction

It is difficult to eradicate cancer cells in vivo because they share with normal cells, for the most part, the same biochemical machinery. There is no cytotoxic substance that is completely selective for malignant cells, and all those presently in use cause dose-limiting toxic side effects (Firestone, 1994). For this reason there is a growing emphasis on the use of delivery vehicles that can specifically target the tumor and bypass normal body tissues. Several different tumor-specific antibodies have been developed and studied, both in experimental as well as clinical studies (Houghton & Scheinberg, 1991). The advent of hybridoma technology has provided a boost to research in this field by rendering the ability to design and make antibodies with predetermined specificity (Kohler & Milstein, 1975). Radiolabeled monoclonal antibodies against tumor-associated antigens have received much attention in cancer research during the last decade or so since they offer the opportunity to specifically target tumor cells (Blumenthal et al., 1990). The tumor uptake of radiolabeled monoclonal antibodies and their fragments is directly related to the integral of blood...
concentration over time, the highest tumor concentrations being obtained with whole antibody which has the longest biological half-life in plasma (Wilbanks et al., 1981; Nelp et al., 1985; Wahl et al., 1983). Unfortunately, the slow passage of the antibody through physiological barriers, as well as blood clearance and uptake of the radioimmunoconjugate by the reticuloendothelial system, results in a very low tumor uptake (0.001% to 0.01% of injected dose/gram of tumor tissue) (Sands, 1988). This leads to a very high background during radioimmunoimaging when the whole antibody is used. Higher tumor to background ratios can be obtained by the use of F(ab')₂ or Fab fragments that disappear more rapidly than whole antibody from the blood (Wilbanks et al., 1981; Wahl et al., 1983). The slow pharmacokinetics and low tumor uptake of whole antibodies also creates problems in radioimmunotherapy. This is because, as long as the percentage uptake by the tumor is very low, the majority of the radioactivity administered will circulate through the body and cause serious radiation damage to healthy tissue (Kuijpers et al., 1993).

The range of antibody fragments that can be obtained by cleavage of an intact antibody are shown in Figure 3.1 (Nezlin, 1994). Fragments are usually generated by protease digestion or chemical cleavage. Papain and trypsin cleave γ-chains at the amino-terminal side of hinge region S-S bridges between the heavy chains, to generate two univalent Fabs with antigen-binding capacity and one Fc. Pepsin cleaves immunoglobulins on the carboxy-terminal side of the hinge region to generate a bivalent F(ab')₂ fragment and the pFc' fragment. After reduction of the S-S bridges, pepsin-derived F(ab')₂ dissociates into univalent Fab'. Reduction of the whole antibody by reducing agents generates the Half Antibody. The minimal antigen-binding fragment is the Fv fragment, which is a heterodimer
Figure 3.1. Range of IgG fragments obtained by enzymatic or chemical cleavage.
of the variable regions of the heavy and light chains.

It has been suggested that F(ab')2 fragments may prove to be more efficacious for radioimmunotherapy than whole monoclonal antibodies because of their greater penetration and more homogenous distribution in tumors (Yokota et al., 1992; Buchegger et al., 1989; Wahl et al., 1983). Using the F(ab')2 fragment of an antibody also provides the advantage of decreased nonspecific binding because of the absence of the Fc portion (Lamoyi, 1986). A major advantage that can be expected from the use of F(ab')2 is that of reduced immunogenicity since intact IgG is much more immunogenic than are similar doses of antibody fragments (Covell et al., 1986). The effect of further size reduction of the F(ab')2 fragment to the monovalent Fab' fragment has also been studied. In a comparative study involving immunoconjugates prepared with intact IgG, or its F(ab')2 or Fab' enzymatic digest products, differences in binding ability were observed (Siegall et al., 1992). The difference in binding was attributed to the differences in avidity due to the monovalence of the Fab' molecule. Because of their smaller size, it has been suggested that Fab' fragments are likely to penetrate the inside of tumors more readily (Jain, 1989). Furthermore, Fab' fragments have a shorter serum lifetime and penetrate into the tumor at a faster rate than IgG or F(ab')2 fragments.

The Fv fragment has also been evaluated for its utility in antibody-mediated delivery (Givol, 1991). Unfortunately, the proteolytic sites for cleaving off V_H and V_L are not particularly preferred (Takahashi et al., 1991) and, therefore, useful access to Fv fragments has been only through gene technology (Skerra & Pluckthun, 1988). Although the Fv fragments reported show full functionality, one complication remains: the affinity of V_H and
$V_L$ for each other is lower than that of the two chains making up the Fab fragment (Glockshuber et al., 1990). A common solution to this dissociation problem is the use of the single-chain Fv (sFv) fragment (Bird et al., 1988). In this case, $V_H$ and $V_L$ are not expressed as two separate proteins, but as a continuous protein with a genetically encoded peptide linker. Another approach that has helped in improving the thermodynamic stability of Fv fragment is by the use of disulfide bonds to connect the two variable domains in an Fv fragment (Glockshuber et al., 1990). It was found that these disulfide-linked Fv fragments could be made in E. coli and were significantly more stable toward irreversible denaturation at 37°C than the sFv fragment or the Fv fragment (Glockshuber et al., 1990). Apart from the advantages related to the small size and lack of Fc region of these molecules, there are other benefits associated with the use of Fv fragments (Pluckthun, 1994). It is generally more efficient to produce smaller molecules. The folding efficiency seems to be often better for Fv-derived fragments than for Fab fragments or larger molecules. Above all, they form attractive agents in antibody-based diagnostics and therapeutics. Fv fragments show better penetration into solid tumors that are often poorly vascularized, where the penetration of large molecules is very inefficient (Pluckthun, 1994).

Of the several agents available for delivery to tumors, radioiodine has been one of the most widely evaluated agent. However, metallic radionuclides have distinct advantages over radioiodine for radioimmunoimaging and radioimmunotherapy due to their superior nuclear properties and simplicity of labeling (Arano et al., 1991). Among the metallic radionuclides suitable for imaging and therapy, copper-67 is very promising because of its 62-hour half-life, which is ideal in terms of the residence time of antibodies (DeNardo et al.,
on tumors, and in terms of its decay pattern which involves both $\beta$- and $\gamma$-emission (Raman & Pinajian, 1969).

Labeling of proteins and other biologically active molecules with metal ions is often accomplished with synthetic chelating agents which ensure that the metal ion remains bound to the carrier molecule under physiological conditions (Meares & Wensel, 1984; Smith-Jones et al., 1991). These molecules must also possess a bioreactive substituent that can be used for cross-linking to the carrier molecule. The first such bifunctional chelators to find widespread use were ethylenediaminetetraacetic acid analogues derivatized at a methylene carbon atom of the diamine backbone (Yeh et al., 1979). In recent years, interest in tumor targeting using radiometal-labeled antibody-chelator conjugates has prompted the development of alternative bifunctional ligands (Brechbiel et al., 1986; Esteban et al., 1987; Kozak et al., 1989; Mathias et al., 1990; Moi et al., 1985; Moi et al., 1988; Cox et al., 1989; Craig et al., 1989; Morphy et al., 1989). Different chelating agents have been reported for chelation of copper (Meares, 1986; Green et al., 1988; Smith-Jones et al., 1991; John et al., 1994). The chelating agent used here was the synthetic porphyrin, N-4-nitrobenzyl-5-(4-carboxyphenyl)-10,15,20-tris(4-sulfophenyl)porphine (N-bzHCS$_2$P), "tailor-made" to incorporate copper under mild conditions (Roberts et al., 1987; Mercer-Smith et al., 1988). An attractive feature of this porphyrin is the presence of an N-benzyl substituent which distorts the planarity of the porphine ring allowing for easy incorporation of copper from the opposite face (Lavallee et al., 1986). Some of the other advantages associated with the use porphyrins in radiolabeling antibodies include: 1) their nontoxic nature at clinically useful concentrations; 2) good stability towards the loss of incorporated metals; and 3)
resistance to metabolism \textit{in vivo}.

Direct conjugation of the porphyrin to polyclonal and monoclonal antibodies and autogenic peptides has been reported (Roberts et al., 1987; Mercer-Smith et al., 1988; Roberts et al., 1989; Roberts et al., 1990; Mercer-Smith et al., 1991). In this study, the porphyrin was conjugated to antibody fragments generated either by chemical or enzymatic cleavage of the whole antibody. The F\((\text{ab}')_2\) fragment used was generated by pepsin digestion of the anti-renal cell carcinoma (RCC) antibody, A6H (Moon et al., 1985; Vessella et al., 1988). The reduced antibody fragment (Half A6H) used was obtained by chemical reduction of hinge region disulfides of A6H. The F\((\text{ab}')_2\) fragment of A6H (henceforth referred to as A6F) has been used in radioimmunoconjugate preparations by labeling with radioiodine (Chiou, 1989; Wilbur et al., 1994), indium-111 (Chiou, 1989), and astatine-211 (Wilbur et al., 1993).

The aim of this study was to use fragments of the whole antibody to prepare immunoconjugates and explore the difference between immunoconjugates prepared using antibody fragments that either lack or possess the Fc portion. It was also of interest to compare the properties of these conjugates with those synthesized using the whole antibody. The idea was to investigate the influence of reduced molecular weight on immunoreactivity, coupling yield, and aggregation of the conjugates. The higher propensity of the Fab fragment to be accumulated in the kidneys made it unattractive for use in this study (Wilbur et al., 1991).
Experimental

Materials and Methods

ACHN cells, originally obtained from American Type Culture Collection (Rockville, MD), were maintained in minimum essential medium, \( \alpha \)-modification (\( \alpha \)-MEM) culture medium supplemented with 10% serum (5% fetal calf serum and 5% \( \alpha \)-calf serum) and antibiotics (10 mL of antibiotic-antimycotic solution per 1 L of medium) by passage every 2 to 3 days. A6F was produced as previously described (Wilbur et al., 1993). Distilled-in-glass grade N,N-dimethylformamide (DMF) from EM Science (Cherry Hill, NJ) was used. \( \text{CuCl}_2 \) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Reagents used for enzyme-linked immunosorbent assay (ELISA) were obtained from Vector Laboratories, Inc. (Burlingame, CA). PD-10 columns were purchased from Pharmacia Labs (Alameda, CA). The 96-well microtiter plates were obtained from Costar Corning (Cambridge, MA). All other reagents, chemicals, and chromatography supplies were purchased from Sigma Chemical Co. (St. Louis, MO). Water purified by a Barnstead Nanopure II system with >17 m\( \Omega \) resistivity was used for all applications. Radiolabeled samples were counted using a Cobra\textsuperscript{TM} Auto-Gamma\textsuperscript{R} counting systems (Model D5003) with the spectral window that extended from 60-210 keV.

Copper-67

Carrier-free copper-67 was obtained as \( ^{67}\text{CuCl}_2 \) in 2 M HCl from Los Alamos National Laboratory (Los Alamos, NM). The acid was evaporated to dryness under a flow of air or argon with gentle heating, and the radionuclide was taken up into DMF for
metalation.

Synthetic Procedures

**Synthesis of the Porphyrin-A6F Conjugate (N-bzHCS\textsubscript{3}P-A6F)**

N-bzHCS\textsubscript{3}P was synthesized and characterized as published elsewhere (Mercer-Smith et al., 1988). Activation of the porphyrin was carried out by mixing 40 µL of N-bzHCS\textsubscript{3}P (1.8 mg, \(1.1 \times 10^{-3}\) mmol) in DMF (200 µL) with 20 µL each of a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (2.6 mg, \(1.3 \times 10^{-2}\) mmol) and a solution of N-hydroxysuccinimide (NHS) (1.5 mg, \(1.3 \times 10^{-2}\) mmol), both in 12.5 mM sodium dihydrogen phosphate, pH 6 (100 µL). After 1 hour of porphyrin activation in the dark at room temperature, 400 µL of a solution of A6F (2.7 mg, \(2.7 \times 10^{-5}\) mmol) in borate-buffered saline (BBS, 0.1 M borate, 0.08 M NaCl), pH 8.5 was added. The coupling step was allowed to proceed for 4 hours in the dark at room temperature.

The porphyrin-antibody conjugates were purified from the excess free porphyrin in the reaction mixture using Sephadex G25 and eluting with BBS. The bright green eluent was stored on ice until further use.

The amount of porphyrin attached to protein was determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions (Laemmli, 1970). A 5 µL aliquot of the reaction mixture was mixed with 5 µL sample buffer (0.6 mM Tris, 10% glycerol, 2% SDS, pH 6.8), and the samples were loaded on a tube gel (6.5 x 0.2 cm) using a running gel of 7% polyacrylamide content at 2 mA per gel for the first 15 minutes and later increased to 8 mA per gel. The gels were scanned at
435 nm for porphyrin absorbance. The area under the peaks was integrated, and the areas were used to calculate coupling yields.

**Synthesis of Porphyrin-Half Antibody Conjugate (N-bzHCS₃P-Half A6H)**

**Generation of half antibody.** The scheme for the preparation of this conjugate has been shown in Figure 3.2. Reduction was carried out by addition of 3.5 mg of dithiothreitol (DTT, 0.023 mmole) to 4.0 mg of A6H (2.7 x 10⁻⁴ mmole). This mixture was dissolved by the addition of 600 µL of 0.1 M sodium phosphate buffer, pH 6.0, and stirring was carried out at 37°C for 1.5 hours. The excess DTT was removed by passing the reaction mixture through a PD-10 column; 2 mL of Half A6H solution was collected. The number of thiols generated were quantitated using Ellman's reagent, [5,5'-dithio-bis(2-nitrobenzoic acid)] (Ellman, 1959). The reagent solution was prepared by dissolving 4 mg of Ellman's reagent in 1 mL of 0.1 M sodium phosphate buffer, pH 8.0. The reduced antibody solution was diluted 25-fold from 20 µL to 500 µL. After measuring the absorbance of the antibody solution at 280 nm, 100 µL of Ellman's reagent solution was added. Following a 15-minute incubation period, absorbance was measured at 412 nm. Using the absorbance values and the extinction coefficients of the Half Antibody (15) and Ellman's reagent (343), the number of thiols per Half Antibody were determined.

**Capping the sulfhydryl groups.** A solution of iodoacetamide was prepared by dissolving 3.4 mg of iodoacetamide (0.0184 mmole) in 500 µL of BBS. The solution was slightly warmed to dissolve the solids. On cooling, 1.98 mL of Half A6H solution was added dropwise to the iodoacetamide solution with constant mixing. Reaction of
Figure 3.2. Scheme for the synthesis of N-bzHCS₃P-Half A6H.

Half A6H-SH + HSC(CHOH)₂CH₂SH → 1.5 hrs, pH 6, 37°C → Half A6H

Half A6H-SH + I-CH₂CONH₂ → 1 hr, pH 8.5, RT → Half A6H-S-CH₂CONH₂

Half A6H-S-CH₂CONH₂ + Activated porphyrin → 4 hrs, pH 8.5, RT → Half A6H-P
iodoacetamide with the sulfhydryl groups was allowed to proceed for 1.5 hours at room temperature in the dark. The solution was then concentrated by dialysis under negative pressure using a membrane with a molecular weight cut-off of 25,000 Da. The thiol content was once again measured using Ellman's reagent as described before.

