



William Wolk. *Sisters*. Oil on canvas, 36" × 60".

Several classes of therapeutic monoclonal antibodies show promise in clinical trials for the treatment of hematologic malignancies.

Future of Monoclonal Antibodies in the Treatment of Hematologic Malignancies

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Background: *The approval of monoclonal antibodies (MAbs) as antibody-targeted therapy in the management of patients with hematologic malignancies has led to new treatment options for this group of patients. The ability to target antibodies to novel functional receptors can increase their therapeutic efficacy.*

Methods: *The authors reviewed improvements in MAb design to enhance their effectiveness over the existing therapeutic MAb currently approved for treating hematologic malignancies.*

Results: *Three classes of therapeutic MAbs showing promise in human clinical trials for treatment of hematologic malignancies include unconjugated MAb, drug conjugates in which the antibody preferentially delivers a potent cytotoxic drug to the tumor, and radioactive immunotherapy in which the antibody delivers a sterilizing dose of radiation to the tumor.*

Conclusions: *A better appreciation of how MAbs are metabolized in the body and localized to tumors is resulting in the development of new antibody constructs with improved biodistribution profiles.*

Introduction

The 1997 approval in the United States of rituximab (Rituxan), a recombinant chimeric anti-CD20 monoclonal antibody (MAb), for drug refractory low-grade or follicular B-cell non-Hodgkin's lymphoma (NHL) has presented new treatment options for many patients using MAbs as therapeutic agents for hematologic malignancies. Since the approval of rituximab, the US Food and Drug Administration (FDA) has recently approved alemtuzumab (Campath-1H), a humanized rat antibody to CD52 for treatment of refractory chron-

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ic lymphocytic leukemia, and gemtuzumab ozogamicin (Mylotarg), a calicheamicin-conjugated humanized mouse anti-CD33 MAb for therapy of drug-refractory acute myeloid leukemia.¹ Currently, the FDA has favorably examined the safety and efficacy of yttrium-90 (⁹⁰Y)-labeled radioactive murine anti-CD20 MAb (Zevalin) for rituximab- and chemotherapy-refractory NHL and is also examining iodine-131 (¹³¹I)-labeled radioactive murine anti-CD20 (Bexxar) following multicenter phase III clinical trials.²

Thus, three main classes of therapeutic MAb have shown utility in human clinical trials for treatment of hematologic malignancies: (1) unconjugated MAb, in which the MAb either directly induces negative growth signal or apoptosis or indirectly activates host defense mechanisms to mediate antitumor activity, (2) drug conjugates in which the antibody preferentially delivers a potent cytotoxic drug to the tumor, thus decreasing the systemic toxicity normally associated with conventional drug therapy, and (3) radioactive immunotherapy in which the antibody delivers a sterilizing dose of radiation to the tumor.

Our recent understanding of how engaging functional receptors leads to tumor growth or death has increased the opportunity to target antibodies to novel functional receptors that can increase their therapeutic effectiveness. Our knowledge of antibody activation of host defense mechanisms permits modifications of antibodies to improve activation and enhance these functions. Furthermore, a better appreciation of how MAbs are metabolized in the body and localized to tumors is resulting in the generation of antibody constructs with improved biodistribution profiles. This review discusses improvements in MAb design to enhance their effectiveness over the existing therapeutic MAb currently approved for treatment of hematologic malignancies.

Alteration of Antibody Properties

Preventing a Host Response to the Monoclonal Antibody

Early clinical trials using rodent MAbs suffered from rapid formation of human anti-mouse antibody (HAMA) or human anti-rat antibody (HARA). These host responses dramatically altered the pharmacokinetic profile of the antibody, leading to rapid clearance of the MAb and preventing repeat dosing. Although many patients with hematologic malignancies have compromised immune systems, HAMA reactions are problematic, and techniques to ameliorate the human antibody responses by developing less immunogenic MAbs have

been required. These have included chimerization, humanization, PRIMATIZATION[®], and the development of human antibodies from transgenic mice or phage display libraries.³⁻⁷

A chimeric antibody is one in which the variable domains that contain the antigen-binding sites are from the species used for immunization, and the constant domains of the protein chains are derived from human isotypes. Rituximab is an example of a chimeric antibody in which the variable domains are derived from the murine parent MAb generated against human CD20 (2B8) and the human constant regions from human immunoglobulin G1 (IgG1) heavy chain and human kappa light chain. This chimeric antibody is less likely than a fully murine MAb to provoke an immune response. In addition, the human constant IgG1 provides human effector functions to the MAb.⁸ Choice of the human isotype (IgG1, IgG2, IgG3, IgG4) as well as alterations of particular amino acids in the human isotype can enhance or eliminate activation of host defense mechanisms and alter biodistribution of the MAb.

Humanized antibodies represent a further improvement over chimeric antibodies. Humanized antibodies are essentially chimeric antibodies in which only the portion of the variable regions of both the heavy and light chain that contain the complementarity-determining regions (CDRs) and a few amino acids in the framework are from the species used for immunization. The remainder of the antibody remains human. Campath-1H is an example of a humanized antibody.⁹

PRIMATIZED[®] antibodies are types of chimeric antibodies in which monkeys used for immunization are the source of the variable domains. Since the variable regions of the monkey antibodies are almost indistinguishable from human, this type of chimeric antibody does not require any changes (Fig 1).

Fully human antibodies have been derived from mice that have had their endogenous immunoglobulin genes inactivated and human immunoglobulin genes placed into their genome.^{6,10,11} In addition, fully human variable domains have been isolated from phage display libraries and then converted into whole MAbs.¹²

These genetically engineered constructs showed large and overlapping variability in their rate of clearance from the circulation in humans. Chimeric antibodies have reported beta half-lives in humans ranging from 4 to 15 days.¹³⁻¹⁶ Humanized antibodies have reported beta half-lives that ranged from 3 to 24 days.¹⁷⁻¹⁹ PRIMATIZED[®] antibodies ranged from 10 to 13 days in humans, and recombinant human antibodies have

