

Antibody-Mediated Drug Delivery in Cancer Therapy

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1. Introduction

The lack of specificity of currently available chemo- and radiotherapeutic agents constitutes a major obstacle to the treatment of cancer. The difference between malignant and normal cells in regards to their sensitivity to cytotoxic therapies is, in most cases, not sufficient to allow potentially curative doses of drugs or radiation to be administered without unacceptable toxicity to normal cells. Thus, the administration of therapeutic doses of these agents during the treatment of cancer patients also damages rapidly proliferating cells of host tissues such as hematopoietic cells, hair follicles, and the epithelial lining of the gastrointestinal tract. It is generally accepted that the usefulness of many cytotoxic therapies would be enhanced if these agents were rendered more tumor-selective.

Targeted therapy of cancer is based on the use of specific carriers to deliver cytotoxic agents, including chemotherapeutic drugs, radioisotopes, or toxins, to their preferred site of action (i.e., tumors). For targeted delivery of cytotoxic agents, it is important to select a carrier that can be delivered selectively to tumor cells. The drug or radioisotope can be attached to this carrier by a number of synthetic or biochemical means to form a tumor-selective drug conjugate. Administration of such conjugate should lead to the accumulation of drug or radioisotope preferentially in the tumor without significant distribution to normal tissues, followed by selective damage to the tumor cells. Alternatively, the drug conjugate may be selectively "activated" in tumor tissue. Several classes of specific carriers have been evaluated for the selective delivery of drugs or radioisotopes to tumors. Such specific carriers include antibodies (1–4), cytokines such as interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (5,6), growth factors such as epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) (7–9), and hormones such as gonadotropin-releasing hormone (10,11). The use of antibodies as carriers of cytotoxic drugs is particularly attractive because of their unique specificity and high affinity for tumor antigens. In this chapter we will review the various classes of immunoconjugates developed for delivery of drugs to tumor cells.

2. Antibodies As Tumor-Targeting Agents

2.1. History of Antibody-Based Tumor Targeting

At the beginning of the 20th century, Paul Ehrlich proposed the use of polyclonal antitumor antibodies bound to diphtheria toxin as a “magic bullet” against malignant cells (12). However, the use of polyclonal antibodies (PABs) for targeted delivery at that time was limited by their heterogeneity with respect to size, charge, antigen specificity, affinity, low immunoreactive fraction, and the contamination of unwanted immunoglobulins (Ig). Furthermore, the production of highly specific polyclonal antitumor antisera/antibodies is difficult and unreliable; different lots of antisera may have various specificity and affinity, and quantity is usually limited. Despite these limitations, several investigators pursued the use of polyclonal antitumor antibodies as specific carriers for drug delivery in cancer therapy. Early studies in experimental animal models involved the use of a few available anticancer drugs available at the time that lend themselves to simple covalent coupling to antibodies. The first report on the use of an antibody-drug conjugate was published in 1958, when Mathe et al. linked methotrexate by diazotization to antibodies raised against murine L1210 leukemia cells, and used the conjugate in the successful treatment of L1210-bearing DBA/2 mice (13). Although the result appeared to be promising, no serious efforts to extend this therapeutic modality to patients were made until 1967, when Ghose and colleagues demonstrated that therapeutic amounts of radioactive ^{131}I could be linked to a PAb, with retention of antibody activity, and that the radiolabeled antibody could eradicate experimental tumors in vivo (14,15). In 1972, the same group coupled chlorambucil to polyclonal antitumor antibodies and used the preparation to treat melanoma patients in the first human study with antitumor antibodies (16–20). Thirteen patients with inoperable recurrent malignant melanoma were treated with chlorambucil bound to polyclonal antitumor antibodies raised in rabbits and goats, and compared to a control arm of 11 patients treated with conventional chemotherapy using dimethyltriazenoimidazole carboxamide. Objective responses were achieved in 2 patients and 5 other patients showed disease stabilization in the antibody/chlorambucil-treated group, whereas all 11 patients in the control arm showed disease progression. The median survival of the responders and the stabilizers were 20 mo, compared to that of 3 mo for nonresponders in the antibody/chlorambucil group and patients in the control group. During the same period, Ghose’s group also demonstrated that radiolabeled antitumor antibodies could localize preferentially not only in tumors in mouse models (both syngeneic and xenografted human tumors), but also in tumors in patients (21–23). More impressively, administration of a radiolabeled antibody led to cure of lymphoma-inoculated mice (24). For excellent reviews on the early history of antibody-based targeted therapy of cancer, see refs. 1 and 23.

In 1976, Kohler and Milstein, by employing a method of somatic hybridization, successfully generated “hybridoma” cell lines producing monoclonal antibodies (MAbs) of defined specificities (25). The principle advantages of mAb over the conventional PABs are obvious, including the defined specificity, homogeneity, and availability of MAbs in practically unlimited quantities. These properties of MAbs render them as the most attractive carriers for the selective delivery of therapeutic agents to malignant tumors. To date, numerous MAbs have been produced against virtually every malignant tumor of human tissues. Many of these MAbs have been used as tumor-specific carriers of cytotoxic agents and evaluated either in animal models and/or patients.

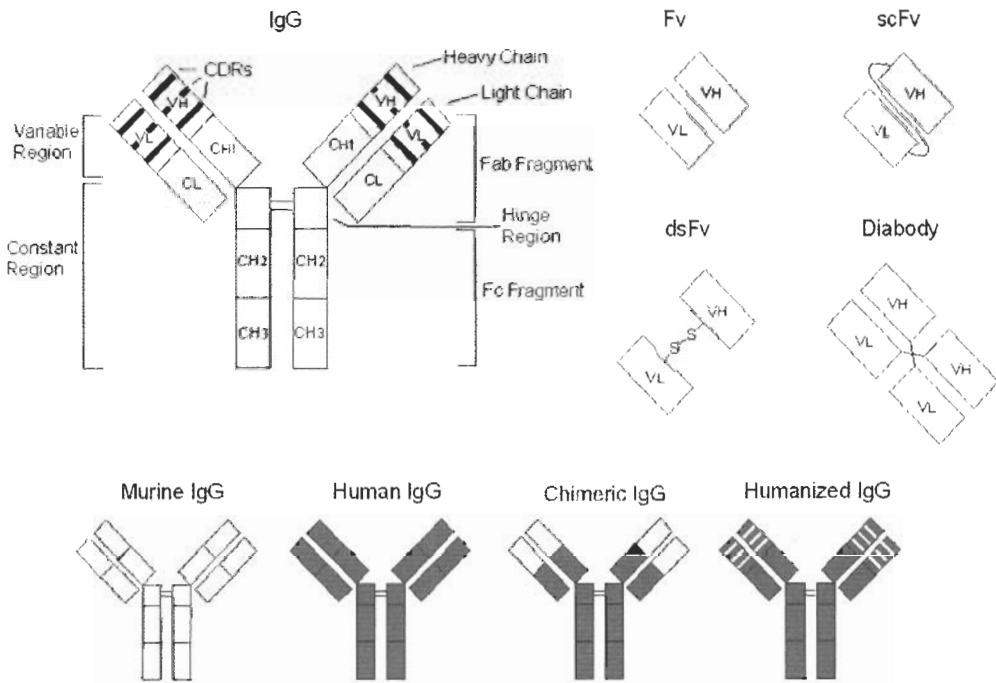


Fig. 1 Schematic representation of an IgG antibody and its fragments. Top panel, Each IgG molecule consists of four polypeptide chains: two identical light and two identical heavy chains paired together by interchain disulfide bonds. The Y-shape IgG contains two Fab (antigen-binding) fragments and a Fc (crystalline) fragment linked together via the hinge region. The smallest module of an antibody required for specific binding is Fv fragment comprising only the variable domains of light (V_L) and heavy (V_H) chains. Each V_L and V_H domains contain three hypervariable regions called complementarity-determining regions (CDRs) that form the antigen-binding surface and are responsible for antibody specificity and diversity. Introduction of a polypeptide linker between the V_L and the V_H domains (in either orientation) results in the formation of a scFv or diabody (or triabody or tetraabody) depending on the length of the linker. Alternatively, a disulfide bond can be introduced into the interface between the V_L and the V_H domain to form a disulfide bond-stabilized Fv (dsFv). Bottom panel, MAbs produced from the traditional hybridoma technique are generally of murine origin. Chimeric antibody is generated by joining the V_L and the V_H domains of a murine MAb to human constant domains: mouse V_L to human C_L and mouse V_H to human C_{H1} -hinge- C_{H2} - C_{H3} , respectively. In antibody humanization, only the CDRs of the murine MAb, along with one to several other mouse residues determined to be critical in maintaining the antibody affinity, are grafted into a human framework. Fully human antibodies can be routinely obtained nowadays with the availability of human antibody phage-display library and human Ig transgenic mouse. (Note: all drawings are not to scale.)