**Coupling with the porphyrin.** Porphyrin was activated by mixing 45 μL of N-bzHCS₃P (1.8 mg, 1.1 x 10⁻³ mmol) in DMF (200 μL) with 22.5 μL each of a solution of EDAC (2.6 mg, 1.3 x 10⁻² mmol) and a solution of NHS (1.5 mg, 1.3 x 10⁻² mmol), both in 12.5 mM sodium dihydrogen phosphate, pH 6 (100 μL). After 1 hour of porphyrin activation in the dark at room temperature, 552 μL of capped Half A6H (2.2 mg, 2.9 x 10⁻⁵ mmol) solution in BBS was added. The coupling step was allowed to proceed for 4 hours in the dark at room temperature. Purification and determination of coupling yield was performed as already described for N-bzHCS₃P-A6F.

**Metalation with CuCl₂ and ⁶⁷CuCl₂.**

All metalation reactions were performed in dim light using acid-washed glassware to prevent inadvertent porphyrin metalation or photooxidation. The purified conjugates were metalated with stable copper (1 mM CuCl₂) or ⁶⁷CuCl₂ using a 1:1 copper to porphyrin ratio at 40°C for 45 minutes. Following metalation, the conjugates were subjected to vacuum dialysis into phosphate-buffered saline (PBS, 10 mM phosphate buffer, 120 mM NaCl, pH 7.4), containing 0.05 M sodium citrate to complex any free copper. Unmetalated preparations were vacuum dialyzed under similar conditions with the exclusion of sodium citrate from the dialysis buffer. Radiometalation yield was calculated for preparations
metalated with copper-67 by counting the sample before and after vacuum dialysis.

**In Vitro Testing**

**Thin Layer Chromatography**

The radiometalated conjugates were analyzed by thin layer chromatography (TLC) to estimate the level of protein-bound activity (Yamazaki et al., 1988). The radiolabeled preparation was spotted on aluminum-backed silica gel plates that were developed in either acetone or 80% methanol. The plates were then cut into the top two-thirds and bottom one-third portions and were counted in the gamma counter.

**Cell Binding Assay**

A cell binding assay was performed to determine the immunoreactivity of the radiolabeled fragments (Vessella et al., 1988). Briefly, approximately 10,000 counts per minute (cpm) of the radioimmunoconjugate (contained in 0.1 mL PBS) were added to 2 x 10^6 RCC (ACHN) cells suspended in 900 µL of 1% bovine serum albumin (BSA) in PBS. Freshly trypsinized cells from a confluent culture were washed three times with 1% BSA in PBS before use. Each tube was counted in a gamma counter to obtain Total Counts. The mixture was incubated at room temperature for 1 hour with rotary inversion mixing. The cells were pelleted by centrifugation, the supernatant was decanted, and the cells were resuspended in 1 mL PBS. The counts remaining associated with the cells, Bound Counts, were quantitated in the gamma counter. The percent immunoreactivity was calculated as Bound Counts/Total Counts x 100.
ELISA was carried out by the ABC method based on use of the high affinity biotin/avidin system. To each well of a 96-well microtiter plate were added 16,000-20,000 antigen-positive ACHN cells. Cell culture medium was added to obtain a final volume of 200 µL per well. The cells were allowed to incubate at 37°C in a 5% CO₂ atmosphere until confluent and attached. The plate was emptied by inversion and cells were fixed by addition of 200 µL of 0.15% glutaraldehyde solution in PBS to each well. After incubation at room temperature for 20 minutes, the plate was again emptied by inversion and washed (five times) with 1% Tween 20 in PBS. Blocking of the free adsorption sites was achieved by the addition of 200 µL of 1% BSA in PBS to each well. After 30 minutes of incubation at room temperature, the plate was emptied and washed as before. Dilutions of the fragment preparations and unmodified antibody and fragments were prepared in 1% BSA in PBS and used in triplicate. The dilutions used were 0.1, 0.3, 0.5, 0.7, 0.9, and 1.0 µg/mL, and the volume added to each well was 50 µL. Once again, wells were emptied and washed after 30 minutes of incubation at room temperature. This was followed by the addition of 50 µL of the biotinylated secondary antibody solution (7.5 µg/mL), an anti-mouse antibody. Incubation time for this step was 30 minutes, following which the plate was emptied and washed as before. The next step was the addition of 30 µL of biotinylated horseradish peroxidase-avidin complex, which was allowed to incubate for 15 minutes at room temperature. The plate was emptied and washed as before. Finally, 50 µL of the substrate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), was added and product formation was monitored at 405 nm. ELISA was carried out on the following preparations: 1)
unmodified A6F; 2) N-bzHCS3P-A6F; 3) CuCS3P-A6F; 4) Goat F(ab')2, as a nonspecific control fragment; 5) unmodified A6H; 6) Half A6H; 7) N-bzHCS3P-Half A6H; and 8) CuCS3P-Half A6H.

Determination of Aggregates by Size Exclusion HPLC

Gel permeation chromatographic (GPC) analysis of all conjugates and unmodified antibodies was carried out using a Hitachi HPLC system equipped with a variable wavelength UV-Vis detector. A Bio-Silect SEC 250-5 column (300 x 7.8 mm) along with a Bio-Select SEC 250 guard column (50 x 7.8 mm) was used. The following preparations were analyzed for their aggregate content: 1) unmodified A6F; 2) N-bzHCS3P-A6F; 3) CuCS3P-A6F; 4) unmodified A6H; 5) freeze-dried A6H; 6) Half A6H; 7) N-bzHCS3P-Half A6H; and 8) CuCS3P-Half A6H. All conjugates were analyzed immediately after vacuum dialysis and several times over a 1-week period. Samples were centrifuged (12,500 x g, 4°C, 2 to 3 minutes) to eliminate any particulate material before injecting on the HPLC column. Analysis was carried out with phosphate buffer (0.1 M sodium phosphate, 1 mM EDTA, 2 mM sodium azide, pH 6.8) as the mobile phase with a flow rate of 1 mL/min. The percent aggregation was calculated using the area under the monomer and aggregate peaks.

Results

Reduction of the whole A6H antibody led to the generation of 5 thiols per half antibody. After capping of the thiols with iodoacetamide, the number of thiols measured were 0.4 thiols per half antibody. The coupling yield for N-bzHCS3P-A6F and N-bzHCS3P-
Half A6H conjugates was 3.9 and 2.9 porphyrins per antibody, respectively. The radiometalation yield was 13% for $^{67}$CuCS$_3$P-A6F and 15% for the $^{67}$CuCS$_3$P-Half A6H conjugate. The specific activities of the $^{67}$CuCS$_3$P-A6F and $^{67}$CuCS$_3$P-Half A6H were 0.47 and 0.54 mCi/mg of protein, respectively.

TLC analysis of radiolabeled preparations showed that majority of the radioactivity remained at the origin. Strips developed in 80% methanol resulted in 90% and 89% activity being retained at the origin for the $^{67}$CuCS$_3$P-A6F and $^{67}$CuCS$_3$P-Half A6H, respectively. TLC plates developed in acetone resulted in > 99% activity being retained at the bottom for both the conjugates under evaluation, confirming the results obtained using 80% methanol, that most of the radioactivity was protein bound and very little free copper-67 or $^{67}$Cu-labeled porphyrin was present.

In the cell binding assay, 9% of the total radioactivity remained associated with the cell pellet when either of the radiolabeled conjugates were tested. The low amount of cell-bound radioactivity indicates that the modified fragments do not possess any antigen-binding capabilities. The results from the ELISA are shown in Figures 3.3 and 3.4. These graphs show that the immunoconjugate preparations, both metalated and nonmetalated, show reduced antigen-binding when compared to the unmodified fragment. Binding of N-bzHCS$_3$P-A6F and CuCS$_3$P-A6F is reduced to 30% and 20%, respectively. Binding of Half A6H is reduced by 52% which may be expected due to the monovalent nature of the Half Antibody as opposed to the divalent nature of whole A6H. Further reduction in binding is observed when Half A6H is linked to porphyrins to prepare the porphyrin-Half A6H conjugate.
Figure 3.3. ELISA performed on A6F conjugates.
Figure 3.4. ELISA performed on Half A6H conjugates.
Aggregation values for each preparation have been shown in Table 3.1. Solutions of A6H and A6F, when thawed and analyzed by HPLC, did not contain any aggregates. Covalent direct attachment of the porphyrin to A6F, however, led to very high aggregation, an average value of 61%. Metalation of the immunoconjugate led to further increase in aggregation by approximately 10%. Reduction of A6H to Half A6H caused minimal aggregation, measured at 3%. However, when this Half A6H solution was used for coupling to porphyrin, the immunoconjugate solution had about 71% of the preparation aggregated. Once again, metalation worsened this effect since the aggregation levels increased to 78% when copper was incorporated in the porphyrin. All aggregation levels remained unchanged over the 1-week period of testing, when conjugates were stored at 4°C (data not shown). Using randomly prepared whole antibody-porphyrin conjugates, it was found that the error associated with the aggregation values was ± 2%.

Discussion

The present study describes a straightforward approach for the preparation of lower molecular weight immunoconjugates radiolabeled with copper-67. Several studies have been performed in the past using antibody fragments as delivery vehicles (Herlyn et al., 1986; Chiou, 1989; Fisch et al., 1992; Siegall et al., 1992; Wilbur et al., 1994). Some of the studies in the literature have suggested that F(\(ab')_2\)) fragments of monoclonal antibodies showed greater selectivity in tumor targeting in vivo as compared with intact antibody (Herlyn et al., 1985; Mach et al., 1983). Other studies, however, such as the one conducted by Murray et al., have found that F(\(ab')_2\)) fragments of an anticarcinoembryonic antigen
Table 3.1

Aggregation levels for the different antibody fragment preparations

<table>
<thead>
<tr>
<th>Sample</th>
<th>Aggregates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6H</td>
<td>0</td>
</tr>
<tr>
<td>Freeze-dried A6H</td>
<td>0</td>
</tr>
<tr>
<td>A6F</td>
<td>0</td>
</tr>
<tr>
<td>N-bzHCS₃P-A6F</td>
<td>61</td>
</tr>
<tr>
<td>CuCS₃P-A6F</td>
<td>71</td>
</tr>
<tr>
<td>Half A6H</td>
<td>3</td>
</tr>
<tr>
<td>N-bzHCS₃P-Half A6H</td>
<td>71</td>
</tr>
<tr>
<td>CuCS₃P-Half A6H</td>
<td>78</td>
</tr>
</tbody>
</table>
monoclonal antibody localized in tumor tissue to an equivalent extent when compared with the whole antibody, and the pharmacokinetics of F(ab')₂ versus whole antibody were virtually identical (Murray et al., 1994). Multivalent antibody fragments with the general structure F(ab')ₙₓ (n = 3 or 4, x = cross-linker) have been prepared and tested in vivo by Schott et al. (1993). In their study, both the fragments showed retention of antigen binding ability in vitro. A large proportion of the ¹⁰⁵Rh-labeled F(ab')₄ₓ (200 kDa) was found to accumulate in the liver, which the authors suggest might be an indication of an upper size limit for the in vivo use of cross-linked fragments. The biodistribution behavior of ¹⁰⁵Rh-labeled F(ab')₃ₓ was intermediate between that of IgG and F(ab')₂ for all organs studied. Kidney localization was reduced, whereas blood circulation time and tumor accumulation was slightly increased, for the trivalent species compared with F(ab')₂.

The use of reduced antibodies in immunoconjugate preparations has been most commonly reported for direct labeling with technetium-99m (Rhodes et al., 1986; Mather & Ellison, 1990). By slowly reducing the protein disulfide groups with tin(II), Rhodes et al. (1986) have been able to achieve around 80% incorporation of technetium-99m. The slow reduction of protein disulfide groups with tin(II) has been advantageously replaced with a fast reduction reaction, using reducing agents such as mercaptoethanol, prior to radiolabeling (Griffiths et al., 1992). Schwarz and Steinstrasser (1987) have pretreated murine monoclonal antibodies with mercaptan-reducing agents and have stabilized the partially reduced IgG by lyophilization and storage of the monoclonals under nitrogen in sealed vials. Arano et al. (1994) reported the mercaptoethanol reduction of the disulfide bonds of a monoclonal antibody against osteogenic sarcoma to generate 6 to 7 thiols per
molecule. This reduced antibody was used in the delivery of $^{67}$Ga chelate of succinyldeferoxamine. Similar to the procedure used here, del Rosario et al. (1990) have reported the use of DTT at 37°C for approximately 5 hours to cleave the hinge region disulfides of antibodies. Upon radioiodination, one of the reduced antibodies had immunoreactivity in the highest range (40-60%) typically obtained for routine radioiodination of this antibody, as determined by a direct cell-binding assay (about 52%). Griffiths et al. (1992) and Mather and Ellison (1990) have demonstrated that reduced IgG used for technetium-99m labeling had several advantages when compared to Fab' fragments. They were easy to label, gave pure products, were resistant to reoxidation, and were stable when challenged with chelators. With intact IgG, from 1 to 9 sulfhydryl groups per antibody molecule, as analyzed by the Ellman reaction (Ellman, 1959), may be generated by the reduction of IgG with reagents such as mercaptoethanol (Griffiths et al., 1991). The theoretical maximum number of sulfhydryl groups which could be produced by cleavage of all intrachain and interchain disulfides is 36 (Pimm et al., 1991). The fact that 5 thiols were generated during the reduction process indicates the success of the procedure without over modification of the antibody. Since it has been shown that partial reduction of a mouse monoclonal antibody generally breaks disulfide bridges in the hinge region of the antibody (Nisonoff et al., 1975; Packard et al., 1986), it is expected that the procedure used here is likely to have generated Half Antibody fragments. Separation of the heavy and light chains of the antibody is also unlikely because of the generally accepted hypothesis that heavy and light chains of an IgG molecule remain intimately associated by noncovalent attractions even after cleavage of all interchain disulfides (del Rosario et al., 1990). The 0.4 thiols per
antibody measured after capping of the thiols correlates well with what has been observed in the past for untreated antibodies. Pimm et al. (1991) have observed that untreated antibodies show less than 1 free sulfhydryl group per antibody molecule when checked by the Ellman's assay. In an effort to radiolabel antibodies with technetium-99m, Pimm et al. found that reduction of four different antibodies with 2-mercaptoethanol led to 29, 22, 19, and 14 sulfhydryl groups, numbers that are much higher than can be accounted for by only limited intrachain cleavage of disulfide bonds. Contradictory to the reports by Griffiths et al. (1992) and Mather and Ellison (1990), the Pimm et al. (1991) report suggests that the reduced antibody may be fragile since they have observed radiolabeled low molecular weight degradation products of the antibodies.