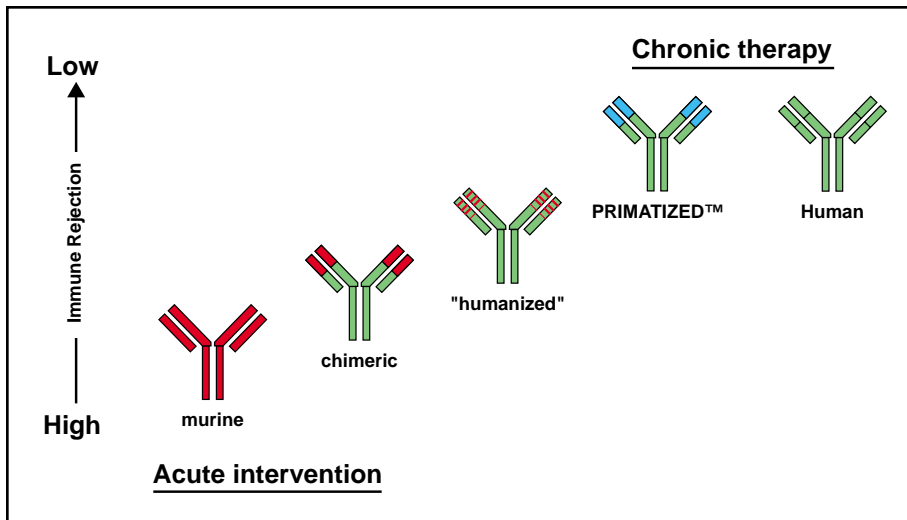


Fig 1. — Generations of antibody technology. Graphic representation of progression of monoclonal antibodies from murine to chimeric to humanized to PRIMATIZED® and human. The more human the antibody is, the less likely it will generate an immune response and the more utility it has for chronic (repeat) therapy.

reported beta half-lives ranging from 11 to 24 days.²⁰⁻²³ A human MAb has been reported to have a beta half-life of 24 days in humans.²⁴

All constructs did prevent, to variable degrees, the formation of human antibody-type responses to the inoculated protein. HAMA is primarily directed to the murine Fc portion of the antibody, although anti-idiotypic antibodies have been reported infrequently among patients demonstrating strong HAMA responses to the murine Fc.^{25,26} Development of genetically engineered antibodies have allowed MAbs to be dosed repeatedly and to high levels needed to achieve therapeutic responses in human clinical trial without provoking HAMA.

Increasing the Affinity of Antibodies

The development of techniques to genetically modify MAbs has also allowed investigators to selectively modify individual amino acids to increase or decrease a desired biological effect. Originally used to modify amino acids in the variable region (CDR) to further reduce immunogenicity of humanized antibody in humans, amino acids in the variable regions have also been mutated to increase the affinity of the antibody for its antigen.

The binding affinity of an antibody for its target is important in determining whether a clinically relevant dose of antibody at reasonable cost can be administered to a patient. Additionally, for drug targeting approaches that typically use much lower MAb doses due to nonspecific toxicity, higher affinity antibodies may improve the amount of cytotoxic drug localized to the tumor. However, too high an affinity of antibody

may impair tumor penetration by localizing the antibody to the periphery of the tumor mass.^{27,28}

Improved affinity has been achieved via directed mutagenesis of individual amino acids in the variable region.²⁹⁻³¹ These improvements in affinity were achieved by genetic approaches in conjunction with combinatorial phage display libraries. In this approach, a single immunoglobulin variable region with affinity for a particular antigen is randomly mutated in one or several positions. A library of mutants is created, and the individual

phage with the highest affinity for the antigen is isolated. Another method to improve affinity of antibodies is chain swapping, whereby a single VH region from an antibody with affinity to a particular antigen is paired with an entire library of VL regions. A new phage display library is constructed and panned on the antigen.³² Either of these enhancement processes generally requires 3 to 6 months to increase the affinity of an antibody approximately 50-fold.

Modifying Antibody Effector Functions Via Heavy-Chain Constant Regions Changes

MAb-based cancer therapies rely on a large variety of mechanisms to kill tumor cells. The amount of activation of host-killing mechanisms is associated with the type of human constant region (antibody isotype) used in the recombinant MAb. These host mechanisms activated by specific sites located in the Fc constant domain include complement-dependent cytotoxicity, antibody-dependent cell cytotoxicity, and phagocytosis (opsonization). Additionally, MAb can directly interfere with tumor growth without activation of host defense mechanisms by induction of apoptosis, by signaling cell cycle blockage, or by blocking growth stimulatory signals through receptor modulation or ligand blocking. These functions are usually independent of the Fc constant domain. Unfortunately, the importance in vivo of any of the individual potential killing mechanisms is unknown, and multiple mechanisms may be involved.

One of the host defense mechanism activated by antibody binding to a membrane antigen is activation of the complement cascade. Activation is initiated by binding of serum complement protein C1q to a mem-

brane antigen/antibody complex. Different human antibody isotypes vary in both binding to the C1q component of complement, as well as to various membrane Fc receptors present on many immunological effector cells. Table 1 summarizes the binding of C1q and binding to the Fc receptors by the four human gamma isotypes (IgG1, IgG2, IgG3, or IgG4). A review of the relationship of human isotype to antibody effector function can be found elsewhere.³³

Antibody that is bound to an antigen on a cellular membrane and binds C1q can lead to direct complement-mediated cell death (CDC) or to phagocytosis by deposition of the C3 component of complement, followed by recognition by C3 receptors on various phagocytic cells. There is evidence that human gamma 2 can be efficacious in phagocytosis because it causes the deposition of more C3 on the surface of bacteria than either human gamma 1 or human gamma 3, even though it does not bind as well to C1q.³⁴ Recently, the C1q binding sites have been

mapped on rituximab, and they differ from those reported in mouse antibodies.³⁵

All human gamma isotypes have an asparagine-linked complex carbohydrate attached to the C_H2 domain that influences C1q binding and binding to Fc receptors. Aglycosylated forms of both antibody have a lower affinity for C1q and Fc receptors.^{33,36} Furthermore, the number of galactose sugars at the terminus of the biantennary chain of the carbohydrate can vary, and small differences in CDC are observed when zero or one or two galactose molecules are present.