2.2. Antibody Structure and Engineering

The majority of antibodies that are used for targeted therapy fall into the IgG class of immunoglobulins. IgG is a tetrameric glycoprotein, consisting of four polypeptide chains: two identical light chains and two identical heavy chains. Each IgG contains two antigen-binding fragments (Fab) and an Fc domain joined together by a hinge region (Fig. 1). The Fab fragment is responsible for specific antigen binding, whereas

the Fc domain binds to the Fc receptors on effector cells, fixes complement, and elicits other *in vivo* biological responses. The variable light (VL) region and the variable heavy (VH) region within the Fab fragment directly contact antigen and are responsible for the unique specificity and diversity of antibodies. Within each VL and VH, there exist three hypervariable regions, also called complementarity determining regions (CDRs), that form the binding surface that contacts the antigen. The smallest functional module of an antibody required for specific antigen binding is the Fv fragment. The Fv fragment is a heterodimer of VL and VH domains; the fragment is usually unstable in solution, because the two domains are noncovalently linked. The instability of Fv fragments was overcome by the invention of single-chain Fv (scFv) in which the VL and the VH domains are connected via a peptide linker (26). The scFv fragment in solution exists mostly as monomer, when the linker is 12–15 or more amino acids, or as a dimer (as so-called “diabody”) when the linker is between 5 and 12 amino acids (27,28). Interestingly, several groups reported recently that the scFv mostly form trimers (“triabody”) and/or tetramers (“tetraabody”) when the VL and VH domains are fused together with a linker of 0–2 amino acids (for review, *see ref.* 29). An alternative approach to increasing the stability of an Fv fragment is to introduce an interchain disulfide bond between the interface of the VL and the VH domains (for review, *see ref.* 30). The residues in the interface to be mutated into cysteine, ideally locate outside the CDR regions, were identified with the aid of computer molecular modeling (31,32). Several disulfide bond-stabilized Fv fragments have been produced with increased stability and/or antigen-binding affinity (33,34).

To date, the majority of hybridoma-derived antitumor MABs are of murine origin. These antibodies are immunogenic in humans and elicit a human anti-murine antibody (HAMA) response. The HAMA can form immunocomplexes with subsequent administered therapeutic antibody, leading to increased clearance accompanied by decreased tumor localization of the antibody, and in some case, serious side effects such as an allergic anaphylactic reaction. Smaller antibody fragments, such as Fab, Fv, and scFv, are usually less immunogenic than the intact IgG in human owing to the lack of the Fc domain. Other approaches attempting to reduce the immunogenicity of rodent-derived antibodies include chemical modification of the antibodies such as conjugation of the antibodies to polyethylene glycol (PEG) or oxidized dextran, and co-administration to patients of immunosuppressive agents such as cyclosporin A, cyclophosphamide, and steroids. Recent advancement in antibody-engineering technologies has not only enabled the ability to tailor-make antibody molecules with predefined characteristics such as size, valency, and multispecificities to suit the intended applications, but also led to the production of chimeric and humanized antibodies with greatly reduced immunogenicity. Chimeric antibodies were the first generation of this genetic-engineering approach where the variable domains (both VL and VH) of a murine MAB were cloned and fused to the constant domains of a human IgG to create a new hybrid IgG molecule that retains the original antigen binding affinity and specificity, but only contains, in theory, one-third of murine amino acid sequences (35) (Fig. 1). Humanization of antibodies takes this approach one step further by genetically grafting only the CDRs of the murine antibody, along with a few murine residues outside the CDRs believed to be important for antigen-binding affinity, into a human IgG framework (Fig. 1). These residues are identified with the help of computer-based modeling and site-directed mutagenesis because of their critical roles in maintaining the correct conformation of the antigen-binding surface and/or direct contacting the antigen (36). Approximately

90–95% of the amino acid sequence of a humanized antibody is of human origin. Since the mid-1990s, with the advancement in human antibody phage-display libraries (37,38) and human Ig transgenic mice (39,40), fully human antibodies with desired specificities can now be readily isolated. This new generation of antibody therapeutics, including chimeric, humanized, and fully human antibodies, has proved to be much less immunogenic in multiple clinical studies (for review, *see ref. 41–45*).

2.3. Classes of MAb Therapy and Mechanisms of Action

Three major classes of MAbs have been developed as cancer therapeutics: (1) antibodies that act as molecular antagonists that modulate the function of key regulatory molecules on tumor cells, such as blocking growth factor/receptor interaction and/or downregulating expression of oncogenic proteins (or receptors) on the cell surface; (2) antibodies that recruit effector mechanisms of the immune system, such as the antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (CMC), and (3) antibodies used as targeting devices (immunoconjugates) to specifically deliver cytotoxic agents to tumor sites. Functional blockade is thought to be one of the main antitumor mechanisms for several antibodies, including those directed against EGF receptor (also called HER1) and HER2 (*erbB2/neu*) on tumor-cell surface (46,47), and receptors for VEGF on endothelial-cell surface (48). By interfering with important growth factor/receptor pathways, these antibodies can influence the growth and survival of tumor cells. In addition, antibodies that inhibit function of regulatory pathways may potentiate the cytotoxic effects of chemotherapeutic drugs and radiation. For example, a number of studies have demonstrated that both RituxanTM and HerceptinTM could significantly enhance the therapeutic efficacy of various cytotoxic agents in both laboratory and clinical settings (49,50). There is evidence to suggest that immune-effector mechanisms may also play an important role in the clinical antitumor efficacy of the antilymphoma antibody RituxanTM and the antibreast-cancer antibody HerceptinTM. For example, both RituxanTM and HerceptinTM have been shown to mediate significant levels of ADCC and CMC effects on a number of malignant cell lines (51,52). Further, the antitumor effect of these two MAbs *in vivo* was severely diminished in mice with deficiency of Fc receptor on its effector-cell surface or when mutants of the MAb with reduced Fc-binding efficiency were used (53).

A large number of MAbs specific for a diverse set of tumor targets have been utilized for the development of immunoconjugates. Several types of cytotoxic agents, including toxins, chemotherapeutic agents and radioisotopes, have been conjugated to antitumor antibodies and tested preclinically and in various clinical studies. For example, clinical trials have been performed using immunotoxins (ITs) in patients with carcinomas of breast, ovary, and colon, as well as several lymphomas and leukemia (for reviews, *see refs. 58–60*). An anti-CD33-calicheamicin conjugate, MylotargTM, has gained FDA approval for the treatment of acute myeloid leukemia (AML) in patients over 60 yr of age (61). In addition, a ⁹⁰Y-labeled anti-CD20 antibody (ZevalinTM) was approved by the US Food and Drug Administration (FDA) for non-Hodgkin's lymphoma (NHL) (62), and a ¹³¹I-labeled anti-CD20 antibody (BexxarTM) has been tested in multiple Phase III clinical trials (63) and is waiting for FDA approval for the treatment of B-cell malignancies.

Other approaches that utilize MAbs in cancer therapy but do not fit into the classes outlined earlier include anti-idiotypic MAbs that mimic tumor antigens to stimulate the anti-idiotypic network to generate antitumor anti-anti-idiotypic antibody response (54);

catalytic antibodies that function as catalysts to induce hydrolysis of cell membrane or proteins, or to convert molecular oxygen into hydrogen peroxide to achieve cell killing (55), antibodies that enhance patient's immune response to tumors by stimulating cytotoxic T lymphocytes via CD40 (56) or by antagonizing endogenous immune inhibitory factors such as CTLA-4 (57).

2.4. Selection Criteria for Antibodies As Immunoconjugates

One of the most important aspects of MAb-based targeting is the selection of an appropriate antibody to be used as the carrier of the cytotoxic agent. The choice of an ideal MAb depends on characteristics of the target antigen, various requirements for the conjugation method, and the mechanism of action for a cytotoxic agent. The choice of MAb also may be dependent on the intended use of the immunoconjugate. For example, for radioimmunoimaging the radiolabeled antibody should specifically and rapidly localize in tumor tissue to create a high tumor/nontumor ratio that is required for ideal imaging soon after the administration of the radioimmunoconjugate. It is also desirable that the radiolabeled antibody be cleared rapidly from the body once the tumor images have been obtained. On the other hand, for targeted antitumor therapy, in addition to its specific localization, the immunoconjugate should remain in the tumor for an adequate period of time to allow the MAb-linked cytotoxic agent to exert its effect. The nature of the cytotoxic moiety used in the conjugate and its mode of action also has significant impact on the selection of antibody carrier. For example, a radioisotope decaying by beta emission can kill cells within an area of several diameters of the targeted tumor cells, whereas most chemotherapeutic agents and toxins require internalization to exert their cytotoxic effect. Thus, a MAb that is ideal for use as a carrier for delivering radioisotopes may not necessarily be a good candidate for the delivery of chemotherapeutic agents or toxins. Several other factors that deserve careful consideration in selecting MAbs as effective carriers for tumor targeting are briefly discussed next.