The synthetic porphyrin used here offers an exceptional chelating system for radiocopper because of the ease of metalation and stability of the metalloporphyrin formed (Roberts et al., 1987). Lavallee et al. (1986) have reported that radiometalation of the porphyrins is almost quantitative under mild conditions. However, radiometalation of the fragment-porphyrin conjugates proceeded in low yields, which may be due to the fact that the porphyrin is now present as part of the immunoconjugate and thus the access of the copper ion is seriously hindered by the large protein molecules. Orientation of the porphyrin is of added importance in the case of N-substituted porphyrins because the metal ion may only complex on one side of the porphyrin (Bain-Ackerman & Lavallee, 1979). Low metalation yields have been previously reported by Mercer-Smith et al. (1988) when the porphyrin was present as part of a conjugate. The whole antibody-porphyrin conjugates discussed in Chapter 2 also showed low radiometalation yields.
The in vitro cell binding analysis of the radioimmunoconjugates suggests that the coupling and purification procedure of the immunoconjugate led to loss in binding ability of the antibody. When the porphyrin is reacted with A6F, there is a good possibility that reaction may occur with amino groups in the antigen binding site or near it, since the size of the molecule is reduced when compared to the whole molecule. Also, the Fc portion that is farther away from the antigen binding site is absent in the F(\(ab\)')\(_2\) molecule. In the case of Half A6H, although Fc portion is available, the number of reactive amino groups available is reduced since the fragment's molecular weight is half that of the intact antibody molecule. Thus, the random nature of the antibody modification chemistry used to conjugate large molecules such as the porphyrin may be responsible for loss in immunoreactivity of the immunoconjugate preparations. ELISA results for the A6F conjugate suggests that 30% of the preparation is still immunoreactive. Even though this result does not indicate as drastic a loss in antigen binding capabilities as suggested by the cell binding assay, it suggests that modification of A6F with the porphyrin does reduce its biological activity. The disagreement in the exact numerical value of the results from the two experiments is not of great significance since in either case the conclusion remains the same: minimal modification of the fragment has led to unacceptably low immunoreactivity. The discrepancy in the results may be explained by considering the differences in the design of the two experiments. The ELISA uses nonradioactive preparations whereas the cell binding assay only uses radiolabeled samples. ELISA offers a direct comparison with the unmodified fragment, whereas the cell binding assay uses the actual radiolabeled preparation of interest. Since no direct comparison to unmodified antibody exists in the cell binding
 assay, it is possible that the amount of radiolabeled preparation used may be higher than the actual available antigens on the cells, especially because the radiolabeled conjugate synthesized had very low specific activity.

Proteins such as antibody molecules and fragments are capable of intermolecular association to form aggregates. Kummer et al. (1993) have found that certain anti-Thy-1 monoclonal antibodies of the IgG2c isotype have the inherent tendency to self-associate, probably through homophilic Fc-Fc contacts, which renders them mitogenic. Aggregation has also been observed in single-chain antibody variable fragment (sFv) proteins (Whitlow et al., 1993; Kortt et al., 1994). In fact, Whitlow et al. (1993) have found that the use of a longer linker (proteolytically stable 18 residue peptide) between the V\textsubscript{H} and V\textsubscript{L} domains of the anti-fluorescein sFv 4-4-20 led to an increased affinity for fluorescein and a reduction in its aggregation tendency. Aggregation observed in the fragment-porphyrin conjugates can be explained on the same basis as that for the whole antibody-porphyrin conjugates. The tendency of porphyrins to stack may be responsible for the intermolecular association properties and may be responsible for aggregation in the immunoconjugates. A detailed study of aggregation kinetics of immunoglobulins was performed by Feder and Joessang (1986). The kinetics of the aggregation process of the fragment conjugates suggest that aggregates are formed soon after conjugate formation, and aggregation levels remain largely unchanged over a 7-day period when the preparation is stored at 4\textdegree C. The 8 to 10% increase in aggregation levels following metalation may be explained on the basis of conformational changes accompanying porphyrin metalation. The metalation mechanism and kinetics to form copper porphyrins from N-benzyl porphyrins have been previously
described (Lavallee et al., 1986; Mercer-Smith et al., 1988; Mercer-Smith et al., 1991; Schulte et al., 1991). Metalation of N-bzHCS₃P leads to the loss of p-nitrobenzyl alcohol, and this causes the otherwise puckered porphyrin to revert back to its planar conformation (Lavallee, 1987). This planar, highly conjugated molecule then has a better opportunity to stack and, consequently, has higher aggregation tendencies leading to higher aggregation levels in metalated conjugates. In addition, reports from Pasternack’s laboratory suggest that porphyrins with negative charges at the periphery tend to localize the electron density near the center. This leads to stronger van der Waals interactions for a stacking type dimer (Pasternack et al., 1973). N-bzHCS₃P, with its negatively charged peripheral substituents, would possess aggregation tendencies which may continue to play a role even after attachment of the porphyrin to the antibody molecule. The higher aggregation of the Half A6H conjugates may be due to the presence of the Fc portion which has been found to play a role in antibody aggregation (Kummer et al., 1993).

The results shown here demonstrate that, although it is possible to link porphyrins to antibody fragments to gain the advantages associated with the use of fragments, there are some problems associated with this approach when A6H fragments are conjugated to porphyrin. Aggregation levels for the conjugates are high and antigen binding properties are compromised.

References


CHAPTER 4

BIOLOGICAL EVALUATION OF POLYAMIDOAMINE

STARBURST™ DENDRIMERS

Introduction

Polyamidoamine (PAMAM) Starburst™ dendrimers are interesting polymers composed of repeating polyamidoamino units (Tomalia et al., 1985; Tomalia et al., 1986; Tomalia et al., 1990; Tomalia, 1991; Alper, 1991). The dendrimers resemble "normal" polymers in some respects but differ from them in important ways. Polymerization processes are usually random in nature and produce a complex mixture of species within a molecular weight range. Starburst™ dendrimers, on the other hand, are prepared using a unique branching strategy that results in polymeric products of specified size, shape, and molecular weight depending on the "generation" of the dendrimer.

Starburst™ dendrimers are synthesized in a step-wise process, which results in molecules with three distinguishing features: (1) an initiator core; (2) layers of repeating units radially attached to the core; and (3) an outer surface of terminal functional groups, primary amines in the case of the PAMAMs (Figure 4.1). Steps A and B of the synthesis can be repeated as desired to form succeeding generations of the PAMAMs, each with twice

1Starburst is a Trade Mark of Dendritech, Inc., Midland, MI.
Figure 4.1. Steps involved in dendrimer synthesis. Adapted from Tomalia, 1990.
the number of terminal functional groups as its predecessor. Table 4.1 summarizes some characteristics of the PAMAMS of generation 0 to 10.

The PAMAM Starburst™ dendrimers, as well as dendrimers prepared using other starting materials, are finding utility in a wide variety of uses, from particle size standards to a number of biological applications. One such application has involved the use of Starburst™ dendrimers in an antibody-targeted drug delivery system (Roberts et al., 1990). The dendrimers admirably fulfill the role of an intermediate linker molecule which can be highly modified with the drug of choice, then attached to a single site on the surface of the antibody. This arrangement was designed to allow maximal delivery of drug to the desired site, while maintaining maximal antibody immunoreactivity, two endpoints that can be mutually exclusive in some antibody modification strategies.

Little is known, however, about the biological properties of the Starburst™ dendrimers. More information is crucial for the continued investigation of the dendrimers in therapeutic (or other in vivo) applications. Preliminary studies on generations 3 (G3; MW=5,147; 24 terminal amines), 5 (G5; MW=21,563; 96 amines), and 7 (G7; MW=87,227; 384 amines) PAMAMs were carried out in V79 cells or in Swiss-Webster mice. A number of biological properties were studied including: (1) in vitro and in vivo toxicity; (2) carcinogenicity; (3) mutagenicity; (4) immunogenicity; and (5) biodistribution.
Table 4.1

Physical characteristics of PAMAM Starburst™ dendrimers Adapted from Tomalia et al., 1990

<table>
<thead>
<tr>
<th>Generation</th>
<th>MW</th>
<th>Diameter (Å)</th>
<th>Amino Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>359</td>
<td>10.8</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>1043</td>
<td>15.8</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>2411</td>
<td>22.0</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>5147</td>
<td>31.0</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>10619</td>
<td>40.0</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>21563</td>
<td>53.0</td>
<td>96</td>
</tr>
<tr>
<td>6</td>
<td>43451</td>
<td>67.0</td>
<td>192</td>
</tr>
<tr>
<td>7</td>
<td>87227</td>
<td>80.0</td>
<td>384</td>
</tr>
<tr>
<td>8</td>
<td>174779</td>
<td>92.0</td>
<td>768</td>
</tr>
<tr>
<td>9</td>
<td>349883</td>
<td>105.0</td>
<td>1536</td>
</tr>
<tr>
<td>10</td>
<td>700091</td>
<td>124.0</td>
<td>3072</td>
</tr>
</tbody>
</table>
Experimental

Materials and Methods

G3, G5, and G7 PAMAM Starburst™ dendrimers were generously provided by the Michigan Molecular Institute (Midland, MI) and were used without further purification. [14C]CH₃I (specific activity: 55 mCi per mM) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Tissue culture supplies came from either Sigma Chemical Co. (St. Louis, MO) or HyClone, Inc. (Logan, UT). All other chemicals were purchased from Sigma Chemical Co.

PAMAM Analysis

The PAMAMs used in these experiments were analyzed by capillary electrophoresis to determine the quality of the preparations with respect to lower and higher molecular weight species.

Cell Culture

V79 cells (Chinese hamster lung fibroblasts) were purchased from the American Type Culture Collection (Rockville, MD) and grown in modified Eagle's medium, α-modification (α-MEM), supplemented with antibiotics and 10% serum, unless otherwise indicated. The cells were grown in a humidified 5% CO₂ atmosphere at 37°C and passaged every 3 to 4 days.
Animals

Male Swiss-Webster mice were purchased from Charles River Laboratories (Wilmington, MA) and used when approximately 20 g. Animals were housed in cedar shavings and were maintained in a 12-hour light/dark cycle in a humidity- and temperature-controlled facility; food and water was available ad libitum. All dendrimer administrations were done by the intraperitoneal route while the mice were hand-held. Unless otherwise noted, no anesthetics were utilized during the injections. Animals were euthanized by CO₂ asphyxiation, followed by bilateral thoracotomy.

In Vitro and in Vivo Studies

In Vitro Toxicity

Duplicate tissue culture flasks were plated with $5 \times 10^4$ V79 cells in 20 mL of medium and allowed to adhere overnight. The cells were then exposed to either 100 nM, 10 μM, or 1 mM of G3, G5, or G7 in medium (pH adjusted to 7.4) for 4 or 24 hours. At the end of the appropriate exposure period, the cells were trypsinized, counted, and seeded into duplicate petri dishes in 5 mL of fresh medium at the level of 1000 cells per dish. After a 6- or 7-day incubation period, the colonies were stained with crystal violet solution (2.5 g crystal violet in 900 mL MeOH plus 100 mL 10% formalin) and colonies composed of 50 cells were counted. The percent surviving cells was calculated in each case and corrected for the plating efficiency of untreated cells, which averaged 27% for these studies. The results are expressed as the mean percent cells surviving ± standard deviation (SD) versus treatment conditions.
In Vivo Toxicity

Sets of five animals each were injected with G3, G5, or G7 PAMAMs dissolved in phosphate-buffered saline (PBS), pH 7.4, at a dose of $5 \times 10^{-6}$, $5 \times 10^{-5}$, or $5 \times 10^{-4}$ mmol/kg. Control animals received vehicle alone. The animals were observed for a period of 2 hours after injection for behavioral abnormalities such as changes in horizontal or vertical motion, level of activity, eating and drinking behavior, etc. Separate sets of animals were monitored for either 7 or 30 days. Changes in body weight over the observation time were recorded as a measure of overall health. After the appropriate time interval, the animals were carefully necropsied to look for internal abnormalities. The results are expressed as the mean change in body weight ± SD for the appropriate observation period.

Immunogenicity

The generation of immune serum was conducted by R&R Rabbitry (Stanwood, WA). Serum samples were obtained from sets of 2 New Zealand white rabbits to serve as nonimmune controls. The animals were then injected subcutaneously with G3, G5, or G7 in PBS, pH 7.4, at a dose of $5 \times 10^{-5}$ mmol, mixed with complete Freund's adjuvant. Two subsequent booster shots, at the same dose as the first, but without the adjuvant, were given at 3-week intervals. Blood samples were collected 10 days after each injection. Each sample was allowed to clot at room temperature for about 1 hour, then was refrigerated for several hours. The samples were centrifuged at 2700 rpm for 6 to 8 minutes. The serum was poured off and recentrifuged for an additional 4 to 6 minutes. The serum was separated from any remaining pellet and stored at -20°C until assayed.
The immunogenicity of the Starburst™ dendrimers was studied using two different methods. First, immunoprecipitation was explored. G3, G5, and G7 solutions were made up in PBS, pH 7.4, at a concentration of 100 μM. Aliquots of 100 μl were placed in wells of a 96-well microtiter plate for a total of 10 nmol/well. Serial dilutions of each serum sample from 1:10 to 1:1,000,000 were made up in PBS. One hundred microliters of each serum dilution was combined with the appropriate antigen in duplicate wells and the plate was incubated at 37°C for 24 hours with shaking. The occurrence of cloudiness was noted, which indicates the formation of an immunoprecipitate and, therefore, a reaction between antibodies in the serum and the dendrimer antigen.

Second, an Ouchterlony double diffusion assay was run. Agar plates were prepared (30 g agar in 1000 mL 0.1 M phosphate buffer, pH 8.0), and a center well and six surrounding wells were cut out of the solidified agar. An aliquot of 1 mL of each serum sample was placed in the center well of duplicate plates. In the surrounding wells, 1 mL of each test solution was placed. The test solutions included: PBS alone, bovine serum albumin (1 mg/mL) as a negative control, and four dilutions of each dendrimer (1, 0.1, 0.01, and 0.001 μmol/mL). The plates were incubated at room temperature for 72 hours, and the incidence of a line of precipitation between various wells was noted at 12-hour intervals, again indicating an immune reaction between antibodies in the serum samples and the dendrimer antigen.
Mutagenicity

V79 cells were grown for 5 days in medium containing HAT (50 μM hypoxanthine, 3.2 μM amethopterin, and 5 μM thymidine) to purge the line of spontaneous mutants at the hypoxanthine ribosyl transferase (HPRT) locus. The cells were then trypsinized, counted, and plated out at the level of $1 \times 10^6$ cells per flask in duplicate flasks containing regular medium and allowed to adhere overnight. The cultures were then exposed to regular medium containing either G3 (0.1 mM), G5 (1 μM), or G7 (10 nM), all adjusted to pH 7.4, for 24 hours at 37°C. (The dendrimer concentrations were chosen based on the results of the in vitro toxicity test above.) The dendrimer-containing medium was replaced with fresh medium, and the cells were allowed to grow normally, with periodic medium replacement, for a 10-day period, which allowed for the expression of mutations. After the expression period, the cells were trypsinized, counted, and seeded out into sets of five petri dishes at the level of $2 \times 10^5$ cells per dish. These cells were grown in medium containing 6-thioguanine (6-TG, 35 μM). Only those cells that have mutated at the HPRT locus are able to grow in the presence of 6-TG. After an additional 10- or 11-day growth period at 37°C, colonies were stained with crystal violet and counted.

Carcinogenicity

Groups of five mice each were injected with $5 \times 10^{-4}$ mmol/kg of either G3, G5, or G7 in PBS, pH 7.4, once a week for 10 weeks. In the case of the G7 group, however, potential acute toxicity of the initial injection led to the administration of a lower dose ($5 \times 10^{-5}$ mmol/kg) for the remainder of the experiment. The animals were observed for a period
of 2 hours after each injection for behavioral abnormalities as described in the toxicity studies above. Observation continued for a 6-month period, with weekly weighings as a measure of overall health. At sacrifice, the animals were examined by Dr. Lorraine Shabestari, attending veterinarian at the Animal Resource Center, University of Utah, for the appearance of tumor nodules or masses. Samples of liver and spleen were removed, fixed in buffered formalin, sectioned, and stained with hematoxylin and eosin for a more careful examination of these organs. The tissue samples were rated for evidence of cancer incidence. Results are expressed as the mean change in body weight ± SD, along with an evaluation of observed tumor incidence at both the macroscopic and microscopic levels.