Substitutions can also be made in the Fc region that increase binding of antibody to Fc receptors for enhanced antibody-dependent cell cytotoxicity (ADCC), increase phagocytosis, or affect the circulating half-life of the antibody.³⁷ Mutations that inhibit binding to FcγRII (primarily an inhibitory receptor) while enhancing binding to FcγRI and FcγRIII have potential to significantly enhance effector function of MAb.³⁷

Table 1. — Effector Function and Pharmacokinetics Related to Antibody Isotype

Receptor or Binding Protein and Function	Cell Types With Receptor	IgG1	IgG2	IgG3	IgG4
C1Q: Complement-dependent by cells with complement receptors (CR1, CR3, CR4)	C1Q: Serum protein CR1, CR3, CR4: Dendritic cells Macrophages Neutrophils	Yes	Yes but weak	Yes	No
FcγRI: Phagocytosis	Dendritic cells Eosinophils Macrophages Neutrophils	Yes	No	Yes	Yes but weak
FcγRII: Ig regulation and other functions	Basophils B cells Eosinophils Langerhans cells Macrophages Mast cells Neutrophils Platelets	Yes	Some binding to a single form of receptor	Yes	No
FcγRIII: Lysis (NK cells) and phagocytosis	Eosinophils Macrophages Mast cells Neutrophils NK cells	Yes	No	Yes	No
FcRn: Pharmacokinetics	Endothelial	Long half-life	Long half-life	Shorter half-life than other IgG	Long half-life
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Recruiting Host Effector Functions With Bispecific Antibodies

The rationale for this approach is that MAb-mediated tumor killing will be enhanced when cytotoxic effector cells are brought in close proximity to tumor cells. Theoretically, a greater number of effector cells can be recruited through the use of bispecific antibodies, where one specificity is to the tumor cell and the other is to an activating receptor on the host effector cell. Bispecific antibodies originally were generated either by chemically linking two purified Fabs, genetically linking Fabs via leucine zippers (amphipathic helices), or engineering one cell to produce two antibodies.^{38,39} Divalent diabodies (two different single-chain Fvs noncovalently associated) are now being used for bispecific applications.^{40,41}

Bispecific molecules are monovalent for each antigen and hence have lower avidity than monospecific antibodies. When bispecific antibodies are administered, binding to the effector cells in the circulation rather than the tumor is a problem that must be resolved. Pharmacokinetic profiles of bispecific antibodies are reported to be only hours compared with weeks with conventional MAb.⁴² In addition, significant human anti-bispecific antibodies have been reported,⁴² although the use of humanized divalent diabodies may eliminate this problem.^{40,41} Several clinical trials in malignancy with bispecific MAb have been reported, the most promising being chemically linked Fabs targeting FcγRI and Her-2.⁴²

Negative Growth Signaling

Binding of an MAb to cell-surface receptors can result in negative growth signaling, thus leading to

either growth inhibition or apoptosis. NHL tumors are sensitive to antibodies directed against the idiotype of their membrane immunoglobulin. Cross-linking of surface immunoglobulin by anti-idiotype (anti-ID) MAb led to tumor regression that was shown to be related to the ability of the anti-ID MAb to induce intracellular signaling.⁴³

Rituximab may also show a similar mechanism. When cross-linked, anti-CD20 has been reported to lead to apoptosis of malignant human B cells.⁴⁴ Similar results have been demonstrated at our institution in which hyper-cross-linking of the CD20 antigen, either by immobilizing rituximab on plastic or by cross-linking membrane-bound rituximab using anti-human IgG, inhibited growth of several human B-lymphoma cell lines in vitro (Table 2). Growth inhibition after cross-linking membrane-bound antigen has also been demonstrated using MAb directed against both CD19 and CD22 antigens.⁴⁵

In vivo, induction of apoptosis is thought to occur via Fc binding to FcR receptors on the host effector cells. Cross-linking of membrane-bound MAb, such as rituximab, via the FcR is thought to mimic the cross-linking observed using a secondary antibody in vitro. Signaling mechanisms involved in apoptosis are not fully elucidated, but they may involve up-regulation of the pro-apoptotic *Bax* molecule in lymphoma cells and down-regulation of anti-apoptotic *Bcl-X_L* molecule.⁴⁶⁻⁴⁸

It is therefore possible to consider novel methods to efficiently design MAb molecules that induce apoptosis without requiring effector cell cross-linking. This was demonstrated by chemically synthesizing IgG/IgG homodimers using MAbs to several B-cell markers

Table 2. — Effect of Cross-linking CD20 on Different B-Lymphoma Cells by Rituximab

Cell Line	Origin	Growth Inhibition*	
		Rituximab (No Cross-linking)	Cross-linking of Rituximab
DHL-4	Diffuse histiocytic lymphoma	+	+++
Ramos	Burkitt's lymphoma	+	+++
SB	Acute lymphoblastic leukemia	–	++
Daudi	Burkitt's lymphoma	–	++
SKW	EBV+ B lymphoma	–	+
Raji	Burkitt's lymphoma	–	++

+++ = >50% inhibition + = 20%-30% inhibition
 ++ = 30%-50% inhibition – = no growth inhibition

* Effect of rituximab on the growth of B-lymphoma cells was determined with and without cross-linking of the cell surface bound rituximab. B-lymphoma cells were coated with saturating concentration (10 µg/mL) of the rituximab and the surface-bound antibody was cross-linked by either secondary anti-human Ig γ chain-specific antibody or by secondary antibody immobilized onto plastic. The cell proliferation was determined by [³H] thymidine uptake and the % growth inhibition was calculated in relation to the proliferation observed in the cells treated with isotope matched irrelevant antibody controls.

(CD19, CD20, CD21, and CD22).⁴⁹ All chemical dimers were growth inhibitory in vitro, and the anti-CD19 homodimer was shown to have antitumor activity when tested in vivo.⁴⁹ Similar results were obtained at our institute using rituximab homodimers linked together via either a reducible disulfide or stable thioether linkage (Fig 2). Rituximab homodimers efficiently induced apoptosis in vitro (Fig 3), were strong-

ly growth inhibitory in vitro, and demonstrated antitumor activity comparable to rituximab when test using Daudi human tumor xenograft models. Growth inhibition and induction of apoptosis could also be mediated IgG/IgG heterodimers using two MABs directed against two different antigens. In this example, rituximab was chemically cross-linked to a PRIMATIZED[®] MAb, p5E8, directed against the human CD23 antigen (Fig 2).⁵⁰

This heterodimer induced apoptosis in vitro while each MAb as a monomer was incapable of apoptosis induction (Fig 3). Formation of MAb dimers by chemically cross-linking is an inefficient method resulting in poor yields and often leads to molecules with reduced affinity. Genetic constructs that are tetravalent have been expressed, and contain a Fc portion of the antibody.⁵¹

Modifying Antibody Biodistribution Via Heavy-Chain Constant Region Changes

The Fc receptors discussed above are all associated with activating the host cell defense mechanisms as well as cell signaling via antibody cross-linking after antibody binding to its cell-surface antigen. Another class of Fc receptor has been described that is involved in antibody catabolism. The receptor FcRn (neonatal receptor, also