2.4.1. Intact Antibody vs Antibody Fragments

The choice of intact antibody or its fragments mostly depends on the intended use of the conjugate. An intact antibody possesses a longer circulation half-life (up to 1–3 wk in humans as demonstrated by several humanized or human antibodies), and is able to elicit effector mechanisms, including ADCC and CMC, that may contribute to the overall antitumor activity of the conjugate. For the purpose of delivering cytotoxic agents, most investigators prefer the intact MAb because, compared to its fragments, intact MAb produces a higher percentage of injected conjugate that localizes in the tumor (64–67). Further, a longer residence time of the intact MAb in tumor may be beneficial for the targeted drug or radioisotope to exert its cytotoxic effects (64–67). In contrast, smaller antibody fragments such as scFv and Fab have a much shorter half-life and these fragments are excreted quickly through the kidney. It is believed that these antibody fragments are, in general, better candidates than intact MAbs for tumor imaging because they penetrate tumor faster and more homogeneously, clear faster from the circulation, and accumulate less in organs of the reticular endothelial systems (such as liver and spleen) (64–67). Another advantage of using antibody fragments is that they are usually less immunogenic than the intact MAb owing to the lack of the Fc domain.

2.4.2. Antibody Specificity

The importance of MAb specificity is obvious because it allows the antibody or its immunoconjugate to bind the target antigen selectively. Although high specificity of

MAbs is universally recognized as an advantageous attribute for MAb-based drug targeting, there are only a handful of antigens that are truly considered tumor-specific, including clone-specific idiotypic Ig on the surface of malignant B cells and T-cell antigen-receptor protein on malignant T cells. Most antitumor antibodies studied to date are, in fact, antibodies directed against tumor-associated antigens (TAA). These antigens are not strictly tumor-specific but they have restricted distribution in normal tissues. Some examples of TAA include carcinoembryonic antigen (CEA), mucin-associated antigen (MUC1), prostate specific membrane antigen (PSMA), HER2/neu, tyrosinase, gp75, and gp100. In most cases, the differential expression of TAA between tumors and normal tissues allows sufficient selectivity for cytotoxic targeting. Sometimes normal organ-specific antigens, like prostate-specific antigen (PSA), or lineage-specific antigen like CD20 and CD19 on B-cells, can also be used for tumor targeting, because damage to these cells or tissues is not life-threatening.

2.4.3. Antibody-Binding Affinity

It is generally accepted that to achieve effective targeting, the affinity of a MAb needs to be of 10^8 M^{-1} or better (68). Although some investigators presume that high-affinity antibody is desirable (69), there is considerable debate whether improving MAb affinity beyond 10^9 M^{-1} would result in a further increase in its tumor localization. Recently, Adams et al. employed several anti-HER2 antibodies of varying affinities derived from the same parent antibody by site-directed mutations, and showed a positive correlation between the amount of antibody localized in tumor and antibody affinity up to 10^9 M^{-1} (70). Further increase in affinity beyond 10^9 M^{-1} did not, however, result in higher tumor localization of the antibody (71). Histological study of the tumor specimens demonstrated that antibodies with moderate affinity (between 10^8 and 10^9 M^{-1}) penetrated deeper in to tumors and percolated more homogeneously into tumor mass than antibodies with high affinity ($>10^{10} \text{ M}^{-1}$), which were trapped around the perivascular tumor cells with much less localization in the distant tumor tissues (71).

2.4.4. Antigen Expression

For antibody-based drug targeting, the antigen should be expressed on the surface of target cells and must be accessible to the administered antibody or its conjugate. Ideally, the target antigen should be present only on tumor cells, and on all tumor cells. Unfortunately, most TAA are expressed in a heterogeneous manner and only a proportion of tumor cells express the antigen. This is not a critical limitation for radioimmunotherapy, especially when beta emitters were used; the radiation can effectively reach and therefore damage cells within an area of several diameters of the targeted tumor cells ("bystander effect"). It is also important that the tumor antigen be expressed on the target cells at high density. The usefulness of targeting would be greatly compromised if the antigen were expressed on the cell surface at a very low level that would not permit the binding of adequate amounts of conjugate needed to achieve a therapeutic response.

An emerging new concept to overcome the heterogeneity of tumor antigen expression is to use antibodies directed against angiogenic markers that are selectively upregulated in tumor vasculature, such as receptors for VEGF (72–74), integrins (75), VE-cadherin (76), and fibronectin isoforms (77,78). Because angiogenesis is required for both tumor growth beyond certain size and metastasis, local damage to tumor vasculature may detrimentally affect all tumor cells that are dependent on the targeted vasculature for nutri-

ents, irrespective of the antigen-expression profile of individual tumor cells. Anti-angiogenic antibody-based therapies should, therefore, be applicable to all types of cancers (for reviews, *see refs. 48,73,74,77,78*). However, it is yet to be established whether true “tumor vasculature-specific” targets can be identified and whether these targets can be exploited for immunoconjugates approaches to cancer therapy.

2.4.5. Antigen Modulation

Ideally, the target antigen should not be secreted or shed from the tumor cell surface into tumor stroma and/or the circulation in amounts that could affect binding of the carrier antibody to tumor cells. Soluble antigen may form immune complexes with administered antibody or its conjugate, leading to rapid clearance from the circulation and thus impeding delivery of the antibody or its conjugate to tumor. The rate of antigen internalization after antibody binding is another critical factor that may influence the effectiveness of antibody-based drug targeting. Because most currently used chemotherapeutic agents and toxins exert their cytotoxic effect on intracellular targets, the ideal antibody conjugate should be rapidly internalized (endocytosed) by the tumor cells and release active form of these cytotoxic agents inside the cells. Thus, antibodies specific for antigens that trigger efficient internalization upon binding may represent more effective carriers for intracellular drug delivery. On the other hand, internalization is not required for the cytotoxic effects of (β and γ) radiation and agents that act through cell membrane such as cytotoxic enzymes. In fact, antibodies with high internalization rate may be poorly retained by the tumor cells after initial binding because of the intracellular degradation and the efflux of lower molecular-weight metabolites following the rapid endocytosis process. This has been shown to be disadvantageous for radioimmunotherapy with ^{131}I -labeled antibodies; the degradation of endocytosed radioiodinated antibody, including dehalogenation and efflux of the catabolic small fragments, resulted in rapid loss of radioactivity from the tumors (79). Novel protein radioiodination techniques have been developed to alleviate the loss of radioactivity owing to dehalogenation (80,81). Alternatively, radiometal isotopes, such as ^{90}Y and ^{111}In , may prove beneficial for targeting rapidly modulating antigens because these isotopes appear to be selectively retained intracellularly after degradation of the carrier antibody (80,81).

2.5. Factors That Influence the Distribution of Immunoconjugates

A key obstacle in MAb-based drug targeting is the inability of the antibody or its conjugate to reach all regions within a tumor in adequate quantity. Despite the fact that accumulation up to 20–30% of injected dose per gram (% ID/g) tissue has been achieved in human tumor xenografts in mice, only very small fractions of the administered MAb or its conjugate accumulated in tumors, usually in the range of 0.001–0.01% ID/g tissues, in most clinical studies (82,83). Several important factors that affect the localization of MAb and its conjugate in tumors are discussed briefly next.

2.5.1. Physiological Factors

Several tumor-related physiological factors can adversely affect MAb localization in tumors (for reviews, *see refs. 84,85*). These factors include the heterogeneity of blood supply, elevated intratumoral interstitial pressure, and the large transport distance, i.e., from the site of antibody transvascular transport to cells in the peripheral region of the tumor.

The tumor vasculature is highly heterogeneous and may be completely different from host's normal vasculature depending on the tumor type, growth rate, and location

(84,85). It consists of vessels recruited from the preexisting network and vessels resulted from the angiogenic response of the host vasculature to growth factors released by cancer cells. One of the key differences between normal and tumor vessels is that the latter are dilated, saccular, and tortuous and may contain tumor cells within the endothelial lining of the vessel wall. The heterogeneity of tumor vasculature would reduce the chance of delivery of MAb or its conjugate to some area of tumor owing to poor blood supply. In addition, an increase in the intercapillary distance would require the MAb or its conjugate to traverse a longer distance in the interstitium to reach tumor cells in the peripheral regions. Tumor necrosis has a similar adverse effect on antibody localization in the tumor because it destroys or narrows intratumoral vessels by thrombosis.

Once the MAb or its conjugate have reached an exchange vessel in tumor, its extravasation occurs by diffusion (solute movement resulting from concentration gradient) and convection (solute movement associated with bulk solvent movement) and to some extent, transcytosis. The rate of extravasation of MAb or its conjugate is dependent on: (1) surface area of exchange; (2) the transvascular concentration and pressure gradient; and (3) other transport parameters, such as vascular permeability and hydraulic conductivity (a constant related to fluid leakage and pressure gradient). Despite higher permeability of vessels in tumors than that in normal tissues, a number of factors can adversely affect extravasation of the circulating immunoconjugate, resulting in poor tumor localization by the antibody (86,87). First, tumors contain regions of high interstitial pressure owing to rapid tumor growth, necrosis, and/or infiltration, and increased concentration of plasma proteins in tumor interstitium resulted from vascular leakage and poor of lymphatic drainage. Because the transvascular passage of macromolecules under normal conditions occurs primarily by convection, a high intratumoral interstitial pressure would impede the fluid extravasation, leading to a decrease in extravasation of MAb or its conjugate. Second, the average area of vascular space per gram of tumor tissues decreases with tumor growth and/or tumor necrosis, therefore resulting in a reduction in the transvascular exchange.