**Preparation of \[^{13}\text{C}]\text{- and }[^{14}\text{C}]\text{-Labeled Dendrimers}\**

The \(^{13}\text{C}\) label was introduced into the dendrimers by reacting about 200 mg of G3, G5, or G7 with a 15-fold molar excess of \[^{13}\text{C}]\text{CH}_3\text{I}\) in the presence of KHCO\(_3\) (Chen & Benoiton, 1976). After stirring for 24 hours, the solvent was removed, and 5 mL of CHCl\(_3\) was allowed to remain in contact with the pale yellow oil overnight. The solvent was removed by rotary evaporation, yielding yellowish, gummy material. \(^{13}\text{C}\) NMR was used to characterize the methylated products.

To prepare the radiolabeled dendrimers, \[^{14}\text{C}]\text{CH}_3\text{I}\) was used. The sealed ampoule in which the radiolabeled reagent was received was frozen in a dry ice/acetone bath and opened. Sixty microliters of unlabeled CH\(_3\)I was added, followed by 1 mL MeOH. The following volumes of this solution were added to the dendrimer solutions: G3, 48 \(\mu\)l; G5, 200 \(\mu\)l; G7, 500 \(\mu\)l. The reaction conditions were chosen to allow high enough reaction
with the $^{14}$C label to produce a preparation with useful specific activity without overmethylating the dendrimers, which could change their physicochemical properties and, thus, their biodistribution.

**Biodistribution Studies**

Injection solutions were prepared in PBS, pH 7.4. The animals were lightly anesthetized with ether and injected with approximately 0.05 to 0.25 μCi $^{14}$C activity (2 to 6 x 10$^4$ mmol/kg dendrimer) and placed, in groups of five, into metabolism cages to allow for the collection of urine. At 2, 4, 8, 24, and 48 hours after injection, samples of blood, liver, kidney, spleen, intestine, heart, lung, pancreas, and bladder were removed and accurately weighed. A single sample of urine, representing all five animals, was also collected. All tissue samples were coarsely homogenized in 1 mL water, 2.5 mL tissue solubilizer were added, and the samples were incubated at 55°C overnight in a water bath to completely dissolve the tissue. One hundred fifty microliters of 30% H$_2$O$_2$ were then added to decolorize the samples, followed by 10 mL scintillation fluid. The samples were counted by liquid scintillation, and the results were expressed as the mean percent injected dose per gram tissue (% ID/g). Because of the unusual results obtained from the initial biodistribution studies, each generation was retested under identical conditions at the 2-hour time point for confirmation.
Statistical Analysis

The mean values for pairs of experimental groups were compared by Student's *t* test; multiple sets of data were compared by a one-way analysis of variance, followed by a Tukey-Kramer multiple comparisons test. Differences were considered significant at a *P* value of \( \leq 0.05 \).

Results

Capillary electrophoretic analysis of the dendrimer samples (data not shown) gave the following yield of the monodendrimer species: G3, 76.7%; G5, 62.7%; G7, 68.5%. Lower molecular weight species arise from incomplete removal of ethylene diamine from the amidation step in the preparation of PAMAM dendrimers. This excess diamine can then serve as a separate initiator core, which is inadvertently alkylated during the next step in the synthesis. Higher molecular weight species presumably arise from an undesired coupling reaction that takes place during the amidation step. The percent monodendrimer species observed is within normal parameters (Ralph Spindler, MMI, private communication).

As seen in Figure 4.2, the PAMAMs exhibited concentration- and generation-dependent toxicity to V79 cells growing in culture. G3 effected maximal cell growth only at the very high concentration of 1 mM, whereas G5 showed toxicity at 10 \( \mu \)M. G7 caused cell death at all the concentrations tested. No significant differences were seen in the results of 4- vs. 24-hour exposures.

Tables 4.2 and 4.3 outline the results of the 7- and 30-day *in vivo* toxicity experiments, respectively. No behavioral toxicity was observed during the 2-hour
Figure 4.2. Percent cell survival (mean ± SD) after dendrimer treatment. V79 cells were exposed to G3-, G5-, or G7-containing medium at 1 mM, 10 µM, or 100 nM for 4 or 24 hours. Clonogenic survival was measured 6 or 7 days later.
Table 4.2

Toxicity of Starburst™ dendrimers--7 days

<table>
<thead>
<tr>
<th>Generation</th>
<th>Dose (mmol/kg)</th>
<th>Dose (mg/kg)</th>
<th>Change in Body Weight (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>5 x 10^{-6}</td>
<td>0.026</td>
<td>11.5 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>5 x 10^{-5}</td>
<td>0.26</td>
<td>8.2 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>5 x 10^{-4}</td>
<td>2.6</td>
<td>8.5 ± 1.2</td>
</tr>
<tr>
<td>5</td>
<td>5 x 10^{-6}</td>
<td>0.1</td>
<td>10.0 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>5 x 10^{-5}</td>
<td>1.0</td>
<td>5.8 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>5 x 10^{-4}</td>
<td>10</td>
<td>9.0 ± 2.8</td>
</tr>
<tr>
<td>7</td>
<td>5 x 10^{-6}</td>
<td>0.45</td>
<td>6.3 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>5 x 10^{-5}</td>
<td>4.5</td>
<td>5.0 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>5 x 10^{-4}</td>
<td>45</td>
<td>3.8 ± 7.5*</td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
<td>7.6 ± 4.6</td>
</tr>
</tbody>
</table>

SD = standard deviation

No data groups were significantly different from each other at a confidence level of 95%.

*One animal died during observation period.
Table 4.3

Toxicity of Starburst™ dendrimers--30 days

<table>
<thead>
<tr>
<th>Generation</th>
<th>Dose (mmol/kg)</th>
<th>Dose (mg/kg)</th>
<th>Change in Body Weight (%) ± SD</th>
</tr>
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<tbody>
<tr>
<td>3</td>
<td>5 x 10^{-6}</td>
<td>0.026</td>
<td>30.8 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>5 x 10^{-5}</td>
<td>0.26</td>
<td>27.5 ± 11.4</td>
</tr>
<tr>
<td></td>
<td>5 x 10^{-4}</td>
<td>2.6</td>
<td>23.4 ± 6.0</td>
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<td>27.3 ± 7.4</td>
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<td>5 x 10^{-5}</td>
<td>1.0</td>
<td>30.0 ± 9.8</td>
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<tr>
<td></td>
<td>5 x 10^{-4}</td>
<td>10</td>
<td>29.8 ± 5.4</td>
</tr>
<tr>
<td>7</td>
<td>5 x 10^{-6}</td>
<td>0.45</td>
<td>27.0 ± 6.7</td>
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<td>4.5</td>
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<tr>
<td></td>
<td>5 x 10^{-4}</td>
<td>45</td>
<td>28.5 ± 6.0</td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
<td>26.2 ± 5.7</td>
</tr>
</tbody>
</table>

SD = standard deviation

No data groups were significantly different from each other at a confidence level of 95%.
observation period following injection. However, one animal in the G7 group receiving the highest dose died at about 24 hours after administration during the 7-day experiment, perhaps suggesting that the larger PAMAMs may lead to some form of biological complication, although this was not experimentally demonstrated. Otherwise, all animals showed a normal growth pattern with no statistically significant differences in body weight changes between treated and control groups.

No evidence of immunogenicity was noted in either the immunoprecipitation or the Ouchterlony double diffusion assays of any dendrimer generation (data not shown). Likewise, no colonies survived the mutagenicity experiment (data not shown).

In the carcinogenicity experiment, one animal died in the G7 group about 24 hours after injection, similar to the situation in the original toxicity experiment. Another animal in that group, along with one in the vehicle-treated group, died of causes unrelated to the experiment (pneumonia caused by soaked bedding). Table 4.4 shows that none of the groups showed changes in body weight after 6 months that were significantly different than controls. Also, no macroscopic or microscopic evidence of cancer was observed. However, all liver samples exhibited some vacuolization of the cytoplasm, which is of unknown importance at the present time.

The procedure to be used for the synthesis of the radiolabeled dendrimers was first carried out using excess $[^{13}\text{C}]\text{CH}_3\text{I}$. $^{13}$C NMR of G3, G5, and G7 preparations clearly showed the presence of methylated dendrimers. All three possible types of methylated amines (mono-, di-, and trimethyl alkylamines) were visible in the NMR by their $^{13}$C chemical shifts (spectra shown in Figures 4.3 to 4.5): mono-, 35.7 to 35.9 ppm; di-, 45.6
Table 4.4
Carcinogenicity of Starburst™ dendrimers--6 months

<table>
<thead>
<tr>
<th>Generation</th>
<th>N Surviving</th>
<th>Change in Body Weight (%) ± SD</th>
<th>Tumors Detected Microscopic</th>
<th>Tumors Detected Macroscopic</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>5</td>
<td>81.2 ± 21.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>61.2 ± 14.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>90.0 ± 1.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Untreated</td>
<td>4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>68.0 ± 5.8</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

SD = standard deviation
No data groups were significantly different from each other at a confidence level of 95%.
<sup>a</sup>One animal died of acute toxicity.
<sup>b</sup>One animal died of causes unrelated to the experiment.
<sup>c</sup>None detected.
Figure 4.3. $^{13}$C NMR spectrum of $^{13}$C-methylated G3 obtained in CD$_3$OD.
Figure 4.4. $^{13}$C NMR spectrum of $^{13}$C-methylated G5 obtained in CD$_3$OD.
Figure 4.5. $^{13}$C NMR spectrum of $^{13}$C-methylated G7 obtained in CD$_3$OD.
to 45.8 ppm; tri-, 51.2 to 54.2 ppm (Pretsch et al., 1989). The $^{13}$C NMR spectra of the $^{13}$C methylated dendrimers were compared to the $^{13}$C NMR spectra obtained from unmodified dendrimers (spectra shown in Figures 4.6 to 4.8). The $[^{14}$C]-labeled syntheses were then carried out with reduced amounts of CH$_3$I. The resulting specific activities of each preparation were: G3, 2.6 mCi/mmol; G5, 3.3 mCi/mmol; G7, 10.8 mCi/mmol.

The initial biodistribution study results are presented for G3, G5, and G7 in Tables 4.5, 4.6, and 4.7, respectively. Blood values were near zero in all cases (data not shown). G3 showed the highest accumulation in liver, kidney, and spleen, which achieved approximately 15% ID/g at their highest levels. Heart, lung, and intestine activity was low. Pancreas showed less than 10% ID/g accumulation in most cases. Urine levels varied from 1 to 5% ID/g. Peak levels were reached at different times after administration depending upon the tissue. G5, in contrast, showed lower liver and kidney localization, but increased spleen uptake, in general. Heart and lung levels were again low, but intestinal accumulation was higher than in G3's case. The most striking feature of G5 biodistribution was the very high levels of activity that accumulated in the pancreas, achieving about 32% ID/g at 24 hours. Urine levels were fairly similar to G3 values. G7's biodistribution generally mimicked that of the other two generations in liver, kidney, spleen, lung, and heart tissue. Intestine and bladder accumulation tended toward the low side for G7. Again very high pancreas uptake was observed with G7, but the peak activity occurred at 2 hours after administration, followed by rapid loss of activity from this tissue. The most striking result of the G7 biodistribution studies was the amazingly high urinary output of radioactivity, which achieved 74% ID/g at 4 hours after compound administration.
Figure 4.6. $^{13}$C NMR spectrum of G3 obtained in CD$_3$OD.
Figure 4.8. $^{13}$C NMR spectrum of G7 obtained in CD$_3$OD.
Table 4.5

Biodistribution of Starburst™ dendrimers--G3

<table>
<thead>
<tr>
<th>Organ</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>8.57 ± 2.5</td>
<td>12.66 ± 2.7</td>
<td>10.26 ± 1.6</td>
<td>14.60 ± 2.5</td>
<td>16.19 ± 3.7</td>
</tr>
<tr>
<td>Kidney</td>
<td>15.25 ± 1.8</td>
<td>16.10 ± 2.6</td>
<td>12.16 ± 0.7</td>
<td>15.35 ± 2.4</td>
<td>13.19 ± 3.7</td>
</tr>
<tr>
<td>Spleen</td>
<td>12.78 ± 1.2</td>
<td>12.93 ± 0.8</td>
<td>9.00 ± 1.4</td>
<td>12.14 ± 2.7</td>
<td>12.03 ± 4.6</td>
</tr>
<tr>
<td>Lung</td>
<td>1.62 ± 0.1</td>
<td>1.42 ± 0.6</td>
<td>1.17 ± 0.5</td>
<td>1.13 ± 0.4</td>
<td>1.59 ± 1.1</td>
</tr>
<tr>
<td>Heart</td>
<td>1.08 ± 0.3</td>
<td>0.65 ± 0.3</td>
<td>0.37 ± 0.1</td>
<td>0.22 ± 0.0</td>
<td>0.18 ± 0.1</td>
</tr>
<tr>
<td>Intestine</td>
<td>4.12 ± 0.7</td>
<td>6.53 ± 0.2</td>
<td>5.26 ± 1.3</td>
<td>4.12 ± 0.6</td>
<td>6.79 ± 1.4</td>
</tr>
<tr>
<td>Bladder</td>
<td>10.44 ± 5.1</td>
<td>8.84 ± 5.3</td>
<td>6.14 ± 1.5</td>
<td>11.50 ± 5.2</td>
<td>5.95 ± 1.7</td>
</tr>
<tr>
<td>Pancreas</td>
<td>7.89 ± 1.6</td>
<td>10.51 ± 2.2</td>
<td>9.17 ± 2.6</td>
<td>8.13 ± 2.1</td>
<td>9.18 ± 3.5</td>
</tr>
<tr>
<td>Urine</td>
<td>4.62</td>
<td>2.75</td>
<td>3.65</td>
<td>3.25</td>
<td>1.13</td>
</tr>
</tbody>
</table>

SD = standard deviation
Table 4.6

Biodistribution of Starburst™ dendrimers—G5

<table>
<thead>
<tr>
<th>Organ</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>5.66 ± 2.5</td>
<td>6.05 ± 1.0</td>
<td>12.21 ± 0.7</td>
<td>10.82 ± 6.0</td>
<td>4.58 ± 2.0</td>
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<tr>
<td>Kidney</td>
<td>6.65 ± 1.5</td>
<td>7.51 ± 1.1</td>
<td>8.07 ± 0.9</td>
<td>7.36 ± 1.6</td>
<td>4.60 ± 0.6</td>
</tr>
<tr>
<td>Spleen</td>
<td>11.78 ± 4.7</td>
<td>16.58 ± 6.4</td>
<td>22.50 ± 10.9</td>
<td>13.10 ± 6.1</td>
<td>6.62 ± 1.8</td>
</tr>
<tr>
<td>Lung</td>
<td>0.62 ± 0.3</td>
<td>0.51 ± 0.2</td>
<td>1.10 ± 1.0</td>
<td>0.52 ± 0.2</td>
<td>0.33 ± 0.1</td>
</tr>
<tr>
<td>Heart</td>
<td>0.25 ± 0.1</td>
<td>0.33 ± 0.2</td>
<td>0.24 ± 0.0</td>
<td>0.17 ± 0.0</td>
<td>0.07 ± 0.0</td>
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<tr>
<td>Intestine</td>
<td>17.22 ± 7.3</td>
<td>12.07 ± 4.5</td>
<td>8.08 ± 1.3</td>
<td>19.48 ± 10.9</td>
<td>8.92 ± 3.3</td>
</tr>
<tr>
<td>Bladder</td>
<td>11.44 ± 2.3</td>
<td>8.31 ± 0.6</td>
<td>10.51 ± 0.8</td>
<td>7.97 ± 3.5</td>
<td>4.06 ± 0.9</td>
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<tr>
<td>Pancreas</td>
<td>20.30 ± 10.2</td>
<td>26.21 ± 15.9</td>
<td>29.73 ± 5.8</td>
<td>31.91 ± 25.3</td>
<td>16.34 ± 8.6</td>
</tr>
<tr>
<td>Urine</td>
<td>0.30</td>
<td>3.20</td>
<td>1.92</td>
<td>4.45</td>
<td>1.31</td>
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</table>