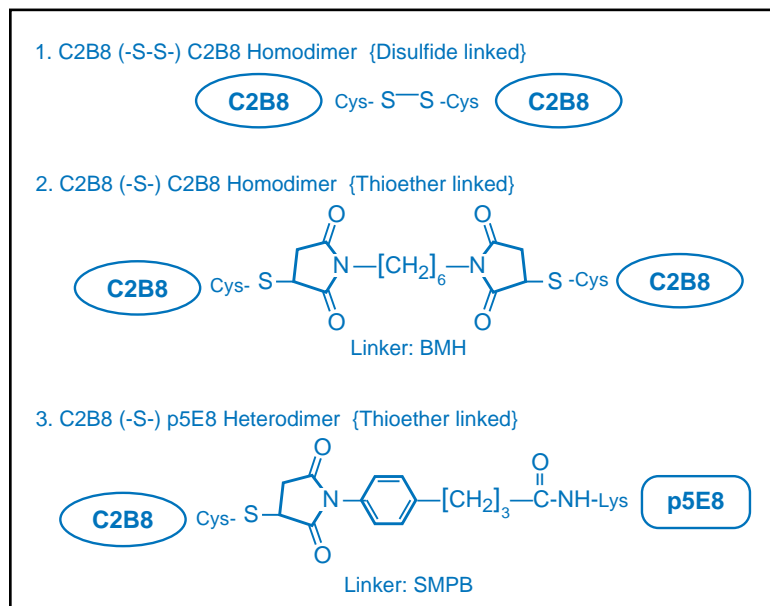


Fig 2. — Structures of C2B8 (rituximab) homodimer and C2B8/p5E8 heterodimer (rituximab/anti-CD23 or IDEC 152). This figure depicts the chemical linkage between the homodimer and heterodimer constructs of MAb rituximab and IDEC 152.

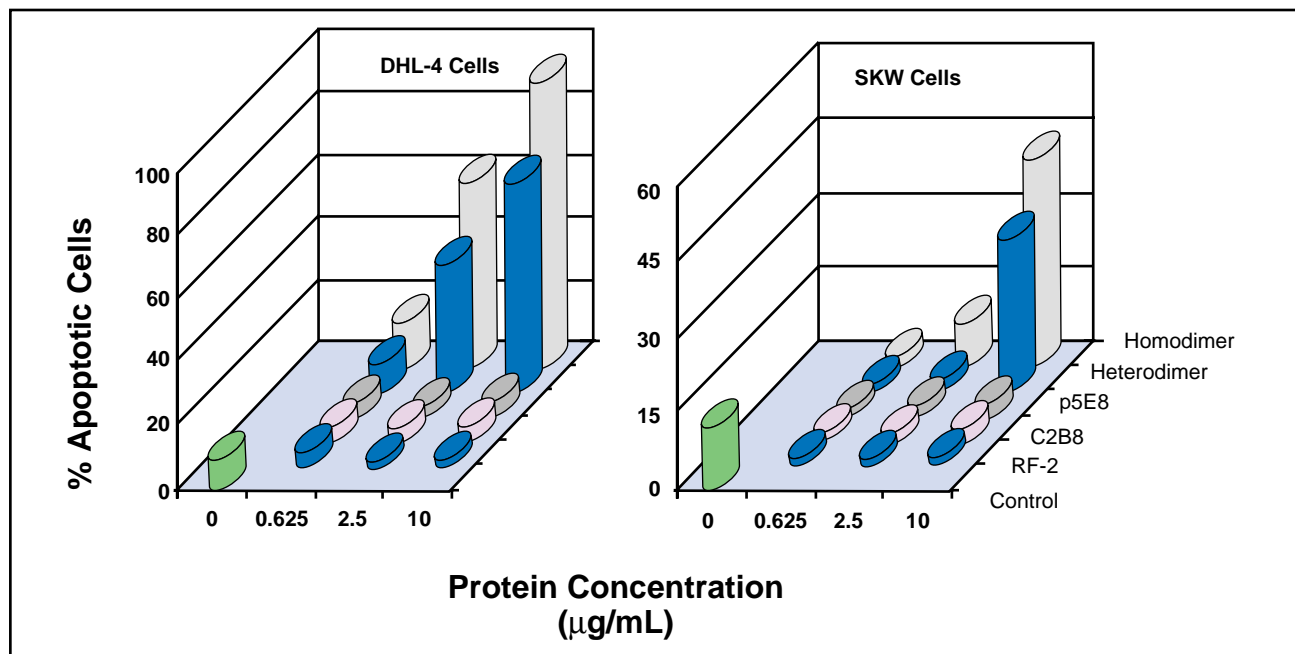


Fig 3. — Apoptotic activity of C2B8 (-s-s-) homodimer and (-s-) C2B8/p5E8 heterodimer on CD20/CD23 positive cell lines. This figure depicts the apoptotic activity at four different MAb concentrations on two different lymphoma cell lines (DHL-4 and SKW) of five different MABs: (1) an isotype matched control antibody RF-2; (2) C2B8 (rituximab, anti-CD20), no cross-linking; (3) p5E8 (IDEC 152, anti-CD23), no cross-linking; (4) Heterodimer (rituximab/IDEC 152); and (5) Homodimer (rituximab). It is clear that both the heterodimer and the homodimer have enhanced apoptotic activity.

called the Brambell receptor [FcRb]) is involved in the maintenance of constant serum IgG levels in the circulation.⁵² It is also the receptor that transfers maternal IgG across the neonatal gut into the newborn serum. There is pH dependence to the immunoglobulin-FcRn interaction, which is mediated in part by histidines in the C_H2 and C_H3 domains.⁵³ FcRn is expressed internally in endothelial cells, which can remove IgG from the blood by pinocytosis. IgG that has been internalized by pinocytosis is present in an internal vesicle in which the pH is being lowered as the vesicle matures. IgG present in this vesicle can bind to the receptor FcRn that is also present in the vesicle at the lower pH. IgG molecules that bind to FcRn in the intracellular vesicle are recycled back to surface of the cell along with FcRn. IgG bound to FcRn is then released at the neutral pH of the blood. This model suggests that antibody that does not bind to the FcRn receptor in the internal vesicle is degraded in lysosomes in the endothelial cell.