Extravasated immunoconjugate could move through the interstitial space by diffusion and convection to reach target tumor cells (87,88). The interstitial space in tumors usually is larger than that in normal tissues. This means immunoconjugates have to transverse longer distance in the tumor interstitium to reach tumor cells in the periphery regions of vascular supply. This may increase the probability of catabolism of MAb or its conjugate in tumor interstitium before it could bind to tumor cells. Tumor metabolism of immunoconjugate may adversely affect the amount of accumulation as well as the residence time of the immunoconjugate within the tumor. Further, in addition to specific binding to tumor cells, the immunoconjugate may also bind non-specifically to proteins or other tissue components during its transportation in tumor interstitium. These binding reactions may not only slower the diffusion rate of immunoconjugate, but also retard its movement towards tumor periphery regions, especially when a high-affinity MAb is used.

2.5.2. Pharmacological Factors

A number of pharmacological factors influence the accumulation of immunoconjugate in target tumors, including the antibody classes (e.g., IgG vs IgM) and the forms (e.g., intact MAb vs fragments) of the antibodies (see discussion in Subheading 2.4.), the dosage and the route of administration, and the metabolism and clearance of immunoconjugate from the circulation.

The effect of the administered dose of an immunoconjugate on the % ID/g tumor localization is difficult to predict. Increase of the dose of radiolabeled MABs given to xenograft-bearing mice has produced conflicting results, with increased, unchanged, or decreased tumor uptake reported (as regard to % ID/g tumor tissues) (89–91). Studies conducted to date in patients have used a wide range of antibody doses varying from less than 1.0 mg to more than 1.0 g (92). The optimal antibody dose may vary depending on many factors, such as the quality of the MAB (e.g., specificity, affinity, and immunoreactivity), the quantity of antigen (e.g., the level of antigen expression and the tumor burden), the rate of antigen/antibody modulation (e.g., shedding and internalization), and different tumor types as well as hosts (92). It should be noted that for a tumor of defined type and size, the number of antigenic sites is limited and may be saturated with appropriate amount of MAB. Once saturated, increasing the dose of MAB given to the tumor-bearing host will not increase the absolute amount of MAB that localized in the tumor. Under this situation, the value of % ID/g tumor tissue decreases, although the absolute amount of tumor-localized MAB remains the same.

The most common route of antibody administration is by intravenous (iv) bolus injection or infusion. Uptake and catabolism by organs of the reticular endothelial system (e.g., liver and spleen) and clearance of small antibody fragments through kidney appear to be the major routes of elimination of circulating immunoconjugate (93). For intracavitary tumors, injection of immunoconjugate via the relevant intracavitary route may provide several theoretical advantages, including increased concentration of MAB at the tumor site and potentially decreased toxicity to those normal organs that are involved in the metabolism of the MAB. For example, studies in both animal models and patients have shown that the intraperitoneal injection was superior to the intravenous route for targeting peritoneal tumors (94).

2.5.3. Immunological Factors

In addition to the properties of MAB such as the quality, the forms, and the species of the antibodies, which were discussed earlier, several important characteristics of antigens may also significantly affect MAB localization in both tumor and normal tissues. For example, high-level circulating antigen, resulted from antigen secretion and/or shedding, may form complexes with administered MAB and prevent the MAB from reaching the tumor sites. Furthermore, for antigen with a rapid shedding or internalization rate, if the shed or internalized antigen is not re-expressed, subsequent administration of immunoconjugate would not produce significant tumor localization. Finally, antigen heterogeneity may also have a significant impact on tumor localization of MAB or its conjugate. Tumor-antigen heterogeneity includes the difference in: (1) the proportion of cells within the tumor expressing the antigen; (2) the levels of antigen expression in different tumor cells within the tumor; and (3) the exact location of the antigen (surface vs intracellular), its accessibility, and its availability in the environment of the cells.

2.5.4. Physical and Technical Factors

In the preparation of an immunoconjugate, the biological properties of the MAB must correlate with the physical properties of the cytotoxic agents chosen for conjugation. For example, a MAB with a high rate of internalization upon binding to target antigen on tumor-cell surface is a desirable carrier for toxins and most chemotherapeutic drugs, because internalization is required for the cytotoxic effects of these agents. In

the cases of radioimaging and radioimmunotherapy, it is important to carefully match the biological half-life of the carrier MAb and the physical half-life of the radioisotope. For example, a long half-life radioisotope should be used when a long time is required for the carrier MAb to accumulate in the tumor, or vice versa, to ensure the best therapeutic effect or quality of imaging. Other limitations that deserve consideration in the construction of radioimmunoconjugates include the availability of radioisotopes, the choice of appropriate methods for radiolabeling, and the availability of instrumentation and facility for the handling of radioisotopes.

3. Antibody-Drug Conjugates (Chemoimmunoconjugates)

Antibody-based targeting of conventional chemotherapeutic agents has been an especially attractive concept. There are several advantages of using chemotherapeutic agents for the construction of immunoconjugates, including: (1) these clinically used agents have established profile of antitumor activity and mode of action; (2) the mechanisms underlying the dose-limiting toxicity and the protocol to control and/or reverse the toxicity have been well-established; and (3) the pharmacokinetics of the agents are known, and the methods to monitor the drug level in circulation and tumors are readily available. Conjugation of chemotherapeutic agents to antitumor antibodies may improve their therapeutic index by increasing both the specific tissue distribution and the retention time of these agents within tumors, and accordingly reducing normal tissue toxicity. A variety of chemotherapeutic agents with different mechanisms of action, including alkylating agents (e.g., chlorambucil and melphalan), DNA intercalators (e.g., doxorubicin and daunorubicin), mitotic inhibitors (e.g., vinblastine and vindesine), and antimetabolites (e.g., methotrexate and 5-fluorouracil [5-FU]), have been conjugated to antitumor MAb and the resulting immunoconjugates tested for antitumor activity both *in vitro* and *in vivo* (for review, *see refs. 1–4*).

Methods for coupling chemotherapeutic agents to MAbs must ensure the retention of activity of both the MAb and the cytotoxic agents. Optimal coupling method should allow controlled drug incorporation, and avoid formation of homopolymers of the MAb or drugs and aggregates of the conjugate. Practically, the methodology for conjugation should be technically feasible and reproducible. Further, the drug must be delivered to the target site in a form that is active or can be activated *in situ*. Because both antibodies and most antitumor drugs have certain functional groups that are essential for binding to target molecule, chemical groups chosen for linkage should not be required for antibody and drug action, or alternatively, these should become available following endocytosis and intracellular catabolism of the immunoconjugate. Drugs have been linked to MAbs using a number of chemical groups including amino, carboxyl, hydroxyl, and sulphhydryl residues (*see reviews in refs. 3,95–97*). In some instance, these chemical groups exist in drugs or MAb molecule, whereas in other cases, they are introduced in the drug and/or MAb molecules by chemical modification. The chemical nature of the linkage commonly used includes peptide bond; aldehyde/Schiff's base (followed by reduction reagents such as sodium borohydride or sodium cyanoborohydride to stabilize the linkage); disulfide bond; and so-called acid-labile linkages, including hydrazone bond and *cis*-aconityl or related linkages (*see reviews in refs. 3,95–97*). It should be considered that, in some cases, a direct coupling between MAbs and drug molecules may create a steric hindrance that can negatively affect the rate of drug release from the immunoconjugate by blocking enzyme access. In this

regard, the introduction of a spacer between the MAb and the drug molecule can overcome this steric hindrance and allow the drug to be released in a given milieu. For example, both lysosomotropic spacer and acid-sensitive spacers have been used as means to release active drug molecules inside lysosomal compartment (98,99).

The site of attachment of the drug molecules to MAbs is also an important point to consider in the preparation of chemoimmunoconjugates. The most commonly used site in an antibody is the ϵ -amino group of the lysine residues, because they are convenient to use for linking to drug molecules via either amide or thiolester bonds. Different MAbs have a variable number of lysine residues, which are spread over the whole antibody molecule. It is difficult to control both the site and the amount of drug attachment to the antibody molecule, thus leading to the potential for production of a heterogeneous population of immunoconjugates. Furthermore, random conjugation of drug molecule to lysine residues within the antibody-binding site may result in the loss of antibody binding to target. In this regard, a site-specific linkage has been exploited by attaching the drug molecules to the sugar residues of the antibody (100,101). Oligosaccharide residues are infrequent in the antigen-binding site, but are universally found in the Fc domain of the antibodies. Thus, site-specific linkage via the sugar residues may avoid drug loading in the antigen binding sites of the antibody. Most chemical reactions for site-specific linkage are based on oxidation of the oligosaccharide residues followed by formation of Schiff's base or hydrazone bond with the drug molecules (100,101).