SD = standard deviation
Table 4.7

Biodistribution of Starburst™ dendrimers--G7

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<tr>
<th>Organ</th>
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<th>4 h</th>
<th>8 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>4.24 ± 0.9</td>
<td>4.13 ± 0.8</td>
<td>4.02 ± 0.9</td>
<td>4.83 ± 0.8</td>
<td>1.87 ± 0.3</td>
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<tr>
<td>Kidney</td>
<td>13.35 ± 4.7</td>
<td>13.08 ± 2.9</td>
<td>12.65 ± 2.3</td>
<td>11.79 ± 2.3</td>
<td>6.42 ± 1.2</td>
</tr>
<tr>
<td>Spleen</td>
<td>15.21 ± 8.2</td>
<td>14.95 ± 7.9</td>
<td>14.14 ± 3.4</td>
<td>10.72 ± 1.8</td>
<td>1.02 ± 0.3</td>
</tr>
<tr>
<td>Lung</td>
<td>1.26 ± 0.5</td>
<td>2.09 ± 1.0</td>
<td>0.72 ± 0.5</td>
<td>0.60 ± 0.6</td>
<td>0.35 ± 0.1</td>
</tr>
<tr>
<td>Heart</td>
<td>0.55 ± 0.1</td>
<td>0.42 ± 0.1</td>
<td>0.09 ± 0.0</td>
<td>0.13 ± 0.1</td>
<td>0.05 ± 0.0</td>
</tr>
<tr>
<td>Intestine</td>
<td>5.26 ± 3.5</td>
<td>4.05 ± 3.2</td>
<td>3.71 ± 2.5</td>
<td>3.48 ± 1.5</td>
<td>0.84 ± 0.2</td>
</tr>
<tr>
<td>Bladder</td>
<td>7.42 ± 2.6</td>
<td>6.27 ± 3.3</td>
<td>6.80 ± 4.3</td>
<td>5.03 ± 1.9</td>
<td>1.27 ± 0.3</td>
</tr>
<tr>
<td>Pancreas</td>
<td>20.26 ± 15.5</td>
<td>11.24 ± 5.7</td>
<td>12.92 ± 6.4</td>
<td>5.76 ± 2.3</td>
<td>3.94 ± 3.4</td>
</tr>
<tr>
<td>Urine</td>
<td>45.92</td>
<td>73.94</td>
<td>20.23</td>
<td>7.99</td>
<td>0.32</td>
</tr>
</tbody>
</table>

SD = standard deviation
In order to better illustrate the differences between the PAMAM generations, Figures 4.9 through 4.13 were plotted to show the % ID/g vs. time for all three generations on one graph in various important or unusual tissues: liver, kidney, spleen, pancreas, and urine, respectively. Liver activity (Figure 4.9) tended to clear after G5 and G7 administration, but G3 activity continued to increase slightly with time in this organ. Kidney localization (Figure 4.10) was fairly comparable for the three generations, with G3 showing higher accumulation in that organ, in general. Likewise, spleen activity (Figure 4.11) over time for the three generations was virtually indistinguishable. In contrast, G5 showed very high, but usually not significantly different, localization in the pancreas (Figure 4.12); G3 and G7 accumulation in this organ tended to be below 10% ID/g with the exception of the 2-hour time point after G7 administration (~21% ID/g). The striking differences in the urinary output of radiolabel after injection of the three generations is illustrated in Figure 4.13. G7 administration resulted in ~46 and ~74% ID/g excreted after 2 and 4 hours, respectively.

Because of the unusual biodistribution characteristics exhibited by the PAMAM dendrimers, a second experiment was performed for the 2-hour time point identical to the first. Figures 4.14, 4.15, and 4.16 illustrate the comparative results from the two experiments for G3, G5, and G7, respectively. As seen in Figure 4.14, much higher pancreas uptake was observed for G3 in the second experiment with respect to the first, which more closely followed the biodistribution pattern of the other generations in that organ at 2 hours after administration. No other significant differences were noted between the two experiments. For G5, statistically significant, but fairly minor differences between
Figure 4.9. Biodistribution pattern (% ID/g ± SD) of $^{14}$C-labeled G3, G5, or G7 in liver tissue.
Figure 4.10. Biodistribution pattern (% ID/g ± SD) of 14C-labeled G3, G5, or G7 in kidney tissue.
Figure 4.11. Biodistribution pattern (% ID/g ± SD) of $^{14}$C-labeled G3, G5, or G7 in spleen tissue.
Figure 4.12. Biodistribution pattern (% ID/g ± SD) of $^{14}$C-labeled G3, G5, or G7 in pancreas tissue.
Figure 4.13. Biodistribution pattern (% ID/g) of $^{14}$C-labeled G3, G5, or G7 in urine.
Figure 4.14. Comparative G3 biodistribution results (% ID/g ± SD) from two separate experiments.
Figure 4.15. Comparative G5 biodistribution results (% ID/g ± SD) from two separate experiments.
Figure 4.16. Comparative G7 biodistribution results (% ID/g ± SD) from two separate experiments.
the studies were seen in heart and bladder accumulation (Figure 4.15). Urine levels cannot be analyzed statistically because the data represent only a single sample. However, considerably higher urinary output of radioactivity after G5 administration was seen in the second experiment, which is the inverse of the bladder situation. Statistically significant, but trivial differences resulted in lung and heart tissues for G7, but all other results were not different between the two experiments (Figure 4.16). Overall, the duplicate studies confirmed the biodistribution pattern originally observed. High pancreas uptake seems to be a characteristic of these dendrimers, regardless of generation. In addition, very high urine activity was observed primarily in the case of the G7 dendrimers.

Discussion

Starburst™ dendrimers were first introduced in 1985 (Tomalia et al., 1985). They were originally under study as molecular mimics for evolutionary chemistry processes such as molecular self-organization; numerous studies reported on dendrimer synthesis and properties (Tomalia et al., 1985; Tomalia et al., 1986; Tomalia et al., 1990; Tomalia, 1991; Alper, 1991; Tomalia et al., 1987a; Padias et al., 1987; Tomalia et al., 1987b; Naylor et al., 1989; Tomalia, 1993). Along with the spherical Starburst™ type of dendritic molecule, other configurations, such as the arborols (Newkome et al., 1990; Serroni et al., 1992), have also been reported. In addition to the PAMAMs discussed here, dendrimers can be synthesized with a wide variety of interior (hydrocarbons, ruthenium, silicon, ethers, thioethers, etc.) as well as exterior functionalities (hydrocarbons, alcohols, thiols, esters, acids, etc.) (Newkome et al., 1990; Serroni et al., 1992; Dubin et al., 1992; Kim, 1992,
Frechet, 1994; Jin et al., 1993). Interesting "biodendrimers" composed of RNA units have recently been reported (Hudson & Damha, 1993). Many mixed systems have also been prepared. Their potential utility is virtually boundless.

Many biological/therapeutic uses for Starburst™ dendrimers are under study. Binding gadolinium atoms to the surface of a dendrimer results in a very effective magnetic resonance imaging contrast agent (Wiener et al., 1994). Recently the PAMAMs have been shown to allow high efficiency transfection of a number of cell types (Haensler & Szoka, 1993). Constructing antibody immunoconjugates using Starburst™ dendrimers as intermediate linker molecules has been reported for the site-specific delivery of boron for boron neutron capture therapy (BNCT) (Barth et al., 1994), as well as radiometals for radioimmunotherapy or diagnosis (Roberts et al., 1990; Gansow et al., 1994). Other PAMAM-antibody conjugates have found utility in immunoassay applications (Singh et al., 1994). Entrapment of "guest" molecules in a dendritic box is another potential use for these molecules (Jansen et al., 1994).

Because of the growing interest in using dendrimers in biological applications, the behavior of the polymers in biological systems is of critical importance. Barth et al. (1994) reported the biodistribution of 125I-dendrimers of generation 1 through 5, administered by intraperitoneal injection, that they are studying for use in BNCT. Only liver, spleen, muscle, and blood levels were reported. The highest level of accumulation of these organs was observed in the spleen, localization increased with increasing generation. The results from G3 and G5 were comparable to the data reported from these generations of iodinated PAMAMs in liver and spleen tissue. No muscle activity was determined in this study.
The biodistribution patterns and/or toxicity of the PAMAMs may solely be a reflection of their polycationic nature. The biological effects observed after administration of polycations are varied and poorly understood, in most cases. For example, tachyplesin I, a polycationic antimicrobial peptide, kills bacteria by causing disruption of the cytoplasmic membrane (a common mechanism of action for this class of antibiotics), which may also occur in mammalian cells (Masuda et al., 1994). Stimulation of protein tyrosine kinases by polycations has been documented (Ruzzene et al., 1994), as has inhibition of skeletal muscle ATPase activity (Hughes et al., 1994). Polycation treatment has resulted in leakage of albumin through glomerular epithelial cells both in vitro (Hammes & Singh, 1994) and in vivo (deBarros e Silva et al., 1992). Intrapleural administration of polycations caused severe lung edema, respiratory insufficiency, and marked mortality in rats (Santana et al., 1993; Coyle et al., 1993). In addition, alterations in the blood-brain barrier after intracarotid infusion of a number of polycations was observed and was a function of molecular weight and number of positive charges (Westergren & Johansson, 1993). In other studies, a toxicity assessment of polycationic peptides, reflecting hemodynamic factors, increased with increased positive charge from +8 to +21 (deLucia et al., 1993). High pancreas uptake of particular chemical compounds, peptides, or proteins has been documented in certain cases, but does not seem to be a pattern associated with polycationic species (Petkova et al., 1993). Interestingly, a recent study reported that synthetic liposomes labeled with $^{99m}$Tc preferentially localized in pancreas after intraperitoneal administration but not after intravenous administration (Goto & Ibuki, 1994). Therefore, the possibility exists that the elevated pancreas uptake observed here was simply a function of the route of administration.
Studies using polycationic peptides possessing large number of amino groups as the dendrimers (poly(L-lysine) polymers) showed accumulation in the liver and spleen after intravenous injection; pancreas was not monitored (Hudecz et al., 1993; Clegg et al., 1990).

From these preliminary studies it is suggested that toxicity of the PAMAMs is dose- and generation-dependent in vitro. G5 or smaller dendrimers do not appear to present any problem in vivo from a toxicity point of view. These results suggest that the larger generations of PAMAM dendrimers may not be a good choice for biological uses, although the smaller generations appear to have little or no deleterious effect at levels to be found in biological applications. Other associated problems with large molecules in in vivo applications may rule out the use of larger generations as well.

In addition, no evidence for mutagenicity, carcinogenicity, or immunogenicity of any of the generations tested was observed. Although the experimental protocols employed for the preliminary determination of these properties do not categorically rule out problems of mutagenicity, carcinogenicity, or immunogenicity, the incidence of these negative qualities must be very low if present at all.

Using the methylated dendrimer preparations, unusual biodistribution patterns, especially with respect to pancreas uptake, were observed, which may dictate caution in certain situations if, in fact, these characteristics apply to unmodified dendrimers. On the other hand, this high pancreas uptake may be capitalized upon for targeted drug delivery in specific disease states such as diabetes or pancreatic cancer. The unexplained high urinary output of activity after G7 administration is curious. At least two possibilities exist allowing for the presence of radioactivity in the urine. The first is that the intact methylated
dendrimer is excreted by that pathway. It is widely accepted that only small molecules (50 to 70 Å) can be filtered by the glomerulus and pass into the urine (Tarloff & Goldstein, 1994). That route seems improbable for a molecule the size of G7, although altered glomerular function, as indicated above by other polycations, may explain this characteristic (Hammes & Singh, 1994; deBarros e Silva et al., 1992). On the other hand, urine radioactivity may be derived from the metabolic demethylation of the [14C]methylated dendrimer derivatives. In preliminary work with methylated dendrimers of all three generations, cytochrome P450 did not appear to mediate any such demethylation (data not shown), leaving this question an open one. The metabolic conversions that the dendrimers undergo, in general, remains a subject requiring careful study.

The different urinary excretion of G7 compared to either G3 or G5 may also be a reflection of a dramatic change in molecular shape. Smaller dendrimers appear to prefer a floppy disc-like arrangement, whereas larger generations favor a hollow ball configuration (Tomalia, 1991). This alteration in topology may affect interaction with cellular targets and/or metabolic enzymes and explain G7's behavior in certain circumstances.

In summary, low generation PAMAM Starburst™ dendrimers appear to possess few problems for in vivo use. The higher generations PAMAMs may not be the best choice of dendrimer for biological applications because of their very high polycationic nature, which may be responsible for some or all of their toxicity and unusual biodistribution; dendrimers constructed with other functional groups may be preferable.
References


Haensler, J. and Szoka, F. C., Jr. (1993) Polyamidoamine cascade polymers mediate


CHAPTER 5

ANTIBODY MODIFICATION STRATEGIES USING POLYMERIC AMPLIFIERS FOR SITE-SPECIFIC DELIVERY OF COPPER-67

Introduction

The search for selective agents for eradication of infectious diseases and cancer is of utmost importance. A rational approach to the development of such selective agents is through the use of antibodies. Radioimmunoimaging and radioimmunotherapy are theoretically feasible techniques providing that monoclonal antibodies conjugated with imaging or therapeutic radionuclides, or anticancer agents, achieve sufficient localization in target sites and maintain their ability to bind antigen (Vaughan et al., 1987; Bradwell et al., 1985; Fritzberg et al., 1988). Several different tumor-specific antibodies have been developed and studied, both in experimental as well as clinical studies (Houghton & Scheinberg, 1991). The advent of hybridoma technology has provided a boost to research in this field by providing the ability to design and make antibodies with predetermined specificity (Kohler & Milstein, 1975). Radiolabeled monoclonal antibodies are under intense scrutiny for use as diagnostic or therapeutic agents in the fight against cancer (Carrasquillo et al., 1986; Macklis et al., 1988).
A promising radionuclide with favorable decay characteristics is copper-67. With a 62-hour half-life, this isotope has great potential in nuclear medicine applications (Raman & Pinajian, 1969) such as antibody-mediated delivery (DeNardo et al., 1983). It decays by β-emission, with particle energies suitable for therapeutic use (395 to 577 keV) (Wessels & Rogus, 1984). Decay by γ-emission involves the loss of a 184 keV photon (48% abundance) that can readily be visualized by Anger cameras in clinical use.