Human gamma 1, gamma 2, and gamma 4 all have high affinities for FcRn, which impacts their 3-week circulating half-life. Human gamma 3 has a lower affinity for FcRn and consequently has a shorter half-life.⁵⁴ The mutation of specific amino acids in the C_H2 or C_H3 domain can result in altered half-lives for immunoglobulins by altering their affinity for FcRn.⁵⁵

Combining Antibodies and Chemotherapy

Rituximab has been reported to enhance the sensitivity of B-cell lymphoma cell lines to killing by cytotoxic drugs, as well as lead to growth inhibition of B-cell lymphoma cell lines.^{56,57} The lack of hematologic toxicity in NHL patients to rituximab and the non-overlapping mechanism of cell killing have allowed investigators to combine unconjugated MAb and traditional chemotherapy.^{58,59} In a trial of 40 patients, rituximab was combined with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy to treat both newly diagnosed (31 patients) or relapsed/refractory low-grade NHL patients showed an overall response rate of 95%.^{58,60} Several of these patients were negative for PCR of the *bcl2* translocation from both blood and bone marrow.⁵⁹ The median time to progression of disease has not been reached in this study at 36+ months.^{59,60} Combinations of rituximab plus chemotherapy have also been used in large blinded trials in older patients with intermediate and high-grade NHL with longer event-free and overall survival over treatment with chemotherapy alone.⁶¹

Combination therapy with conventional cytotoxic drug therapy has also been shown to synergize with carcinoma reactive MAb such as humanized anti-Her-2

receptor antibody (trastuzumab) in advanced breast carcinoma.⁶² Using human tumor xenografts, MAb directed against the human epidermal growth factor receptor now in late-stage clinical development also strongly synergized with the cytotoxic drug paclitaxel.⁶³ In addition to using cytotoxic drugs, therapeutic MAbs have also demonstrated strong synergy with external-beam radiation in human tumor xenograft models.⁶⁴

Combination therapy with conventional cytotoxic drugs or radiation therapy in combination is one of the more exciting areas of improving the efficacy of MAb therapy in both hematologic malignancies and carcinoma. A new area currently being explored in clinical trials is the use of therapeutic MAb with immunomodulatory drugs that can boost the host immune system such as granulocyte-macrophage colony-stimulating factor (GM-CSF) or interferon resulting in longer duration of response.⁶⁵⁻⁶⁷ It has also been suggested that the continued tumor repression, long after rituximab has disappeared from the circulation, might be related to the development of an effective cytotoxic T-cell response to tumor-associated antigens. The use of immunomodulators to boost the T-cell response following rituximab administration may lead to more durable clinical responses.

Combining Therapeutic MAb

In the treatment of hematologic malignancies, the option to combine more than one antibody to improve the overall therapeutic efficacy will become a realistic goal when many of the antibodies in clinical development obtain regulatory approval. The main advantage of using antibody combinations compared with the antibody-chemotherapy or the antibody-radiotherapy is the low toxicity of many MAbs, which should not change in combinations. Criteria for the selection of antibodies for combination therapy will vary depending on the tumor type and the biological function of the antibody, eg, targeting differentially expressed antigens on the same tumor type to increase the percentage of antibody-targeted cells or to increase the amount of cell-surface MAb on each tumor cell. Alternatively, therapeutic MAbs may synergize with each other, especially if they mediate different mechanisms of tumor killing. Preclinical studies at our institution have demonstrated that combinations of rituximab with anti-CD80 MAb IDEC 114 or anti-CD23 MAb IDEC 152⁵⁰ result in synergistic antitumor response in a human lymphoma/SCID (severe combined immunodeficient) mouse model (Table 3). Each MAb was capable of activating both ADCC and CDC mechanisms of tumor-cell killing in vitro. Additionally, both rituximab and IDEC 152 were capable of initiating apoptotic activity after

cross-linking with anti-human MAb in vitro. All of the MAbs showed strong antitumor activity in a disseminated human B-cell lymphoma/SCID mouse model when tested individually. Antitumor activity of each antibody was similar, showing a 152% to 178% increase in the 50% survival rate over untreated animals. However, most of the animals still succumbed to disease. In contrast, when rituximab was combined with either IDEC 114 or IDEC 152, there was a significant increase ($P \leq .01$) in the number of disease-free animals over the monotherapy groups.

Targeted MAb Therapy

Radiolabeled MAb for Radioimmunotherapy

Hematologic malignancies are excellent candidates for radioimmunotherapy (RIT) because of the sensitivity of the malignant cell to radiation and the easier accessibility of the tumor to MAb. Also, the normal differentiation antigens often used as target antigens are expressed at high antigen densities with little tumor heterogeneity. The most common isotopes for therapy have been the high-energy β -emitters. They offer the advantage of inducing lethal damage over a relatively long path length and kill bystander cells that may not express high levels of target antigen. They also have deeper tissue penetration, thereby addressing the issue of poor tumor penetration of IgG molecules in therapy for bulky disease. ^{131}I and ^{90}Y remain the most common high-energy β -emitter isotopes used and have generated therapeutic anti-CD20 MAb (Zevalin and Bexxar). However, RIT has not proven as effective in treating carcinomas or patients with large tumor burdens. Limitations include (1) tumor target antigens with low level or heterogeneous expression, (2) poor tumor penetration of large molecules such as MAb, and (3) relative resistance of the tumor to radiation therapy and dose-limiting bone marrow toxicity, preventing higher input doses.

Newer approaches utilize α -emitters that deposit larger amounts of energy over a much smaller distance. They show promise in targeting small hematologic tumors where tissue penetration and antigen heterogeneity are not as great an issue as with large bulky tumors. They offer the advantage of efficiently killing the malignant cell with minimal nonspecific toxicity, due to their short path length and half-life. Recently, the overall safety of administering ^{213}Bi -labeled anti-CD33 humanized MAb was demonstrated in a phase I clinical trial.⁶⁸ Further clinical evaluation in acute myeloid leukemia will have to be conducted to determine if α -emitters coupled to MAbs can generate a therapeutic response rate greater than the more commonly used isotopes (^{131}I or ^{90}Y).

Pretargeting Approaches for RIT

Pretargeting is another strategy for RIT because of the potential to achieve higher intratumor concentration of isotope than achieved with conventional RIT. Pretargeting strategies use an MAb directed against a tumor-associated antigen and have a high-affinity non-covalent binding site for a small molecule.^{69,70} The small molecule can be conjugated to an isotope (eg, ^{90}Y) and administered after the tumor-targeted MAb has localized at the tumor site and cleared from the circulation. Since the pretargeted antibody is nontoxic, high doses can be administered to saturate antigenic sites at the tumor. Often a polyvalent "chase" is given to rapidly remove the pretargeting MAb from the circulation. The aggregated MAb created by cross-linking with the chase is rapidly removed from the circulation and degraded in the liver, directly before administration of the isotopically labeled small molecule. A study using this approach of 10 patients with relapsed or refractory NHL taking rituximab/streptavidin was recently reported.⁷¹ Transient grade III hematologic toxicity was obtained in 5 of 7 patients treated with 30 or 50 mC/m^2 ^{90}Y DOTA-biotin. Six patients achieved an objective clinical regression. However, 6 of the 10 patients developed antibody responses to the inoculated streptavidin portion of the rituximab conjugate.