To achieve therapeutic benefit in chemoimmunoconjugates, it is essential that they deliver sufficient cytotoxic load to the target tumor cells. The number of molecules required for each cytotoxic agent to kill a tumor cell varies depending on its potency. For example, although a single toxin molecule is enough to kill a cell upon entering the cytosol, approx 10^6 and 10^7 molecules of methotrexate and doxorubicin or daunorubicin, respectively, are needed to accomplish the same task (3,102). Because most of the chemoimmunoconjugates studied to date have a low drug to MAb ratio (in the range of 3–10 drug molecules per antibody molecule), combined with the fact that the number of antigen sites on each tumor cell is usually in the range of 10^5 – 10^7 , it is likely that most antitumor MAb cannot deliver adequate cytotoxic molecules necessary for cell killing under steady-state conditions. Several strategies have been exploited to enhance the effectiveness of MAb-drug conjugates. One of the strategies is to construct chemoimmunoconjugates with highly potent cytotoxic agents so that even a small number of targeted molecules can achieve cell killing. Examples of these highly toxic agents include maytansinoids (microtubule-targeting agent) (103) and the enediyene family of antibiotics (DNA-targeting agents) (104); these compounds are approx 100–1000-fold more toxic than conventional chemotherapeutic agents. Several MAb-maytansinoids immunoconjugates have been successfully constructed and have demonstrated potent antitumor activity in eradicating established xenografts in animal models (105). Two compounds of the enediyene family of antibiotics, neocarzinostatin and calicheamicin, have been used in MAb-based targeting (106,107). In fact, an anti-CD33 MAb-calicheamicin conjugate, Mylotarg, has demonstrated significant activity in clinical trials and was approved by the FDA in year 2000 for the treatment of AML (61).

Another strategy to enhance the antitumor activity of chemoimmunoconjugates is to increase drug loading, or incorporation, on each antibody molecule. The antibodies tend, however, to lose their immunoreactivity and solubility after incorporation of about 10 moles of drug per mole of IgG under direct conjugation (108,109). The most commonly used method to address this limitation is the use of an intermediate carrier; the

drug molecules are first loaded at high levels of drug substitution onto a multivalent intermediate carrier, such as oxidized dextran and human serum albumin, followed by conjugation of one or two of the preloaded intermediate carrier to an antibody molecule. For example, doxorubicin was conjugated to an antihuman chronic lymphocytic leukemia (CLL) MAb via oxidized dextran at a drug/MAb ratio of approx 25–40 without affecting antibody activity, compared with a drug/MAb ratio 4–6 when using a direct linkage. The MAb-dextran-doxorubicin conjugate was much more potent than the direct conjugate in inhibiting the growth of human leukemia cells both *in vitro* and *in vivo* in an animal model (110). Incorporating a spacer between the antibody and the intermediate carrier may prove to be further beneficial; the spacer can be specifically designed to not only alleviate the potential for steric hindrance caused by the MAb and the intermediate carrier, but also to provide a preferable cleavage site, e.g., lysosomotropic and pH-sensitive spacers, to control the release of the conjugated drug in a given milieu, such as in the lysosomal compartment (110). The criteria for the selection of an intermediate carrier include the molecular size and shape (e.g., globular or linear), homogeneity, charge, solubility, ease of handling, number of available functional groups, coupling stability, toxicity, and biodegradability.

Recently, liposomes have attracted considerable interest in serving as the intermediate drug carrier, mainly owing to their ability to load a large amount of drug molecules without the requirement for chemical modification, i.e., the drug molecules packed inside the liposome remain in their native form. In the preparation of immunoliposomes, the cytotoxic agents are first loaded into the liposome followed by chemical conjugation of the liposome to MAb molecules via the functional groups that are pre-incorporated in the liposome surface (for reviews, *see refs. 111–113*). A promising method developed recently involves a “postinsertion” technique by which the clinically approved liposomal drug, such as Doxil, can be easily converted into a tumor-targeting preparation by a simple step of incubation with the antitumor antibodies or its fragments (114,115). The advantage of this approach is that by using clinically approved liposomal drugs, it not only confers tumor specificity to the nontargeted liposomal drug, but also theoretically can be applied to a variety of tumor-targeting antibodies and also avoids issues associated with the manufacturing of multiple chemoimmunoconjugates. However, there are also several limitations associated with liposome therapy, including slow tumor penetration owing to the large size of liposome and premature removal of liposome from circulation by macrophages in the liver and spleen (111–113). Increased tumor localization of immunoliposome, as well as other MAb-based conjugates, can be achieved by enhancing tumor vascular permeability by methods such as hyperthermia (116), external radiation (117), and administration of pharmacological agents or cytokines including IL-2 and tumor necrosis factor- α (TNF- α) (118,119). Furthermore, the invention of sterically stabilized liposomes, or “stealth liposomes,” has greatly improved the pharmacokinetics and tumor localization of immunoliposomes by reducing their uptake and degradation by macrophages (120,121). Several promising immunoliposome preparations, including those targeting HER2 and CD19, have been studied in animal models and shown potent antitumor activity (122–124).

Mylotarg (Gemtuzumab ozogamicin, also called CMA-676) is the first, and to date, the only chemoimmunoconjugate to receive FDA approval for clinical application in patients of over 60 yr of age with AML (61,125). Mylotarg consists of a humanized anti-CD33 MAb (HP67.6) coupled to calicheamicin. In a Phase I clinical trial, 40 patients

with refractory or relapsed CD33+ AML were treated with escalating doses, from 0.25 mg/m² up to 9.0 mg/m², of Mylotarg as 2-h iv infusions at 2-wk intervals for one to three cycles. Three patients achieved blood count normalization (complete response [CR]) and leukemia cells were eliminated from the blood and bone marrow of another five patients (partial response [PR]) (126). All the doses were well-tolerated, with side effects including reversible fever, chills, hypotension, and hepatic transaminasemia and hyperbilirubinemia. No antibody responses to the carrier humanized anti-CD33 antibody were observed. In several Phase II studies, 142 patients with relapsed CD33+ AML were given 9.0 mg/m² on d 1 and 15. CRs were seen in 23 patients, and PRs were achieved in another 19 patients, for an overall response rate (ORR) of 30% (42/142) (127). The progression-free survival at 30 mo was 35%, and overall 1-yr survival was 31%; both rates compare favorably to rates of other salvage therapy for relapsed/refractory AML. Again, no antibody responses to the carrier humanized anti-CD33 antibody were observed in these trials. Apart from those toxicities seen in the Phase I trial, grade 3–4 level of myelosuppression was observed in 98% of patients (127).

Another chemoimmunoconjugate that has been studied extensively is BR96-doxorubicin conjugate (BR96-DOX) (for review, *see ref. 128*). BR96 is an anti-Le^y MAb that internalizes following binding to cell-surface antigen. In the preparation of BR96-DOX conjugate, a chimeric version of BR96 was used coupled with doxorubicin-6-maleimido-caproylhydrazide derivative via reduced thiol groups on the BR96 antibody molecule. Approximately eight molecules of doxorubicin were linked to each MAb molecule. Preclinical evaluation of BR96-DOX was carried out in human tumor xenograft-bearing nude mice and rats (129,130). At doses of 200 mg/kg or more, the conjugates led to regression and cures of mice or rats bearing various human xenografts including lung, colon, and breast carcinomas, without any signs of toxicity. On the other hand, doxorubicin either linked to a control antibody, or used by itself at the maximum tolerated dose (10 mg/kg), had little antitumor activity. Unmodified BR96, alone or together with doxorubicin, was not curative (129,130). Toxicology studies performed on dogs that expressed Le^y antigen in the epithelial cells from the gastrointestinal tract that was cross-reactive to BR96, showed that BR96-DOX and unmodified BR96 had the same dose-limiting toxicity, i.e., severe vomiting and bloody diarrhea, when given at doses greater than 400 mg/kg (128). In view of the promising animal studies, two Phase I clinical trials and a Phase II clinical trial were performed in patients with advanced solid tumors. In the Phase I trials, the patients were given BR96-DOX either by iv infusion over 24 h every 3 wk or by bolus injection weekly. The maximum tolerated doses were established as approx 700 mg/m² (~18 mg/kg) and 200 mg/m² (~5 mg/kg) for the infusion and the bolus injection protocols, respectively (131,132). In the Phase II trial, 14 patients with metastatic breast cancer were given BR96-DOX at 700 mg/m² every 3 wk. Only one patient showed PR after treatment with the conjugate, compared with 4 PRs in 9 patients treated with free doxorubicin (133). The dose-limiting toxicity was, as observed in the dogs, gastrointestinal, particularly vomiting and bloody diarrhea. Biopsy studies demonstrated significant BR96-DOX binding to epithelial cells in the gastrointestinal tract, with the damage most likely a result of complement activation (133). Further dose escalation was limited because of the gastrointestinal toxicity. Several approaches are being investigated, aiming to enhance the therapeutic index by increasing the potency of BR96-DOX conjugate and/or reducing its toxicity. For example, new coupling chemistries have been explored to increase drug incorporation and conjugate stability. In addition, genetic

modifications of BR96 have been made to abolish its capacity to fix complement (128). Another approach is to combine the BR96-DOX with other conventional chemotherapeutic agents, such as Taxotere and Gemzar. It has been observed that BR96-DOX may synergize with these chemotherapeutic agents, so that a lower dose of the conjugate can be used to achieve the same curative effect observed in animal models (134). In this regard, several Phase I and II studies have been completed in patients with breast and colon cancers using BR96-DOX in combination with Taxotere. Other Phase II trials are being conducted in patients of prostate cancer and nonsmall cell lung cancer (NSCLC) using BR96-DOX in combination with Taxotere or Gemzar.