Since in vivo stability of the metal ion-antibody conjugate is very important, a macrocyclic chelating agent has been used which, under physiological conditions, prevents loss of the metal. The chelating agent selected was a synthetic porphyrin, N-4-nitrobenzyl-5-(4-carboxyphenyl)-10,15,20-tris(4-sulfophenyl)porphine (N-bzHCS₃P), "tailor-made" to incorporate copper under mild conditions. Use of this particular porphyrin provides the ability to design a synthetic scheme with copper-67 labeling as the final step, a desirable sequence for radiopharmaceutical preparation (Roberts et al., 1987; Mercer-Smith et al., 1988; Roberts et al., 1989; Roberts et al., 1990; Mercer-Smith et al., 1991). The highlight of this porphyrin is the presence of an N-benzyl substituent which distorts the planarity of the porphine ring. This feature allows for rapid incorporation of copper from the opposite face (Lavallee et al., 1986) and under relatively mild conditions which are unlikely to affect antibody integrity. In addition, the sulfonyl groups impart water solubility to the otherwise hydrophobic porphyrin.

Direct conjugation of the porphyrin to polyclonal and monoclonal antibodies and autogenic peptides has been reported (Roberts et al., 1987; Mercer-Smith et al., 1988; Roberts et al., 1989; Roberts et al., 1990; Mercer-Smith et al., 1991). However, if several
porphyrin molecules must be attached to the protein and the advantages of single-site attachment to protein are to be preserved, then it is necessary to construct a carrier molecule. An approach that involves the incorporation of polymeric amplifiers as intermediate linker molecules for the synthesis of antibody-porphyrin conjugates was developed. Three conjugates were synthesized and were subjected to in vitro testing. Polymeric amplifiers have been used in this study because of their ability to carry a large load of cytotoxic molecules without overmodifying the protein itself. This approach is particularly attractive since it involves coupling several cytotoxic molecules to a linker molecule, which has multiple reactive sites, and then coupling this complex to a monoclonal antibody. The polymeric amplifiers used were Starburst™1 dendrimers and a linear polypeptide polymer, poly(lys,ala)2:1 (K2A).

Starburst™ dendrimers are macromolecules that differ from classical polymers in certain respects. Polymers synthesized by the normal polymerization processes give rise to molecules with different degrees of polymerization and hence their molecular weight is expressed as a range. In contrast, Starburst™ dendrimers are highly ordered macromolecules with a well defined architecture. Immunoconjugates were prepared using polyamidoamine Starburst™ dendrimers of generations 3 and 4 (G3 and G4). These homogenous molecules were used in an attempt to improve the homogeneity of the final bioconjugate. The linear random polymer, K2A, composed of lysine and alanine in a 2:1 proportion (MW ~ 50 kDa), was included for comparison with the spherical Starburst™ dendrimers.

1Starburst is a Trade Mark of Dendritech, Inc., Midland, MI.
Usually labeling of proteins involves the modification of amino acid side chains. These include tyrosines, the ε-amino groups of lysines, N-terminal amines, carboxyl groups of glutamic and aspartic acids, and sulfhydryl groups generated by mild reduction of cystines (Pietersz et al., 1994). The site at which modification takes place has a special significance when labeling of immunoglobulins (Ig) is involved. Certain polypeptide moieties of immunoglobulins impart their specific antigen binding ability, and modification of the amino acid side chains in this region may result in partial or complete loss of immunological activity. In contrast, the carbohydrate moieties of immunoglobulins are not involved in the antigen binding properties of these molecules, and modification should not affect antigen binding (O'Shannessy et al., 1984). One immunoconjugate was synthesized by modification of the carbohydrate residues, whereas two others were synthesized by random modification of the amino groups. Different coupling strategies were used in order to compare the properties of the different immunoconjugates and determine the ones that exhibited good retention of immunoreactivity and maximal porphyrin loading. Immunoconjugates were synthesized by coupling porphyrin-modified polymers to the anti-renal cell carcinoma (RCC) antibody, A6H (Moon et al., 1985; Vessella et al., 1988).

RCC is a clinically relevant tumor model, constituting approximately 85% of all primary kidney tumors and 2 to 3% of all cancers in adults (Millan, 1989). La Vecchia et al. have reported a periodic increase in mortality resulting from cancer of the kidney from 1955 to 1990 in most European countries (La Vecchia et al., 1992). Chemotherapy has been largely unsuccessful, with vinblastine being the most active agent, but response rates rarely exceed 16% (deKernion, 1983). Immunotherapy and hormonal therapy have also
been largely unsuccessful; several studies are still in progress (Wirth, 1993). With surgical
tumor resection being the only curative therapy available for RCC at this time (Wirth, 1993),
new systemic therapies and specific diagnostic tools for RCC are critically needed.

The aim of this study was to develop antibody-porphyrin conjugates with
intermediate linkers synthesized using different linkages for connecting the porphyrin­
bearing polymer to the antibody. The idea was to investigate the difference in the use of
spherical vs. linear polymers as intermediate linkers in antibody-porphyrin conjugates. This
study was also designed to determine the influence of the chemical bond between the
polymer and antibody on the immunoreactivity, coupling yield, and aggregation of the
conjugates.

Experimental

Materials and Methods

ACHN cells, originally obtained from American Type Culture Collection (Rockville, MD), were maintained in minimum essential medium, α-modification (α-MEM) culture medium supplemented with 10% serum (5% fetal calf serum and 5% α-calf serum) and antibiotics (10 mL of antibiotic-antimycotic solution per 1 L of medium) by passage every 2-3 days. A6H was produced as previously described (Moon et al., 1985; Vessella et al., 1985). Distilled-in-glass grade N,N-dimethylformamide (DMF) from EM Science (Cherry Hill, NJ) was used. CuCl₂ was obtained from Aldrich Chemical Co. (Milwaukee, WI). N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB) and 2-iminothiolane (Traut's reagent) were purchased from Pierce Chemical Co. (Rockford, IL). Reagents used for enzyme-
linked immunosorbent assay (ELISA) were obtained from Vector Laboratories, Inc. (Burlingame, CA). PD-10 columns were purchased from Pharmacia Labs (Alameda, CA). 96-well plates were obtained from Costar Corning (Cambridge, MA). All other reagents, chemicals, and chromatography supplies were purchased from Sigma Chemical Co. (St. Louis, MO). Water purified by a Barnstead Nanopure II system with > 17 mΩ resistivity was used for all applications. Radiolabeled samples were counted using a Cobra™ Auto-Gamma® counting systems (Model D5003) with the spectral window that extended from 60-210 keV.

Copper-67

Carrier-free copper-67 was obtained as \( ^{67}\text{CuCl}_2 \) in 2 M HCl from Los Alamos National Laboratory (Los Alamos, NM). The acid was evaporated to dryness under a flow of air or argon with gentle heating, and the radionuclide was taken up into DMF for metalation.

Synthetic Procedures

Synthesis of the Porphyrin-G4-Antibody Conjugate (P-G4-A6H)

Preparation of porphyrin-G4 complex (P-G4). The synthetic scheme for synthesis of P-G4-A6H is shown in Figure 5.1. N-bzHCS\(_3\)P was synthesized and characterized as published elsewhere (Mercer-Smith et al., 1988). For activation, 480 \( \mu \)L of N-bzHCS\(_3\)P (1.8 mg, 1.1 x 10\(^{-3}\) mmol) in DMF (200 \( \mu \)L) was mixed with 240 \( \mu \)L each of a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (2.6 mg, 1.3 x 10\(^{-2}\) mmol) and a
Figure 5.1. Scheme for the synthesis of the conjugate P-G4-A6H.
solution of N-hydroxysuccinimide (NHS) (1.5 mg, 1.3 x 10^{-2} \text{ mmol}), both in 12.5 mM sodium dihydrogen phosphate, pH 6 (100 \mu\text{L}). After 1 hour of porphyrin activation in the dark at room temperature, 1296 \mu\text{L} of G4 (MW = 10,619 Da; 24 terminal amines; 2.2 mg, 2.1 x 10^{-4} \text{ mmol}) was added and the mixture stirred in the dark at room temperature for 18-20 hours. The P-G4 complex thus prepared was used for coupling with the oxidized antibody without any further purification.

**Preparation of oxidized A6H (A6H-CHO).** Oxidation of A6H was carried out by mixing 5.0 mg of salt-free A6H with 667 \mu\text{L} of a 30 mM solution of sodium m-periodate in 0.1 M sodium phosphate buffer, pH 6.0. Oxidation was carried out at 4\degree\text{C} for 4 hours in the dark. Excess sodium periodate and generated fragments were removed by passing the mixture through a PD-10 size exclusion column. The eluent collected was dialysed under negative pressure using a membrane with a molecular weight cut-off of 25,000 Da.

**Coupling of A6H-CHO to P-G4.** For the final coupling step, 265 \mu\text{L} of A6H-CHO (1.18 mg) was mixed with 1316 \mu\text{L} of P-G4 solution and allowed to stir at room temperature for about 12 hours. For reducing the Schiff's base, 12 mg of NaCNBH\textsubscript{3} was added and stirring was continued for an additional 12 hours to form P-G4-A6H.

Before purification, the mixture was subjected to buffer exchange by dialyzing it into 10 mM Tris, pH 8.5, for 24 hours, using a membrane with a molecular weight cut-off of 3500 Da. Purification was carried out by ion exchange chromatography using preswollen DEAE-cellulose as the stationary phase and 1 M NaCl for elution. The eluent was monitored at 435 nm for porphyrin absorbance. Fractions containing the conjugate were then pooled and dialyzed into water at 4\degree\text{C}. After 10 hours of dialysis, the water was
replaced and dialysis was continued for an additional 10 hours. The essentially salt-free solution was then freeze-dried and reconstituted with 2 mL of borate-buffered saline (BBS, 0.1 M borate, 0.08 M NaCl), pH 8.5. (Phosphate buffers interfere with the later metalation step by producing insoluble salts.) The bright green solution was stored on ice until further use.

The amount of porphyrin attached to protein was determined by UV-Vis spectrophotometric analysis using the extinction coefficients for the antibody at 280 nm (14.3) and for the porphyrin at 435 nm (3000) for quantitation of protein and porphyrin. Using these values, the number of porphyrins per antibody molecule was determined.

Synthesis of Porphyrin-G3-Antibody Conjugate (P-G3-A6H)

Preparation of G3-SIAB complex (G3-SIAB). The synthetic scheme followed has been shown in Figure 5.2. To 380 μL of a solution of G3 (MW = 5,147 Da; 12 terminal amines, 3.6 mg, 7 x 10⁴ mmol) in BBS (1000 μL) was added 2.66 μL of a solution of SIAB (20 mg, 5 x 10⁻² mmol) in DMF (1000 μL) and allowed to react for 1 hour at room temperature in the dark. The excess unreacted SIAB was quenched by addition of 2040 μL of BBS. The final mixture was allowed to stir for 1.25 hours at room temperature in the dark.

Preparation of porphyrin-G3-SIAB complex (P-G3-SIAB). The porphyrin was activated by mixing 480 μL of a solution of N-bzHCS₃P (1.8 mg, 1.13 x 10⁻³ mmol) in DMF (200 μL) with 240 μL each of a solution of EDAC (2.6 mg, 1.3 x 10⁻² mmol) and a solution of NHS (1.5 mg, 1.3 x 10⁻² mmol), both in 12.5 mM sodium dihydrogen phosphate, pH 6
Figure 5.2. Scheme for the synthesis of the conjugates P-G3-A6H and P-K2A-A6H.
\[
\text{NH}_2 + \text{A6H-SH} \xrightarrow{\text{BBS 8.5}} \text{A6H-SH}
\]

\[
\text{A6H-SH} + P_L \xrightarrow{\text{pH 9.2}} P-G3-A6H \quad \text{or} \quad P-K2A-A6H
\]

\[
\text{P-L-SIAB} \quad \text{24 hrs, RT}
\]

Figure 5.2. (continued).
(100 μL). After 1 hour of porphyrin activation in the dark at room temperature, 4800 μL of G3-SIAB was added and the mixture was stirred in the dark at room temperature for 18 to 20 hours.

**Thiolation of the antibody (A6H-SH).** To 3.6 mg of A6H was added 0.3 mg of iminothiolane (2.6 x 10⁻² mmol). This mixture was dissolved by the addition of 500 μL of BBS and was stirred in the dark at room temperature for 1 hour. The unreacted iminothiolane was removed by passing the mixture through a PD-10 column and 2 mL of the thiolated antibody was eluted with BBS, pH 11. The number of thiols generated were quantitated using Ellman's reagent [5,5'-dithio-bis(2-nitrobenzoic acid)] (Ellman, 1959). The reagent solution was prepared by dissolving 4 mg of Ellman's reagent in 1 mL of 0.1 M sodium phosphate buffer, pH 8.0. The thiolated antibody solution was diluted 20-fold from 20 μL to 400 μL. After measuring the absorbance of the antibody solution at 280 nm, 100 μL of Ellman's reagent solution was added. Following a 15-minute incubation period, absorbance was measured at 412 nm. Using the absorbance values and the extinction coefficients of the antibody (14.3) and Ellman's reagent (343), the number of thiols per antibody was determined.

**Coupling of P-G3-SIAB and A6H-SH.** In the final step, 2310 μL of P-G3-SIAB was added in 100 μL aliquots to 1980 μL of A6H-SH (2.8 mg) over a period of 2 hours at 4°C. Stirring was then carried out at room temperature for 24 hours. Purification and quantitation of the coupling yield were carried out as already described for the P-G4-A6H conjugate.
Synthesis of Porphyrin-K2A-Antibody Conjugate (P-K2A-A6H)

**Preparation of K2A-SIAB complex (K2A-SIAB).** The scheme for the synthesis of this conjugate has been shown in Figure 5.2. A solution of the polymer was prepared by dissolving 6 mg of K2A (1.2 x 10^4 mmol) in BBS (1500 μL). Of this solution, 500 μL was used for reaction with 48 μL of a solution of SIAB (20.11 mg, 5 x 10^2 mmol) in DMF (1000 μL). The solution immediately turned hazy and an additional 250 μL of BBS was added to clarify the solution. The mixture was allowed to react for 1 hour at room temperature in the dark. The excess unreacted SIAB was removed by passing the solution through a PD-10 column, yielding 2 mL of purified K2A-SIAB eluted in BBS.

**Preparation of porphyrin-K2A-SIAB complex (P-K2A-SIAB).** Porphyrin activation was carried out by mixing 400 μL of a solution of N-bzHCS₃P (1.8 mg, 1.1 x 10^3 mmol) in DMF (200 μL) with 200 μL each of a solution of EDAC (2.6 mg, 1.3 x 10^2 mmol) and a solution of NHS (1.5 mg, 1.3 x 10^2 mmol), both in 12.5 mM sodium dihydrogen phosphate, pH 6 (100 μL). After 1 hour of porphyrin activation in the dark at room temperature, 1050 μL of K2A-SIAB was added and the mixture was stirred in the dark at room temperature for 20 to 22 hours.

**Thiolation of the antibody (A6H-SH).** To 3.4 mg of A6H was added 0.2 mg of iminothiolane (1.5 x 10^3 mmol). This was followed by the addition of 500 μL of BBS to dissolve the solids, and the mixture was allowed to stir in the dark at room temperature for 1 hour. The unreacted iminothiolane was removed by passing the reaction mixture through a PD-10 column and 2 mL of the thiolated antibody was eluted with BBS, pH 11.