Repeating dosages to maximize the antitumor effect without eliciting an immune response would be ideal. Mutating surface residues of streptavidin capable of forming high-energy ionic or hydrophobic interactions has been explored as a method to decrease immunogenicity.⁷² Alternatively, a high-affinity-binding site for a small molecule could be constructed as part of the pretargeting MAb. The Affinity Enhancement System (Immunomedics, Inc, Morris Plains, NJ) uses a bispecific MAb and a radiolabeled bivalent hapten.⁷³ The bispecific MAb recognizes both the target cell and the radiolabeled hapten. The hapten is bivalent to increase the binding avidity of the radiolabeled small molecule to the pretargeted bispecific antibody.

Linking Cytotoxic Molecules to MAb

An alternative strategy is use of the MAb to deliver a cytotoxic drug or toxin rather than a radioisotope. Unlike therapy with naked antibodies such as rituximab, which must be directed against surface noninternalizing antigens where they remain available to activate host effector functions, MAb used for delivering cytotoxic drugs or toxins must be directed against internalizing antigens. Therefore, MAb-targeted drug therapies are cell-specific in that they are designed to efficiently kill the target cells that bind and internalize the drug or toxin conjugate. This is in contrast to RIT,

which efficiently kills both target and bystander populations. Selecting an appropriate target antigen for drug or toxin targeting is therefore critical to avoid the emergence of antigen negative-phenotypes.

Bacterial toxins (diphtheria toxin [DT] or *Pseudomonas exotoxin* [PE40]) or plant toxins (ricin or gelonin) are the most commonly used toxins for antibody targeting. Toxins also offer the advantage of preparing genetically engineered fusion proteins, in which genes encoding for the MAb antigen recognition sites (Fv) are fused to the genes encoding the toxin.⁷⁴ Examples include anti-CD22 and anti-CD19 MAb-ricin immunoconjugates for the treatment of NHL and a single-chain Fv (scFv) fusion protein derived from the MAb BR96 and PE40.^{75,76}

The common features of human clinical trials using immunotoxins either as MAb conjugates or fusion proteins have included (1) liver toxicity or vascular leak syndrome that was dose limiting⁷⁷⁻⁸⁰; the cause of the syndrome remains unknown but has been related to the toxins' cytotoxic effects on vascular endothelial cells,⁸¹ (2) a rapid appearance of a human antitoxin antibody resulting in both neutralization of the toxin and rapid elimination of the conjugate from the serum on repeat administration, and (3) limited clinical activity. Using humanized MAb and toxins of human origin

rather than bacterial or plant origin (eg, human tumor necrosis factor) may reduce or eliminate the host antibody responses, allowing repeated courses of therapy.⁸²

Conjugation of the MAb to low-molecular-weight cytotoxic drugs with potencies of the same magnitude as the bacterial or plant toxins (eg, enediynes and maytansinoids) avoid the problems caused by using highly toxic foreign proteins. A large variety of low-molecular-weight cytotoxic drugs have been conjugated to MAb or other proteins.⁸³ One enediyne conjugate (Mylotarg, a recombinant humanized anti-CD33 MAb-calicheamicin immunoconjugates) has recently been approved for drug-refractory acute myeloid leukemia.⁸⁴

Linker designs must be stable enough to allow the MAb to target the drug to the tumor site and then release active drug intracellularly after internalization on the target cell. MAb directed against internalizing antigens on B-cell lymphomas conjugated to doxorubicin (ADM) via a hydrolytic hydrazone bond demonstrated strong inhibition of tumor growth in preclinical animal models.^{85,86} ADM-hydrazone MAb conjugates prepared with a non-internalizing anti-CD20 MAb was not effective.⁸⁶ Similar conjugates were tested in metastatic breast carcinoma using MAb BR96.^{87,88} Doses as high as 700 mg/m² (corresponding to 20 mg/m² conjugated drug) were safely administered

Table 3. — Antitumor Response of Anti-lymphoma/Leukemia Antibody Combination

Antibody	Mechanism of Tumor Killing ^a			Antitumor Response ^b		
	Apoptosis	Antibody-dependent Cell Cytotoxicity	Complement-Mediated Cytotoxicity	Dose	% Disease Survival	Increased Life-span ^c
Rituximab (anti-CD20)	+	+	+	400 µg	10	172
IDEC-114 (anti-CD80)	-	+	+	400 µg	0	178
IDEC-152 (anti-CD23)	+	+	+	400 µg	40	152
Rituximab + IDEC-114	+	+	+	200 µg + 200 µg	80	-
Rituximab + IDEC-152	+	+	+	200 µg + 200 µg	70	-

^a Three different mechanisms of antibody-mediated tumor killing were evaluated in vitro. The apoptosis was determined by the ability of the cross-linked antibody to activate caspase-3 in B-lymphoma cells and/or to transform cells to be terminal deoxynucleotidyl-transferase-dUTP nick-end labeling (TUNEL) positive. The ADCC activity of antibody and their combinations was determined with 4-hour ⁵¹Cr-release assay using B-lymphoma cells (SKW) as targets and activated host-effector cells from peripheral blood of donors at 50:1 effector-to-target ratio. The CDC activity of antibody and their combinations was determined using ⁵¹Cr-labeled targets and human complement.

^b The antitumor response was evaluated in a disseminated human B lymphoma/SCID mouse model. On day 0, groups of mice (n=10) were inoculated intravenously with 4 × 10⁶ human B lymphoma, SKW cells (CD20⁺, CD80⁺, CD23⁺). Mice in the combination treatment groups were injected intraperitoneally with 200 µg each of IDEC-114 or IDEC-152 and rituximab. Mice in the monotherapy treatment groups received 400 µg of IDEC-114 or IDEC-152 or rituximab. All antibody injections were given in a final volume of 200 µL. Antibody injections were given on days 1, 3, 5, 7, 9, and 11 after tumor inoculation. Mice were monitored for disease development and death. On day 30, all 10 mice in the control group that were injected with a formulation buffer died of disease. The % survival represents the data on day 60.