4. Antibody-Radioisotope Conjugates (Radioimmunoconjugates)

The use of radiolabeled MAb for cancer treatment offers several theoretical advantages over that of chemoimmunoconjugates and the immunotoxins to be discussed later. Once bound to tumor cells, the radioimmunoconjugates can kill not only the MAb-coated cells, but potentially also those surrounding tumor cells ("bystander effect") via radioisotope emission within an area defined by the path length of the isotope and the composition of the tumor tissues. This allows the killing of antigen-negative tumor-cell variants and tumor cells that are inaccessible to the radioimmunoconjugate. Furthermore, internalization of the MAb, which is necessary for the cytotoxicity of most chemoimmunoconjugates and immunotoxins, is generally not required for that of the radioimmunoconjugates. One exception to this is radioimmunoconjugates containing alpha emitters that have a very short path length (approximately the distance of one cell).

Several important issues to consider in choosing an appropriate radioisotope for preparation of radioimmunoconjugate include the properties of the carrier MAb and the intended use of the conjugate. It is important to carefully match the physical properties of the radioisotope to the biological properties of the carrier MAb. For example, for radioimmunoimaging purpose, an antibody fragment with short circulation half-life coupled with a gamma emitter of matched physic half-life would be the ideal choice. On the other hand, for radioimmunotherapy using an intact MAb, the half-life of the radioisotope should be long enough to allow the MAb to localize and accumulate in the tumor to exert its cytotoxic effect. There is still considerable debate regarding the best class of radioisotopes suitable for MAb-targeted cancer therapy. Beta emitters such as ^{131}I , ^{90}Y , and ^{188}Re release radiation that penetrates several millimeters and therefore could be beneficial in killing tumor cells that are either antigen-negative or inaccessible to the radiolabeled MAb (135–137). However, use of beta emitters somewhat compromises the selectivity of the targeted therapy by damaging nearby normal cells. Alpha emitters, such as ^{211}At and ^{212}Bi , are more effective in inducing cell damage once delivered to the vicinity of the tumor cells, but because their energy dissipates over a very short range, the damage is likely to be limited to a single cell (138,139). Some isotopes decay by electron capture and subsequent Auger cascade, which is believed to be highly efficient in producing lethal effect (by damaging DNA molecules) if initiated near the cell nucleus. The antitumor activity of Auger emitters, such as ^{125}I , therefore could be enhanced considerably when linked to a carrier MAb that undergoes active internalization once binding to tumor-surface antigen (140,141). Some of the radioisotopes, along with their relevant properties, that are being used or that are believed to have potential use in radioimmunotherapy are listed in Table 1.

Among these radioisotopes, ^{131}I has been used most often because of its availability, ease of manipulation for MAb labeling, and favorable emission characteristics. Most

Table 1
Radioisotopes Used for Radioimmunotherapy of Cancers

Isotope	Mode of decay	Half-life (h)	maximum tissue path length (mm)	Particle energy (MeV)
^{131}I	β, γ	193	2.4	0.61
^{90}Y	β	64.2	11.9	2.28
^{186}Re	β, γ	91.2	5.0	1.07
^{188}Re	β, γ	16.7	11.0	2.12
^{67}Cu	β, γ	58.6	2.2	0.577
^{177}Lu	β, γ	160.8	2.2	0.5
^{212}Bi	α	1.01	0.04–0.08	6.09
^{211}At	α	7.2	0.04–0.08	5.8
^{125}I	Auger	60.2 d	0.01–0.02	0.35

of the currently used iodination methods, including chloramine T and the iodogen methods, involve covalent attachment of radioiodine to the tyrosine residues in the antibody (142,143). These methods oxidize iodine to a cationic species capable of replacing a hydrogen atom in the phenolic ring of tyrosine in the ortho position. They are relatively easy to perform and can yield high iodine incorporation, but may damage MAb activity by iodinating the tyrosine residues in the CDRs that are important for antigen binding (144). One nonoxidative method for protein iodination is the Bolton-Hunter method, which attaches the radioiodine to the lysine residues in the antibody. A common major drawback of the aforementioned methods is the relative instability of the radiolabel in vivo owing to a process called dehalogenation (142,143). This rapid degradation of iodinated MAb can lead not only to decreased localization of the MAb in tumor, but also to unwanted accumulation of radioactivity in the thyroid and stomach. It has been postulated that the dehalogenation of radiolabeled MAb is mediated via enzymes involved in thyroid hormone metabolism, such as deiodinases, that cannot distinguish iodine-labeled tyrosine from thyroxine (145). Therefore, it is plausible that dehalogenation could be minimized if the iodine is linked to proteins in a way that is not susceptible to deiodinases (146–148). In this regard, several methods have been developed for protein iodination via nonphenolic aromatic rings. In these methods, a radio-labeled intermediate, such as *N*-succinimidyl (tri-*n*-butylstannyl) benzoate, 3-tri-*n*-butylstannylphenylisothiocyanate, or *N*-succinimidyl 3-iodobenzoate, is first prepared, followed by covalent conjugation to MAb, usually via the lysine residues (149–152). Antibodies iodinated via these methods were shown to be superior to those labeled by chloramines T method in regard to immunoreactivity, stability both in vitro and in vivo, and localization in tumor xenografts, along with reduced iodine accumulation in the thyroid and stomach (149–152).

An undesired issue in using ^{131}I -labeled MAb for cancer therapy is that the radioisotope, in addition to its therapeutic beta radiation, also emits long-range gamma radiation that may pose an unwanted risk to patient's family members and health care workers. In this regard, a pure beta emitter, such as ^{90}Y , has gained considerable interest in recent years (135–137). In contrast to ^{131}I , ^{90}Y is a metallic radioisotope that is usually linked to MAb via the use of bifunctional or heterobifunctional chelating agents. A chelator, such as DTPA and EDTA, is first labeled with the metallic radioisotopes

followed by its attachment to the MAb by a reactive functional group (for reviews, *see* refs. 153,154). Although bifunctional chelators can be designed for linkage to different functional groups in the antibody molecule, e.g., amino group, sulfhydryl group, or tyrosines, the most commonly used bifunctional chelators are those designed specifically for coupling through lysine residues. A major obstacle to the extensive use of metallic radioisotopes has been the difficulty in the development of high-affinity chelators that can form stable bonds to the carrier MAb without affecting its antigen-binding activity (153–155). The main drawback associated with the use of metallic isotope-labeled MAb is their tendency for accumulation of radioactivity in the liver and bone (156).

Zevalin™, a ^{90}Y -labeled murine anti-CD20 antibody (Ibritumomab Tiuxetan, or IDEC-Y2B8), is the first radiolabeled MAb to receive approval for therapeutic applications from the FDA (62). Zevalin™ is composed of a murine anti-CD20 MAb (IgG1, κ), Ibritumomab, covalently bound to the metal chelator tiuxetan, which links ^{90}Y . The use of a murine MAb rather than a human or humanized version in the conjugate proved to be advantageous; the circulation half-life of Ibritumomab in human beings is approx 48–72 h (compared with that of 1–3 wk for most humanized and human MAb) (157,158), which matches very well with the physical half-life of ^{90}Y (~64 h). Thus, the use of a murine MAb minimizes nonspecific radiation to nontarget cells/organs. Extensive clinical trials have been performed using Zevalin™ in patients with relapsed/refractory low- and intermediate-grade NHL. Several Phase I/II studies demonstrated that the optimal regimen for Zevalin therapy consists of two pretreatments with Rituxan, at 250 mg/m² 7 d prior to and immediately prior to the administration of Zevalin. The maximum tolerated doses of Zevalin are 0.4 mCi/kg for patients with platelet count higher than 150,000 and less than 25% bone-marrow involvement with NHL, and 0.3 mCi/kg for patients with platelet count between 100,000 and 149,000 (159,160). In a randomized Phase III trial, 143 patients were treated with Zevalin (73 patients, 0.4 mCi/kg) or Rituxan alone (70 patients) in which the patients were given 4 weekly doses of 375 mg/m². The ORRs was 80% in the Zevalin treatment group, compared with 56% in the Rituxan group, with CRs of 30% vs 16%, respectively (161). In a Phase II trial in patients with mild thrombocytopenia (platelet count from 100,000 to 149,000), Zevalin, given at 0.3 mCi/kg, yielded an ORR of 82% with 37% CRs (162). Furthermore, in an open-label, nonrandomized trial, 57 patients who had failed prior Rituxan therapy, Zevalin regimen (0.4 mCi/kg) demonstrated an ORR of 74%, which was significantly higher than the ORR obtained from the last cycle of Rituxan therapy (32%) (163). More importantly, 51% (19 out of 37) patients who did not respond to their last Rituxan therapy showed a response to the Zevalin regimen. The primary toxicities associated with Zevalin treatment are hematological, including neutropenia, thrombocytopenia, and depletion of peripheral B lymphocytes, all of which are transient and reversible. Nonhematologic toxicities are similar between Zevalin and Rituxan, which include asthenia, nausea, chills, fever, throat irritation, headache, and vomiting. It is noteworthy that plasma immunoglobulin levels remain in the normal range during Zevalin therapy, and the overall incidence of serious clinical infections was low. Further, HAMA was only observed in three out of 211 patients tested (1.4%) (62).