**Coupling of P-K2A-SIAB and A6H-SH.** To prepare the desired conjugate, 1938
µL of P-K2A-SIAB were added in 100 µL aliquots to 1950 µL of A6H-SH (2.2 mg) over a period of 2 hours at 4°C. Stirring was then carried out at room temperature for 24 hours. Purification and quantitation of the coupling yield was carried out as already described for the P-G4-A6H conjugate.

**Metalation with CuCl₂ and ⁶⁷CuCl₂**

All metalation reactions were performed in dim light using acid-washed glassware to prevent inadvertent porphyrin metalation or photooxidation. The purified conjugates were metalated with stable copper (1 mM CuCl₂) at a 1:1 copper:porphyrin ratio at 40°C for 45 minutes. Metalation with ⁶⁷CuCl₂ was also carried out at 1:1 copper:porphyrin ratio at 40°C for 90 minutes. Following metalation, the conjugates were subjected to vacuum dialysis into phosphate-buffered saline (PBS, 10 mM phosphate buffer, 120 mM NaCl, pH 7.4), containing 0.05 M sodium citrate to complex any free copper. Unmetalated preparations were vacuum dialyzed under similar conditions with the exclusion of sodium citrate from the dialysis buffer. Metalation yield was calculated for preparations metalated with copper-67 by counting the sample before and after vacuum dialysis.

**Control Preparations**

In order to pinpoint the steps that were responsible for loss of biological activity of the antibody, a step-wise analysis of the reaction sequence was carried out. The procedure involved treatment of the antibody using identical reaction conditions as were used in the synthesis with the exclusion of the porphyrin, dendrimers, K2A, and the coupling reagents,
EDAC and NHS. Following antibody modification steps, ELISA was performed to determine immunoreactivity of modified antibody.

**Oxidation of A6H.** For the oxidation, 3 mg of salt free A6H (2 x 10^-5 mmol) were mixed with 390 µL of a 30 mM solution of sodium m-periodate in 0.1 M sodium phosphate buffer, pH 6.0. Oxidation was carried out at 4°C for 4 hours in the dark. Excess sodium periodate and generated fragments were removed by passing the mixture through a PD-10 desalting column and 2 mL of oxidized antibody solution was collected by elution with BBS, pH 9.2. The eluent was concentrated by vacuum dialysis using a membrane with molecular weight cut-off of 25,000 Da. Part of the dialyzed solution (A6H-CHO) was used for ELISA.

**Reaction with methylamine.** To simulate the reaction with the dendrimer-porphyrin complex, 530 µL of A6H-CHO (1.5 x 10^-5 mmol) was stirred with 528 µL of DMF, 528 µL of 12.5 mM sodium dihydrogen phosphate, pH 6, and 1426 µL of BBS. Approximately 0.2 mg of methylamine hydrochloride was added to block the reactive aldehydes generated on the antibody by oxidation and to mimic the actual reaction with amino groups from the dendrimer. The mixture was stirred at room temperature in the dark for 13 hours. For reduction of the intermediate Schiff's base, 4.5 mg of NaCNBH₃ were added and stirring was continued for an additional 12 hours to obtain A6H-NHCH₃ solution.

**Preparation of A6H-SH.** For thiolation, 1.9 mg of A6H (1.3 x 10^-5 mmol) were mixed with 0.2 mg of iminothiolane and 500 µL of BBS was added. After reaction for 1 hour at room temperature, the reaction mixture was purified by passing through a PD-10 column. Fractions of A6H-SH were collected using BBS, pH 11, for elution.
**Reaction with iodoacetamide.** To prevent oxidation of the free sulfhydryl groups, A6H-SH was reacted with iodoacetamide. The solution was prepared by mixing 4.3 mg of iodoacetamide with 928 µL of BBS. Slight warming was required to dissolve the iodoacetamide. Upon cooling, the iodoacetamide solution was mixed with 186 µL of DMF and 186 µL of a solution of 12.5 mM sodium dihydrogen phosphate, pH 6. This mixture was added to 1965 µL of A6H-SH (1.57 mg, 1 x 10⁻⁵ mmol) at 4°C in 100 µL aliquots over a 2-hour period. The mixture was allowed to stir at room temperature for 24 hours. This solution (A6H-CONH₂) was used for ELISA. The mixture was then subjected to the regular purification protocol. The solution was dialyzed in 10 mM Tris, pH 8.5, at room temperature for 24 hours using a membrane with a molecular weight cut-off of 3500 Da. Following purification by ion exchange chromatography and dialysis of the pooled fractions into water, the purified antibody solution (pure A6H-CONH₂) was tested by ELISA.

**In Vitro Testing**

**Thin Layer Chromatography**

The radiometalated conjugates were analyzed by thin layer chromatography (TLC) to estimate the level of protein-bound activity (Yamazaki et al., 1988). The radiolabeled preparation was spotted on aluminum-backed silica gel plates that were developed in either acetone or 80% methanol. The plates were then cut into the top two-thirds and bottom one-third portions and were counted in the gamma counter.
Cell Binding Assay

A cell binding assay was performed to determine the immunoreactivity of the radiolabeled antibody (Vessella et al., 1988). Briefly, approximately 10,000 counts per minute (cpm) of the radioimmunoconjugate (contained in 0.1 mL PBS) were added to 2 × 10^6 RCC (ACHN) cells suspended in 900 μL of 1% bovine serum albumin (BSA) in PBS. Freshly trypsinized cells from a confluent culture were washed three times with 1% BSA in PBS before use. Each tube was counted in a gamma counter to obtain Total Counts. The mixture was incubated at room temperature for 1 hour with rotary inversion mixing. The cells were pelleted by centrifugation, the supernatant was decanted, and the cells were resuspended in 1 mL PBS. The counts remaining associated with the cells, Bound Counts, were quantitated in the gamma counter. The percent immunoreactivity was calculated as Bound Counts/Total Counts x 100.

ELISA

ELISA was carried out by the ABC method based on use of the high affinity biotin/avidin system. To each well of a 96-well microtiter plate were added 16,000-20,000 antigen-positive ACHN cells. Cell culture medium was added to obtain a final volume of 200 μL per well. The cells were allowed to incubate at 37°C in a 5% CO₂ atmosphere until confluent and attached. The plate was emptied by inversion and the cells were fixed by addition of 200 μL of 0.15% glutaraldehyde solution in PBS to each well. After incubation at room temperature for 20 minutes, the plate was again emptied by inversion and washed (five times) with 1% Tween 20 in PBS. Blocking of any free adsorption sites was achieved
by the addition of 200 μL of 1% BSA in PBS to each well. After 30 minutes incubation at room temperature, the plate was emptied and washed as before. Dilutions of the antibody preparations and unmodified antibody were prepared in 1% BSA in PBS. The concentrations used were 0.1, 0.3, 0.5, 0.7, 0.9, and 1.0 μg/mL, and the volume added to each well was 50 μL. Once again, wells were emptied and washed after 30 minutes of incubation at room temperature. This was followed by the addition of 50 μL of the biotinylated secondary antibody solution (7.5 μg/mL), a mouse specific antibody. Incubation time for this step was 30 minutes, following which the plate was emptied and washed. The next step was the addition of 30 μL of biotinylated horseradish peroxidase-avidin complex, which was allowed to incubate for 15 minutes at room temperature. The plate was emptied and washed as before. Finally, the substrate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), 50 μL, was added and product formation was monitored at 405 nm. ELISA was carried out on the following preparations: (1) unmodified A6H; (2) freeze-dried A6H; (3) P-G4-A6H; (4) Cu-G4-A6H; (5) P-G3-A6H; (6) Cu-G3-A6H; (7) P-K2A-A6H; (8) Cu-K2A-A6H; (9) A6H-CHO; (10) A6H-NH-CH₃; (11) A6H-SH; (12) A6HCONH₂; (13) pure-A6HCONH₂; and (14) MOPC-21, which served as a nonspecific control antibody.

**Determination of Aggregates by Size Exclusion HPLC**

Gel permeation chromatographic (GPC) analysis of all conjugates and unmodified antibodies was carried out using a Hitachi HPLC system equipped with a variable wavelength UV-Vis detector. A Bio-Silect SEC 250-5 column (300 x 7.8 mm) along with
a Bio-Select SEC 250 guard column (50 x 7.8 mm) was used. The following preparations were analyzed for their aggregate content: (1) unmodified A6H; (2) freeze-dried A6H; (3) P-G4-A6H; (4) Cu-G4-A6H; (5) P-G3-A6H; (6) Cu-G3-A6H; (7) P-K2A-A6H; and (8) Cu-K2A-A6H. All conjugates were analyzed immediately after vacuum dialysis and several times over a 1-week period. Samples were centrifuged (12,500 x g, 4°C, 2 to 3 minutes) to eliminate any particulate material before injecting on the HPLC column. Analysis was carried out with phosphate buffer (0.1 M sodium phosphate, 1 mM EDTA, 2 mM sodium azide, pH 6.8) as the mobile phase with a flow rate of 1 mL/min. The percent aggregation was calculated using the area under the monomer and aggregate peaks.

Molecular Modeling

Molecular modeling studies were performed on G3 and G4 on Silicon Graphics Iris or Indigo work stations. The molecules were constructed and visualized using the builder module of the Insight II software obtained from Biosym Technologies, Inc. (San Diego, CA). Minimizations and dynamics were performed using Discover software which was also obtained from Biosym Technologies, Inc. The dendrimer structures were first minimized by using the steepest gradient for 2000 iterations. To obtain a global minimum, molecular dynamics simulations were used. Dynamics was initialized at 2000 K with gradual reduction in temperature to 300 K over a period of 30 picoseconds. The lowest energy conformer obtained in the molecular dynamics simulation was further minimized by 3750 iterations. Since no crystal co-ordinates or detailed NMR data were available for these dendrimers, the images generated were used only to obtain a qualitative judgement of proximity of the
amino groups on the surface. Energy minimized porphyrin molecules were also attached
to the G4 to gain an estimate of amino group accessibility after porphyrin attachment.

Results

P-G4-A6H conjugates were prepared with a coupling efficiency of 2.7 porphyrins
per antibody as determined by UV-Vis spectrophotometry. The coupling yield for P-G3-
A6H and P-K2A-A6H conjugates was 1 and 0.8 porphyrins per antibody, respectively. The
radiometalation yield was 16% for $^{67}$Cu-G4-A6H, 45% for $^{67}$Cu-G3-A6H, and 22% for the
$^{67}$Cu-K2A-A6H conjugate. The specific activities of the $^{67}$Cu-G4-A6H, $^{67}$Cu-G3-A6H, and
$^{67}$Cu-K2A-A6H were 0.27, 0.24, and 0.11 mCi/mg of protein, respectively.

TLC analysis of radiolabeled preparations showed that the majority of the
radioactivity remained at the origin. Strips developed in 80% methanol resulted in 82%,
86%, and 64% activity being retained at the origin for the $^{67}$Cu-G4-A6H, $^{67}$Cu-G3-A6H,
and $^{67}$Cu-K2A-A6H, respectively. TLC plates developed in acetone resulted in 99% activity
being retained at the bottom for all the three conjugates under evaluation, confirming
the result that most of the radioactivity was bound to the protein and very little free copper-
$^{67}$ or copper-$^{67}$-labeled porphyrin was present.

In the cell binding assay, 15% of the total radioactivity remained associated with the
cell pellet when $^{67}$Cu-G4-A6H was tested. Testing of $^{67}$Cu-G3-A6H resulted in 10% cell
bound radioactivity; 9% of the total radioactivity was found to be cell-bound when $^{67}$Cu-
K2A-A6H was tested. The low amount of cell-bound radioactivity indicates that the
antibody does not possess any antigen-binding capabilities. The values are significantly
lower that the minimal immunoreactivity (50%) typically desired for injection (Vessella et al., 1988). The results from the ELISA are shown in Figures 5.3 through 5.5. These graphs show that the immunoconjugate preparations, both metalated and nonmetalated, show drastically reduced antigen-binding activity when compared to the unmodified antibody. Thus, the ELISA results confirm the loss in biological activity of the immunoconjugate preparations indicated by the cell-binding assay.

To determine the cause of this total loss of biological activity, the antibody was subjected to all the steps involved in the synthetic scheme. On performing ELISA after each step in the procedure, it was observed that the oxidation of A6H led to total loss of binding capability (Figure 5.6). However, thiolation had almost no effect on the immunoreactivity of A6H (Figure 5.6). As expected, when oxidized A6H was reacted with methylamine, and tested for its immunoreactivity, the preparation was still devoid of any biological activity and did not bind to the ACHN cells (Figure 5.7). Reaction of thiolated antibody with iodoacetamide followed by stirring at room temperature for 24 hours led to almost 50% loss in binding ability. Purification by ion exchange chromatography and dialysis of the pooled fractions into water led to total loss of binding ability (Figure 5.8).

Aggregation values for each preparation have been shown in Table 5.1. A frozen solution of A6H, when thawed and analyzed by HPLC, did not contain any aggregates. Desalting and freeze-drying A6H also did not cause aggregation. The P-G4-A6H conjugate and its metalated counterpart did not show any aggregation. On the other hand, conjugates prepared by the thioether linkage showed considerable aggregation. Almost 43% of P-G3-A6H was aggregated immediately after preparation, and this amount remained unchanged
Figure 5.3. ELISA performed on G4-containing immunoconjugates.
Figure 5.4. ELISA performed on G3-containing immunoconjugates.
Figure 5.5. ELISA performed on K2A-containing immunoconjugates.
Figure 5.6. ELISA performed on oxidized and thiolated A6H.
Figure 5.7. ELISA performed on A6H-NH-CH$_3$ and A6HCONH$_2$. 
Figure 5.8. ELISA performed on purified A6HCONH$_2$. 
Table 5.1

Aggregation levels for the different immunoconjugates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Aggregates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A6H</td>
<td>0</td>
</tr>
<tr>
<td>Freeze-dried A6H</td>
<td>0</td>
</tr>
<tr>
<td>P-G4-A6H</td>
<td>0</td>
</tr>
<tr>
<td>Cu-G4-A6H</td>
<td>0</td>
</tr>
<tr>
<td>P-G3-A6H</td>
<td>42</td>
</tr>
<tr>
<td>Cu-G3-A6H</td>
<td>43</td>
</tr>
<tr>
<td>P-K2A-A6H</td>
<td>55</td>
</tr>
<tr>
<td>Cu-K2A-A6H</td>
<td>56</td>
</tr>
</tbody>
</table>
over the 1-week period of testing (data not shown). Metalation of the conjugate did not alter the level of aggregates present in solution. Aggregation was also seen in P-K2A-A6H and Cu-K2A-A6H. Both these preparations showed about 53% aggregation irrespective of the presence or absence of copper. Once again, aggregation levels remained unchanged over a 7-day period when conjugates were stored at 4°C (data not shown). Using randomly linked antibody-porphyrin conjugates, it was found that the error associated with the aggregation measurements was ± 2%.

**Discussion**

Due to the lack of suitable diagnostic or therapeutic agents, the overall survival rate in RCC patients has not improved in the past 10 years (Couillard & deVere White, 1993). The continuing need for therapeutic and diagnostic agents has led to an extensive evaluation of radiolabeled antibodies that might be useful in RCC patients (Wilbur et al., 1993; Vessella et al., 1988). Isotopes that decay by β- and γ-emission are good candidates for radiolabeling antibodies since they can be used for both imaging (γ-emission) and therapy (β-emission). Copper-67 offers the potential for both uses and, in particular, has an excellent gamma photon (185 keV), which could result in valuable images for diagnostic purposes using conventional γ cameras, due to their operation in this range.