^c Increased life-span was calculated as number of days for 50% survival of the treated group divided by the number of days for the 50% survival of the untreated group.

every 3 weeks. Localization of doxorubicin was observed in tumor biopsies, but clinical responses were not observed.^{87,88}

Conjugating a more toxic drug, such as the enediyne that produce single- and double-stranded breaks in the DNA or the maytansinoids that cause metaphase arrest similar to the vinca alkaloids, has the potential to increase potency.⁸⁹⁻⁹¹ These drugs have in vitro potencies in the subnanomolar range compared with the micromolar potency of Adriamycin. Several enediyne immunoconjugates have been evaluated in preclinical models. These include calicheamicin linked through a hydrolytic hydrazone linkage or reducible disulfide bond.^{84,92-94} Additionally, maytansine can be conjugated via a reducible disulfide linkage to MAb.⁹⁵⁻⁹⁷

These highly potent MAb drug immunoconjugates have shown impressive antitumor activity in preclinical animal models where cross-reactivity to normal populations expressing low levels of target antigen are not present. Lode et al⁹³ demonstrated that calicheamicin conjugated to the antiganglioside-GD2 MAb 14G2a efficiently targeted tumor cells expressing both high and low antigen densities. It remains to be determined if these potent MAb-drug immunoconjugates will target and kill normal cells expressing low antigen levels, which would contribute to increased toxicity and reduced therapeutic window.

Targeting Antibody-Enzyme Complexes

In this approach, the pretargeting antibody is used to preferentially localize an enzyme molecule rather than a drug or radioactive isotope at the tumor site. The enzyme is designed to rapidly convert a large number of a low-molecular-weight prodrug (inactive drug) to its active (cytotoxic) form. Once the MAb/enzyme conjugate is cleared from the host circulation but remains localized at the tumor site, a high dose of prodrug can be administered where activation would preferentially occur at the tumor site rather than systemically. Since the drug is released extracellularly, it has the advantage over drug-targeting approaches in killing bystander cells as well as the MAb-targeted cell.

The choice of prodrug/enzyme system is important. Endogenous enzymes that may be present in the blood or tissues cannot be considered for MAb conjugation since prodrug activation would also occur systemically.

Clinical trials have been limited to humanized anti-CEA F(ab')₂ antibody conjugated to the bacterial enzyme carboxypeptidase G2.⁹⁸ Since the drug is activated extracellularly and can easily diffuse from the tumor mass, solid tumors rather than hematologic

malignancies remain the target of choice. Additionally, the conjugate was strongly immunogenic, demonstrating a rapid human antibody response to the bacterial enzyme. Therefore, as with the use of immunotoxins, humanized MAb coupled to a human intracellular enzyme could potentially minimize the immunogenicity problem. Human β -glucuronidase has been directly conjugated to MAb or expressed as fusion proteins using a humanized anti-CEA fusion protein.^{99,100} The divalent fusion construct and its small size were designed to accumulate and be retained sufficiently at the tumor site while allowing the nonlocalized protein to rapidly clear from the circulation.¹⁰⁰ Human β -glucuronidase is an intracellular enzyme that can rapidly convert the doxorubicin prodrug (glucuronyl-doxorubicin) to its active form. Unlike the parent compound, doxorubicin, the prodrug is water-soluble and does not efficiently transport across the cell membrane into the intracellular environment. Since β -glucuronidase is not present extracellularly, little systemic activation occurs, thus allowing high input doses of the prodrug.

Another interesting approach substitutes a humanized catalytic antibody for the enzyme component in the fusion protein.¹⁰¹ The catalytic antibody can activate prodrugs derived from a variety of potent cytotoxic molecules such as doxorubicin, camptothecin, or a nitrogen mustard prodrug.^{101,102} Since the activation site is unique to the catalytic MAb, no systemic activation can occur, allowing high input doses of extremely potent cytotoxic molecules that are activated at the tumor site.

Changing the Pharmacokinetic Profile

The difficulty in finding antibodies efficacious in tumors is partly due to the fact that whole antibodies cannot efficiently penetrate large tumors masses. Effective treatment using MAb-targeted therapies requires that sufficient amounts of MAb be localized within the tumor. Intratumoral hydrostatic pressure increases as the tumor become progressively larger and antibodies localize less efficiently.¹⁰³ Patients in the phase I/II study of ibritumomab tiuxetan (⁹⁰Y-2B8, Zevalin) had an overall response rate of 67% (82% in low-grade NHL), which dropped to 41% in patients with large (≥ 7 cm) tumor burdens.¹⁰⁴ With unconjugated MAb, large doses can be safely administered (approaching kilogram quantities over a course of treatment) to achieve favorable tumor localization. However, radioactive or drug conjugate therapies are more toxic and result in dose-limiting toxicities that preclude higher dosing. Engineering longer half-life into the molecule would increase the radiation exposure to normal tissues, especially bone marrow, which is dose-limiting. The challenge for targeted MAb strategies would be to decrease

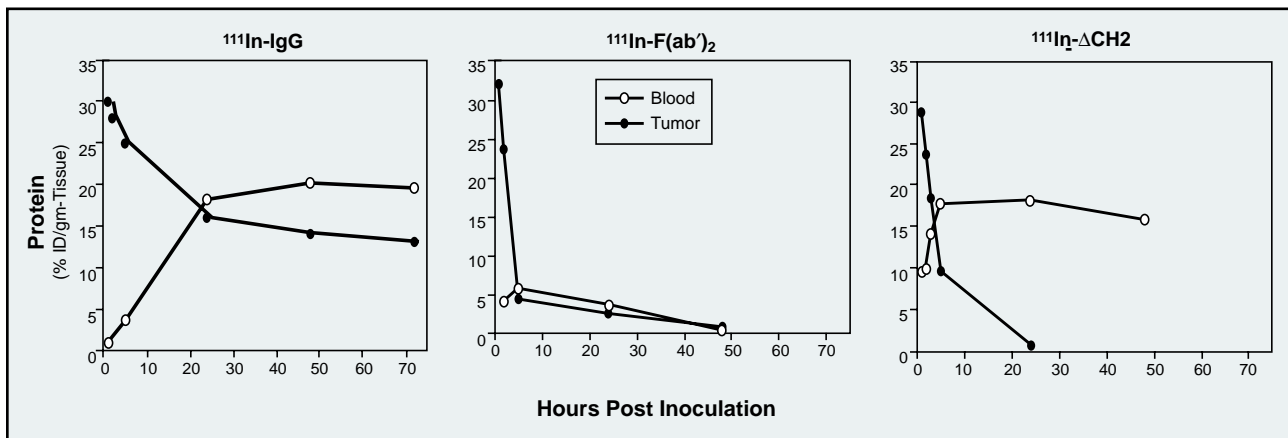


Fig 4. — Localization and blood clearance of Ig constructs. Plasma clearance and tumor localization profile of ^{111}In -labeled anti-CD20 IgG (Zevalin, ibritumomab, 2B8), compared to its ^{111}In -labeled $\text{F}(\text{ab}')_2$ and $\text{C}_{\text{H}2}$ domain-deleted fragments. Labeled constructs were injected intravenously into BALBc (nu/nu) mice bearing Daudi (CD20 positive) human tumor xenografts. Reprinted from *Clinical Reviews in Oncology/Hematology*, Vol 40. Reff ME, Heard C. A review of modifications to recombinant antibodies: attempt to increase efficacy in oncology applications. 25-35, copyright 2001, with permission from Elsevier Science.

the circulating half-life of the conjugated protein (allowing higher input dose) without negatively affecting tumor localization.