Another radioimmunoconjugate that has been tested extensively in clinical trials is Bexxar™, a ^{131}I -labeled murine anti-CD20 MAb (also known as anti-B1 antibody, or Tositumomab) (63). Bexxar, like that of Zevalin, is given to patients in three stages over 1–2 wk, but with various doses calculated for each individual patient according to

the pharmacokinetic (PK) parameters measured prior to the therapeutic intervention. The patient is administered 450 mg of unlabeled MAb on d 0, along with 5 mCi of Bexxar (35 mg MAb) to determine the PK of the antibody. Whole-body gamma counting is performed on d 0, 2, 3, or 4, and d 6 or 7, to calculate the radiation dose delivered to whole body and bone marrow. On d 7–14, the therapeutic dose, comprising 450 mg unlabeled MAb with sufficient dose of Bexxar (35 mg of labeled MAb), is given to deliver the predetermined maximum-tolerated radiation dose to the whole body and bone marrow, which is 75 cGy in patients with a normal platelet count and 65 cGy in patients with platelet count between 100,000 and 149,000 (63,164). Accordingly, the actual ^{131}I radioactivity administered to each patient varies from 45–177 mCi. In two Phase II trials in patients with relapsed/refractory low-grade or transformed NHL, Bexxar regimen yielded ORRs of 71% and 60%, with CRs of 34% and 32%, respectively (165,166). When given as the initial therapy to 76 patients with follicular NHL, Bexxar treatment resulted in an ORR of 97% with 63% CRs (167). In a multicenter pivotal Phase III trial in patients with low-grade or transformed NHL, and who had failed at least two prior regimens and had relapsed within 6 mo of completing those therapies, ORRs to the Bexxar regimen was reported in 65% of the patients with 20% CRs (168). In a Phase II trial in patients who had failed Rituxan therapy, Bexxar treatment yielded 57% ORRs with 14% CRs (169). The major toxicities of Bexxar are mild to moderate transient neutropenia, thrombocytopenia, and anemia. Approximately 6% of patients developed AML and myelodysplasia syndrome, but this may be related to prior chemotherapy (63). Other common side effects of Bexxar are similar to those of Zcvalin, including asthenia, fever, nausea, and headache. Bexxar gained approval recommendation from The Oncologic Drugs Advisory Committee in December 2002 and is awaiting marketing approval from the FDA.

5. Antibody-Toxin Conjugates (Immunotoxins)

One of the advantages of using toxins as the cytotoxic moiety in MAb-based tumor targeting is their extreme potency. In many cases, a single toxin molecule is able to kill a cell once entering the cytosol (170). Three major types of toxins are used in the preparation of immunotoxins: bacteria toxins, plant toxins, and fungal toxins. All three types of toxins catalytically inhibit protein synthesis in eukaryotes, but each at a distinct step during translation. Based on their biochemical characteristics, plant toxins can be grouped as: type I, single-chain ribosomal inhibitory polypeptides such as pokeweed antiviral protein and saporin; and type II, such as ricin and abrin, which are heterodimers. The A chain of type II toxins are the toxic moiety, whereas the B chain contains the binding site for carbohydrates on cell surface through which A chain gains access to cell cytosol. Type I plant toxins only contain the catalytic domains without the binding domains. Bacteria toxins such as pseudomonas exotoxin A (PE) produced by *Pseudomonas aeruginosa* and diphtheria toxin (DT) produced by *Corynebacteria diphtheria*, are produced as single-chain polypeptides, each containing a binding and a catalytic domains separated by a translocation domain. Fungal toxins such as alpha sarcin are also single-chain proteins, but are functionally different from the type I plant toxins. For example, α sarcin is a phosphodiesterase, whereas type I plant toxins are *N*-glycosidases.

Originally ITs were constructed via chemical coupling methods using the intact toxin molecules. These ITs were extremely cytotoxic to target cells, but the specificity was

suboptimal, because the binding moiety of the toxins, e.g., the B chain of ricin or the Ia (binding) domain of PE, may bind to the surface of normal cells via carbohydrate residues (171,172). Several approaches have been developed to circumvent this problem: (1) Construction of ITs that contain only the toxic moiety, such as the A chain of ricin or the catalytic domain of PE. While enhancing the specificity, the potency of these ITs was significantly reduced owing to lack of the binding and translocation unit (173,174). Furthermore, the glycosylated side residues of toxin A chain may still cause nonspecific uptake by hepatic nonparenchymal cells via the mannose receptor and by macrophages (175,176). Chemical deglycosylation has been used to modify the A chain in order to reduce the nonspecific uptake (177,178). (2) Construction of ITs with the intact toxins that have been chemically modified to block the oligosaccharide-binding sites on the B chains, for example, blocked ricin (179,180). (3) Delivery of the B chain together with the A chain-containing ITs either directly or indirectly via a second MAb homing to the same target cells (181,182). (4) Construction of ITs using single-chain ribosomal inhibitory proteins, such as pokeweed antiviral protein and saporin, that do not contain any cell-binding components (183,184). Several deglycosylated ricin A chain (dgA)-based ITs, for example, the anti-CD22 ITs, RFB4-dgA, and RFB4-Fab'-dgA; the anti-CD25 IT, RFT5-dgA; and the anti-CD19 IT, HD37-dgA, have been produced via chemical coupling and tested in both animal models and limited Phase I trials. Furthermore, an anti-CD19 IT, comprising of the anti-B4 antibody coupled to an intact ricin with blocked B chain, was also constructed and tested in at least two Phase I trials. (For detailed reviews on the preclinical development and clinical investigation on these ITs, *see* refs. 58–60.)

Several major drawbacks in preparation of ITs via chemical coupling include low efficiency of the coupling processes, loss of antibody and/or toxin activity owing to chemical modification, generation of heterogeneous products, and difficulties in large-scale production. Recent developments in general molecular-biology techniques and antibody engineering, along with the structural knowledge of several toxin molecules including PE, DT, and ricin, has made it possible to produce second-generations ITs via recombinant methods. In this approach, the MAb or its fragment is fused genetically to the catalytic domain of a toxin, followed by expression of the fusion protein in *Escherichia coli* or other hosts. For example, in the preparation of PE-based ITs, the recombinant antibody fragment, either as a scFv or a disulfide bond-stabilized Fv fragment, is fused to the N-terminus of the truncated derivatives of PE, e.g., PE40 (amino acid 253–613, with the binding domain Ia [amino acid 1–252] deleted) or PE38 (a PE40 derivative with additional deletion of portion of domain Ib [amino acid 365–379]) (185–187). Interestingly, ITs produced by fusion of the antibody fragment to the C-terminus of PE40 or PE38 are not active (188). For DT-based ITs, the inverse arrangement is required for activity. In this case, the antibody fragment must be fused to the C-terminus of DT to free the N-terminus for translocation and catalytic activity (189).

DAB389IL2 (Ontak™), a recombinant fusion protein comprising IL-2 fused to the first 389 amino acid of DT, is the first and the only IT approved so far by the FDA for clinical application in the treatment of cutaneous T-cell lymphoma (CTCL) (190,191). In a Phase I trial, Ontak produced 5 CRs and 8 PRs in 35 CTCL patients and 1 CR and 2 PRs in 17 patients with NHL (192,193). In a Phase III trial of 71 CTCL patients, 7 CRs and 14 PRs were achieved (194). Several PE40 or PE38-based recombinant ITs have also been successfully produced and tested in a number of clinical studies, including the anti-Le^y antibody B3(dsFv)-PE38 (also known as LMB-9) and BR96(sFv)-

PE40, the anti-^{erb}B2 (HER2/neu) antibody c23(dsFv)-PE38, the anti-CD25 antibody anti-Tac(Fv)-PE38 (also known as LMB2), and the anti-CD22 antibody RFB4(dsFv)-PE38 (also known as BL22) (for reviews, *see* refs. 58–60). LMB2 is very potent against human leukemia cells both *in vitro* and in animal models. In Phase I clinical trials, LMB2 was given to 35 patients for a total of 59 treatments, and resulted in CR of one patient with hairy-cell leukemia (HCL) and PRs in seven other patients (195). It is noteworthy that all four HCL patients responded to LMB2 (one CR and three PRs) therapy. A Phase II trial of LMB2 is planned in patients with CD25+ hematologic malignancies. BL22 is a disulfide bond-stabilized IT, and is currently in a dose-escalating phase I trial (196). Of 16 HCL patients who were resistant to cladribine, 11 had CRs and 2 had PRs; the remaining three non-responders either received low doses of BL22 or had neutralizing antibodies to the toxin (197).