The synthetic porphyrins offer an exceptional chelating system for radiocopper because of the ease of metalation and stability of the metalloporphyrin formed (Roberts et al., 1987). The stability of the metalloporphyrins is comparable to other copper chelating systems currently under study (Fujiwara et al., 1985; Meares, 1986). Other attractive
features of these porphyrins include their nontoxic nature at clinically useful concentrations and the range of functional groups possible for conjugation with proteins.

Polylysine and branched polypeptides have been used as intermediate linkers in several different studies (Pietersz, 1990; Ryan et al., 1992; Caneva et al., 1993). Oligonucleotide-polylysine-heparin complexes have been shown to be potent sequence-specific inhibitors of HIV-1 infection (Degols et al., 1994). Succinylated polylysine has been used as a linker for the attachment of deferroxamine to antibodies (Slinkin et al., 1990). In a study designed to investigate the antigenicity of a predicted epitope region of herpes simplex virus, small peptides were conjugated to a branched polypeptide with a polylysine backbone, poly[L-Lys-(DL-Ala),], where \( m \approx 3.4 \) (Hudecz et al., 1993).

The use of dendrimers in drug delivery is also on the rise. Roberts et al., (1990) and, more recently, two other groups have reported the use of dendrimers as linkers molecules in the design of drug delivery agents (Barth et al., 1994; Gansow et al., 1994). Dendrimer-based metal chelates have also been recently reported as a new class of magnetic resonance imaging contrast agents (Wiener et al., 1994). The polyamidoamine dendrimers are spherical molecules with amino groups present at the periphery and ammonia as the initiator core (Tomalia et al., 1990). Attachment of the large porphyrin ring to amino groups on the dendrimer surface is likely to hinder the access to the maleimido or unreacted amino groups and may be responsible for the low coupling efficiency. It is known that polyanionic molecules form a very stable \( \alpha \) helix with polymers such as poly-L-lysine (Degols et al., 1994). Such interaction could be responsible for the low porphyrin loading that was observed in preparation of the K2A-containing conjugate. The low coupling efficiency
observed for the conjugate prepared using K2A may also be due to steric hinderance caused by the porphyrin. Hydrophobic interactions of the porphyrin ring with the alanine side chains could also be responsible for reduced accessibility of the porphyrin to the amino groups and may lead to reduced coupling efficiency as well.

Due to the lack of structural details, the molecular modeling studies performed were only used to procure qualitative information. Images generated from the molecular modeling studies have been shown in Figures 5.9 to 5.11. As seen from Figure 5.11, due to the large size of the porphyrin, attachment of only two porphyrins can cover a large span of the dendrimer surface and reduce accessibility. Such interactions may be responsible for the reduced coupling to more porphyrins and antibodies that was observed when the dendrimers were used as linkers. Interaction of the porphyrin with the hydrophobic alanine side chains may be responsible for reduced coupling in the K2A conjugate.

Lavallee et al. (1986) have reported that radiometalation of the porphyrins is almost quantitative under mild conditions. However, radiometalation of the A6H conjugates proceeded in low yields, which may be due to the porphyrin being part of the immunoconjugate limiting accessibility of the copper ion. It is also likely that the low metalation observed is due to the presence of the intermediate linkers and the interaction of the porphyrin with these linker molecules. Highest metalation yield for the G3-containing conjugate may be because of minimal steric hinderance caused by the smaller size (5,147 Da) of G3. Orientation of the porphyrin is of added importance in the case of N-substituted porphyrins because the metal ion may only complex on one side of the porphyrin (Bain-Ackerman & Lavallee, 1979). Similar low metalation yields have also been previously
Figure 5.9. Molecular modeling image generated for G3.
Figure 5.10. Molecular modeling image generated for G4.
Figure 5.11. Molecular modeling image of G4 attached to two porphyrins.
reported by Mercer-Smith et al. (1988) when the porphyrin was present as part of a conjugate. The random conjugates reported in Chapters 2 and 3 also showed reduced metalation yields ranging from about 13 to 41%.

The *in vitro* cell binding analysis of the A6H radioimmunoconjugates suggests that either method of antibody modification and processing leads to loss in binding ability of the antibody. Random thiolation of the antibody involved modification of approximately 15 to 20 amino groups, and since the modification was small in size, it is likely that the antigen-recognition capabilities were not affected to a great extent. This was confirmed by the ELISA performed on the thiolated antibody (Figure 5.6). Ion exchange chromatography, dialysis, and lyophilization are operations routinely carried out on proteins, including antibodies. Since the immunoconjugate preparations bearing the thioether linkage were devoid of antigen-binding properties after purification of the preparation, it is likely that excessive handling of the sensitive antibody was responsible for such loss in biological activity.

The average carbohydrate content of IgGs is 3% and consists of two oligosaccharide chains (O'Shannessy & Quarles, 1985). The main part of the sugar of IgG occurs in the C_H domain and is N-linked to Asn-297 of both heavy chains (Rademacher & Dwek, 1984). A small amount of additional N-linked sugars has been found in human IgG (Parekh et al., 1985) and in rabbit IgG (Taniguchi et al., 1985). These sugars are situated in the Fab fragment and their presence depends on the availability of an Asn-X-Ser(Thr) sequence in the hypervariable region (Savvidou et al., 1981). O-Linked oligosaccharides that are species- and allotype-specific may be attached to the hinge region of IgG (Rademacher &
Dwek, 1984). Several groups have performed periodate oxidation of the oligosaccharide chains to generate reactive aldehydes which have been used for conjugation of cytotoxic agents to produce immunologically active conjugates (Nakane & Kawaoi, 1974; Hoffman & O'Shanessy, 1988; Laguzza et al., 1989; Roberts et al., 1990). Gaivoronskaya et al. (1981) have shown that immunologic activity in periodate-treated antibodies is not affected when treated with low concentrations of periodate, but decreases significantly with an increase in periodate concentration.

The loss in immunological activity and generation of aldehydes appears to be dependent largely upon the antibody used. For example, Abraham et al. (1991) have detected 25.5 aldehyde groups on the periodate-oxidized antibody, 96.5, measured by borohydride reduction, whereas the same reaction conditions led to only 9.6 aldehydes per antibody L6 of the same IgG2a isotype. The fact that A6H lost complete binding ability when subjected to sodium periodate oxidation could be a consequence of the sensitive nature of the antibody along with a combination of the specific conditions used, or even the presence of some oligosaccharides in the Fab portion as has been observed in case of some human and rabbit IgGs. It is also possible that amino acids such as methionine, tyrosine, histidine, phenylalanine, and tryptophan present in the antigen-binding site were oxidized (Atassi, 1977) and are responsible for the inability of oxidized A6H to bind to its antigen.

The action of sodium periodate is largely dependent up on the exact conditions of pH, temperature, and reagent excess that are used (Atassi, 1967). For example, oxidation of tyrosine and tryptophan residues in apomyoglobin was eliminated, and reaction at pH 5 and 0°C was entirely specific for the two methionines, when periodate was present in
equimolar amount relative to the number of methionine residues in the protein (Atassi, 1967). In the presence of a larger periodate excess at 32°C and pH 8.5, oxidation of the glycoprotein, ovatransferrin, at three tyrosines and one tryptophan was reported (Azari & Phillips, 1970) without effect on the carbohydrate moiety. Thus, either one or more of these factors could be responsible for loss of immunoreactivity observed during oxidation of A6H. Roberts et al. (1990) have reported the use of 5 mM sodium periodate at pH 5.5 for 8 hours at room temperature for oxidation of an anti-conalbumin antibody with 90% retention of immunoreactivity. Rodwell et al. (1986) report the use of oxidation conditions that are similar to the ones described here. They carried out oxidation of the oligosaccharide moieties by incubation of the antibody in the dark with 10 to 30 mM sodium periodate in PBS, pH 6.0, on ice for 1 hour. Using these conditions they report that binding of the oxidized antibody was similar to that of the unmodified antibody. Periodate oxidation of the enzyme, horseradish peroxidase, led to inhibition of enzyme activity. However, most of this activity was recovered upon reaction with IgG (O'Sullivan & Marks, 1981). Such recovery of activity was not observed in A6H, suggesting that the loss in binding activity was not temporary or transient.

Aggregation of antibody molecules has been reported on several occasions in the past. Kummer et al. (1993) have found that certain anti-Thy-1 monoclonal antibodies of the IgG2c isotype have the inherent tendency to self-associate, probably through homophilic Fc-Fc contacts, which renders them mitogenic. Absence of aggregates in the amine-linked conjugate may be caused by the presence of linker conjugated porphyrins present in the Fc region, preventing Fc-Fc contacts. It is also likely that conformational constraints prevent
the interaction of porphyrins present in this region and hence they are unable to participate in intermolecular association leading to the formation of aggregates. This may also explain the aggregation observed in the case of thioether-linked conjugates. These conjugates are nonspecific with respect to their porphyrin localization and it is likely that porphyrins can participate in intermolecular association owing to their tendency to stack. The presence of such interactions may be responsible for the aggregation observed. This is similar to the aggregation of randomly linked porphyrin-antibody conjugates that was discussed in Chapters 2 and 3. From a detailed study of aggregation kinetics of immunoglobulins performed by Feder and Joessang (1986), it was suggested that aggregation of IgG molecules is an irreversible process. The aggregation observed in thioether-linked conjugates confirms this finding since the aggregation levels were found to remain constant over a 1-week period. This corroborates with what has been observed in the past with respect to aggregation of randomly linked antibody-porphyrin conjugates (Chapter 2). It was also observed that the randomly prepared conjugates were not seriously devoid of immunoreactivity, suggesting that aggregation is not directly responsible for loss in binding capability of the immunoconjugates. In the case of these random conjugates, attempts involving the use of higher salt concentrations, the nonionic surfactant 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), and glycercol did not help in reversing the aggregation process.

The results shown here demonstrate that, although it is possible to link porphyrins to antibodies using linear or spherical polymers, the coupling scheme needs significant alteration to obtain immunologically active conjugates. The thioether-linked conjugates may
be prepared with retention of immunological activity if the number of manipulations carried out is decreased. A6H is apparently very sensitive to oxidation and thus a different coupling strategy needs to be designed in order to obtain site-specific linking of porphyrins. The carbohydrate oxidation strategy should be avoided for preparation of conjugates with A6H. Clearly, there are a number of features of these conjugates that are poorly understood and require further explanation. The results shown here do not suggest any difference in the properties tested for the conjugates prepared with linear or spherical polymers. However, there is a difference in the properties of conjugates prepared using the two different coupling strategies. Whereas the thioether-linked conjugates show variable amounts of aggregates, the carbohydrate oxidation method gives conjugates that are free of aggregates. This later method needs to be pursued with other antibodies that are less sensitive to oxidation, since conjugates prepared by carbohydrate oxidation approach were free of aggregates, as desired.

References


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CHAPTER 6

SUMMARY AND CONCLUSIONS

Because cancer is very diverse in its expression, it appears that disparate paths may be required to fight it. Selective targeting of therapeutic agents to tumors, to reduce toxicity to normal tissues, has been a major focus of cancer research. The natural barriers which have to be overcome before the targets are really reached are significant. Several strategies have been developed to increase the amount of cytotoxic agent reaching the target site. Of the different reagents that have been used against cancer, certain radioisotopes possess a distinct advantage, in that they can be used for therapeutic as well as diagnostic purposes.

Antibodies and their immunologically reactive fragments, raised against tumor associated antigens, have shown promise as carriers for a wide variety of different molecules. To explore the specific localization ability of these antibodies, a number of studies has been carried out for tumor-specific delivery of cytotoxic agents. Some of the major drawbacks that have impeded the use of antibodies as drug carriers are the low dose of the antibody that reaches the tumor, the immunogenicity of these preparations, and the lack of suitable chemical modification strategies to prepare radioimmunoconjugates with high radioisotope loading and maximal retention of immunoreactivity. Although the use of humanized monoclonal antibodies may address the immunogenicity problem, poor antibody localization continues to be a problem. Also newer bioconjugation techniques are required
to obtain preparations with better properties.

A panel of copper-67 immunoconjugates was synthesized with the aim of preparing immunoconjugates which had high specific activity, so that the problem of poor tumor dosimetry might be addressed. Different coupling strategies were developed to generate a set of unique immunoconjugates. The tumor model used was renal cell carcinoma, and studies were carried out with an antirenal cell carcinoma antibody, A6H. Direct attachment of a copper-67 chelator, a synthetic porphyrin, to antibody or antibody fragments led to conjugates with low specific activity. The conjugate prepared with the whole antibody showed good \textit{in vivo} tumor localization. Biodistribution of the conjugate was compared with that of the unconjugated copper-67-labeled porphyrin, and it was observed that the immunoconjugate preparation had a better tumor to blood ratio, suggesting potential advantages of the antibody-mediated delivery approach. However, conjugates involving the use of fragments had reduced immunoreactivity and were not tested \textit{in vivo}.

All the conjugates prepared by direct, random attachment of the porphyrin to antibody or its fragments showed high levels of aggregation, probably due to the stacking nature of porphyrin molecules. This was substantiated by studies carried out using preparations with varying number of porphyrins per antibody, as well as metalated versus nonmetalated immunoconjugates. Use of higher salt concentrations as well as non-ionic surfactants could not reduce aggregation to acceptable levels.

Using intermediate polymeric linkers for the preparation of immunoconjugates did not help increase the number of porphyrins per antibody, and again resulted in conjugates with low specific activity. Unlike the conjugates prepared by direct attachment of
porphyrins to antibodies, these conjugates were completely devoid of immunoreactivity. A detailed investigation led to the suggestion that A6H was possibly a particularly sensitive antibody and had lost its binding potential as the number of manipulations carried out during the course of immunoconjugate preparation was increased. Oxidation of carbohydrate residues on A6H led to immediate loss in binding activity. Intermediate polymer-containing conjugates that did not involve modification of the carbohydrate residues were found to contain aggregates. Modification of the carbohydrate regions of the antibody, however, had a different effect on aggregation properties of the immunoconjugate. The conjugate prepared by oxidation of the carbohydrate portion of the antibody was free of any aggregates, suggesting the potential utility of this approach for development of aggregate-free immunoconjugates using other antibodies.

During the biological evaluation of the polyamidoamine dendrimers, it was observed that in vitro toxicity increased as the generation number and concentration increased. Generation 7 was toxic to V79 cells even at a concentration of 100 nM. When the dendrimers were studied for their in vivo toxicity in mice, once again a dose- and generation-dependency was observed. Only generation 7 dendrimers showed any evidence of toxicity when injected at a dose of 5 x 10^4 mmol/kg. However, none of the generations tested showed any evidence of immunogenicity, mutagenicity, or carcinogenicity. In biodistribution studies, generations 5 and 7 dendrimers were found to show high localization in the pancreas. Overall, it appears that lower generation dendrimers in low doses may be used in vivo without any unwanted effects.

The results obtained here suggest that the use of immunoconjugates for cancer
imaging and therapy can provide promising results with the appropriate choice of components in the delivery system. The chemical methodology developed here can be used for synthesis of conjugates using different antibodies and different chelating agents. The carbohydrate modification strategy may help provide conjugates that are free of aggregates and help in solving the problem of reticuloendothelial system uptake of these conjugates.