Intact recombinant antibodies (150 kd) with human gamma constant regions have long circulating half-lives ranging from 7 to 21 days.^{13,15,16,18,19,21,24,105-113} A variety of smaller recombinant MAb fragments that retain binding to tumor target populations have been prepared and include Fab 55 kd, $\text{F}(\text{ab}')_2$ (110 kd), single-chain Fv (25 kd), and double-chain Fv (50 kd). Larger antibodies with selected regions in the constant region deleted have been evaluated in animal models as well as mutations in the FcRn binding region located in the Fc portion of the MAb heavy chain.

The smaller molecules that are devoid of FcR binding tend to have a short circulating half-life, usually measured in hours. An example is an anticarcinoembryonic antigen $\text{F}(\text{ab}')_2$ (100 kd) with a half-life of 16 to 24 hours in humans.¹¹⁴ Radiolabeled anticarcinoembryonic antigen Fab' has a reported half-life of 4 hours, and a Fab immunotoxin (antibody-staphylococcal enterotoxin A recombinant fusion protein) demonstrated a half-life of 2 hours in humans.^{114,115} An scFv (25 kd) specific for carcinoembryonic antigen has been reported to have a half-life of 5 hours in humans.¹¹⁶

The smaller antibody fragments clear the circulation quickly, and their small size allows them to penetrate solid tumors more easily.²⁷ However, the smaller MAb fragments — Fab, scFv — by themselves have not proven useful for RIT due to a significant decrease in their ability to bind (as a percentage of the inoculated dose) and be retained at the tumor site when compared with intact immunoglobulin.¹¹⁷ In addition, recombinant proteins that are smaller than 60 kd are absorbed

by the kidney. In the case of Fab and $\text{F}(\text{ab}')_2$, this kidney uptake can be dose-limiting.¹¹⁸ In order to decrease kidney uptake, investigators have modified the molecules. An example is the charge modifications that were performed by covalently linking antibody fragments to polyethylene glycol in order to block kidney reabsorption.¹¹⁹

Recombinant proteins with a molecular weight of greater than 60 kd may be taken up by the reticuloendothelial system and catabolized by the liver. This includes modified antibody molecules such as the anti-B4 ricin immunotoxin (full murine IgG coupled to the ricin A chain) that have half-lives of hours in humans, and antibodies with linked cytotoxic drugs such as calicheamicin that have half-lives of 2 to 3 days.^{84,120}

Newer approaches to improve MAb delivery systems have used selective deletion of individual regions (domains) on the MAb or point mutations in FcRn recognition sites on the MAb.⁵⁴ $\text{C}_{\text{H}2}$ domain-deleted (120 kd) and $\text{C}_{\text{H}1}$ -Ck domain-deleted (130 kd) MAbs have been prepared and analyzed in human tumor xenograft models.¹²¹⁻¹²³ Deletions of the $\text{C}_{\text{H}1}$ region showed good tumor localization, but serum half-life was prolonged. In contrast, $\text{C}_{\text{H}2}$ domain deletions showed good tumor uptake and retained a short circulating serum half-life comparable to that of $\text{F}(\text{ab}')_2$ molecules. Fig 4 compares the serum clearance and tumor localization of three MAb forms of ^{111}In -labeled 2B8 (anti-CD20) in Daudi (CD20+) human tumor xenografts: IgG (Zevalin), $\text{F}(\text{ab}')_2$, and $\text{C}_{\text{H}2}$ domain-deleted MAb.

To obtain maximal tumor targeting, the MAb must balance rapid blood clearance with similar or improved biodistribution profiles, which is not necessarily related to the size of the MAb fragment alone. In Fig 4,

^{111}I -labeled F(ab')_2 was cleared rapidly from the blood but did not appreciably accumulate or remain the tumor when compared to the tumor localization of intact MAb. The domain-deleted antibody also cleared rapidly from the blood but showed tumor accumulation (as a percentage of the inoculated dose) and tumor retention comparable to that of intact IgG. Similar results were obtained using "minibodies" that used an 80-kd scFv- $\text{C}_{\text{H}}3$ fusion protein.¹²⁴ Minibodies, like the $\text{C}_{\text{H}}2$ domain-deleted constructs, remain dimeric through normal binding interaction between the two $\text{C}_{\text{H}}3$ domains or by inserting a gamma 1 hinge between the scFv and $\text{C}_{\text{H}}3$ domain giving a disulfide-linked molecule. These new constructs offer the potential of delivering a greater dose to the tumor than that achieved with conventional MAbs.

Conclusions

MAbs are having a significant impact on the treatment of many types of cancers, especially those of hematologic origin. MAb-based therapies may use different mechanisms of action than cytotoxic drugs by either acting through activation of host defense molecules or sending apoptotic or growth inhibitory signals. The efficacy of rituximab and trastuzumab may be attributable in part to having such an effect.^{44,48,125,126} MAbs have been shown to be effective as single-agent therapies and in many cases are synergistic with conventional drug approaches or other therapeutic MAbs as presented in this review.

Conjugation of MAbs with radionuclides or cytotoxic drugs offers promising new therapeutic approaches to increase clinical activity of unconjugated MAbs. For RIT, high clinical response rates with manageable toxicity profiles have been reported. These MAb-targeted strategies offer potential as single-agent therapies or in patient groups with low response rates following unconjugated MAb treatment. The lack of overlapping toxicity may also allow the potential for increased clinical activity in combination with unconjugated MAbs. The combination of RIT and rituximab has been shown to be more efficacious than rituximab alone.

Finally, the development of genetic engineering techniques to reduce immunogenicity, alter half-life, increase efficacy, and increase tumor targeting has provided the new classes of antibodies that have moved from the bench to the clinic. During the next decade, as new tumor-specific proteins are discovered, new MAb targets will be identified for regulating tumor cell growth or inducing apoptosis. Additionally, changes in MAb will allow for more efficient radionuclide or cyto-

toxic MAb drug targeting or lead to more efficient activation of host effector mechanisms leading to better therapeutic antibodies.

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