The dose-limiting toxicity of many IT therapies is vascular leak syndrome (VLS), with symptoms ranging from weight gain and mild peripheral edema to hypertension and pulmonary edema (198). Several mechanisms have been proposed to be responsible for VLS, including cytokine-induced activation of macrophages and endothelial cells, inflammation, and direct damage to the endothelial cells by the ITs (199–201). Several recent studies suggested that a three amino acid motif present in both protein toxins and IL-2 might be responsible for VLS (202–204). Mutation of this sequence in toxin molecules, or use of anti-inflammatory agents and/or cytokine antagonists, therefore, may prove to be beneficial in future clinical trials (201,204). Other side effects associated with IT therapies include: (1) nonspecific toxicity resulted from binding of either the MAb or the toxin molecule, and (2) immunogenicity of the bacteria or plant-derived toxin molecules. Molecular modeling and engineering of the MAb and/or the toxins may help to construct ITs with reduced nonspecific binding to normal cells. Concurrent administration of immunosuppressive agents such as CTLA-4, anti-CD20 MAb, or steroids may alleviate the immune response to the ITs (201,205). Further, the use of human-derived toxin-like molecules, such as RNase, along with humanized or human antibodies, may prove to be nonimmunogenic in human therapy (206,207).

6. Antibody-Directed Enzyme-Prodrug Therapy (ADEPT)

ADEPT, the use of antibodies to carry enzymes to tumors for *in situ* generation of cytotoxic molecules from relatively nontoxic precursors, was first proposed by Philpott et al. in 1973 (208,209), and further developed by Bagshawe and Senter and their colleagues (210,211). In theory, an enzyme molecule can activate an unlimited number of precursors leading to the local amplification and accumulation of cytotoxic molecules within a tumor. Thus, ADEPT would potentially overcome the problem associated with low uptake of the chemoimmunoconjugates by tumor cells. Furthermore, because the cytotoxic molecules are generated within the tumors, nonspecific toxicities generally associated with conventional chemotherapies are minimized owing to low drug distribution to normal tissues such as bone marrow and intestinal epithelium. ADEPT usually involves two steps: first, the antitumor antibody-enzyme conjugate (or fusion protein) is given to patients. After sufficient amount of time to allow adequate tumor localization and clearance of the conjugate from the circulation (for an optimal tumor/nontumor localization ratio), a prodrug (or precursor) is administered for its conversion into an active cytotoxic molecule by enzyme pre-localized in the tumors. Activation of prodrug at tumor sites results in a high local concentration of the cytotoxic

molecules, which may not only kill the antigen-positive tumor cells, but also reach and kill nearby antigen-negative tumor cells (the "bystander effect") (for reviews, *see refs. 212–215*). An additional step can be added between the administration of antibody-enzyme conjugate and the prodrug; that is, to give the patients a clearing agent that will remove the circulating conjugate at a faster rate, thereby further minimizing prodrug activation outside of tumors.

Unlike for chemoimmunoconjugates and immunotoxins, the ideal carrier MAb for ADEPT approach should be a noninternalizing antibody because antigen modulation upon antibody binding will decrease the enzyme concentrations on tumor-cell surface, and as a result, reduce the rate of prodrug activation. The MAb should also possess high specificity to tumor antigens (ideally surface antigens) with high binding affinity. To minimize nonspecific toxicity, it is essential that the prodrug-activating enzyme is not present in any normal tissues. Other criteria for enzyme selection include high substrate specificity, optimal enzyme activity at or near physiological pH and temperature, nonrequirement of cofactors, absence of enzyme inhibitors in tumors, and stability during conjugation and in circulation. So far, a number of enzymes have been employed in the ADEPT approach, including carboxypeptidase, alkaline phosphatase, aminopeptidase, cytosine deaminase, α -galactosidase, β -glucuronidase, β -lactamase, nitroreductase, and penicillin amidase (for reviews, *see refs. 212–215*). Most of these enzymes are of plant or bacteria origin, thus are immunogenic in humans. The use of PEG-modified enzyme, or administration of immunosuppressive agents such as cyclosporin and FK506, may reduce the immunogenicity of the MAb-enzyme conjugate. The ultimate resolution to the immunogenicity issue is to use humanized or human antibodies (216) and human enzymes, e.g., human glucuronidase and carboxypeptidase (217,218). Finally, catalytic MAb, or abzyme, can be used to replace the enzyme in the conjugate (219,220). Catalytic antibodies are antibodies against antigens that mimic the transition-state of a chemical reaction and thus they catalyze a given reaction by preferentially binding to and stabilizing the transition state configuration (221). In ADEPT, the catalytic MAb is coupled to an antitumor MAb to form a bispecific antibody molecule: one arm of the molecule binds to tumor cells while the other arm catalyzes prodrug into active agent (222,223). One of the advantages of this approach is its high specificity because an abzyme can be generated to catalyze a chemical reaction that is not mediated by a naturally occurring enzyme (223). Further, the immunogenicity of the MAb-enzyme fusion protein can be greatly reduced by using a humanized or a human abzyme as the catalytic unit.

A number of MAb-enzyme conjugates have been produced, either by chemical coupling or by recombinant methods, and tested both *in vitro* and *in vivo* in animal models (for reviews, *see refs. 213–215*). A limited number of clinical trials have been performed to date, mostly with conjugates containing the enzymes of bacteria origin. For example, in a Phase I trial in 10 patients with advanced colorectal cancer, the patients were treated with an anti-CEA antibody (A5B7) F(ab')₂ fragment conjugated to carboxypeptidase G2. A benzoic acid mustard-glutamate prodrug was given and converted by CPG2 in the tumor into a cytotoxic form. PR was seen in one patient, with stable disease seen in six other patients (224). In another study, where 27 cancer patients were given A5CP antibody-carboxypeptidase G2 conjugate and ZD2676P prodrug (bis-iodo phenol mustard), disease stabilization was seen in three patients (225). In both studies, significant HAMA and antibody to carboxypeptidase G2 were developed in all patients. Current studies in ADEPT focus on the use recombinant fusion protein comprising of

small antibody fragments, e.g., scFv, Fv, or Fab, fused to relatively nonimmunogenic enzymes (213–215). The clinical utility of these new generation MAb-enzyme fusion proteins remains to be demonstrated in future trials.

7. Bispecific Antibody-Mediated Drug Delivery

Bispecific antibodies (BsAb) are immunoglobulin-based molecules that bind to two different epitopes on either the same or distinct antigens (226,227). The majority of studies with BsAb have been focused on their use to redirect cytotoxic immune effector cells to kill tumor cells. In this context, one arm of the BsAb binds to antigen expressed on the tumor-cell surface, whereas the other arm binds to a determinant expressed on effector cells. Cross-linking between the tumor and the effector cells triggers the activation of the effector cells, resulting in killing of the tumor cell. A number of cell-surface molecules expressed by effector cells have been explored for efficient cell activation in order to achieve potent tumor-killing. For example, CD3, CD16, and CD64 expressed on T lymphocytes and natural killer (NK) cells/macrophages and other mononuclear cells (MNCs), respectively (228–230). Preliminary results from a number of early-stage clinical trials using these antitumor/anti-effector cell BsAb appeared to be very promising. Interested readers are referred to several excellent recent reviews on this topic (for reviews, *see* refs. 231–234). In addition to effector cell-targeting, BsAb has also been used to target cytotoxic agents, such as radionuclides, drugs, and toxins, to tumors. In this approach, the effector-cell specificity of an antitumor BsAb is replaced by an antibody directed against either the cytotoxic agent directly or the carrier of the agent, such as a hapten or liposome (235–240). Like the ADEPT, the BsAb-based targeting approach usually involves two steps: the BsAb is first administered to the patients, and after an adequate interval for the systemic clearance of the antibody to allow an optimal tumor/normal tissue localization ratio, the cytotoxic modalities are given, which leads to rapid accumulation of the cytotoxic agent within the tumor owing to its binding to the tumor-bound BsAb. A variety of cytotoxic modalities have been tested in BsAb-based targeting, including chemotherapeutic agents such as vinca alkaloids (235) and anthracycline antibiotics (236); toxins such as ricin A chain, saporin, and gelonin (237); chelated radioisotopes (238); prodrug-activating enzymes (239); and drug-loaded liposomes (240). A similar approach to the BsAb-based targeting is the use of a biotinylated antitumor MAb, followed by a streptavidin-labeled carrier, such as a heptan or a liposome preloaded with cytotoxic agents (241,242).

A major obstacle in the development of BsAb has been the difficulty in producing the antibody in sufficient quantity and quality for clinical studies via traditional hybrid hybridoma methodology or chemical conjugation (243). Coexpression of two different sets of IgG light and heavy chains in hybridomas may produce up to 10 different light-and-heavy-chain pairs, with only one of these pairs the functional bispecific heterodimer (244). On the other hand, chemical crosslinking of two IgGs or their fragments is often inefficient and can lead to the loss of antibody activity (245). In both methods, purification of the BsAb from the nonfunctional species, such as homodimers and mispaired heterodimers of noncognate Ig light and heavy chains produced by the hybridoma, and multimeric aggregates resulting from chemical conjugation, is often difficult and the yield is usually low (246). In recent years, a variety of recombinant methods have been developed for efficient production of BsAb, both as antibody fragments (for reviews, *see* refs. 29,243,247) and full-length IgG formats (248). In addition, production up to

one gram of antibody per liter of culture via *E. coli* fermentation has been reported for a number of BsAb fragments (249–251). Taken together, these novel technologies in BsAb production will greatly facilitate its application in tumor-targeting drug delivery.

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