# The Evolution of Antibodies into Versatile Tumor-Targeting Agents

**Michael Z. Lin,[1] Michael A. Teitell,[2] and Gary J. Schiller[1]**

[1]Departments of Medicine and [2]Pathology and Laboratory Medicine, David Geffen School of Medicine at University of California at Los Angeles, Los Angeles, California

## ABSTRACT

**In recent years, monoclonal antibodies have become important weapons in the arsenal of anticancer drugs, and in select cases are now the drugs of choice due to their favorable toxicity profiles. Originally developed to confer passive immunity against tumor-specific antigens, clinical uses of monoclonal antibodies are expanding to include growth factor sequestration, signal transduction modulation, and tumor-specific drug delivery. In this review, we shall present the origins of antibody therapeutics within the field of immunotherapy and their evolution into effective anticancer agents, then discuss their multiple mechanisms of action, the basis of their tumor selectivity, and their therapeutic properties compared with traditional therapies. Antibodies are complex molecules whose efficacy and toxicity depend on the antigen, the antibody, any conjugated groups, and even the patient. Finally, we shall present new technologies being developed to increase the efficacy and selectivity of antibody-based therapeutics. Interestingly, many of the new approaches straddle the middle ground between immunotherapy and the traditional modalities of chemotherapy and radiotherapy, and can be seen as ways of combining the selectivity of the former with the efficacy of the latter.**

## INTRODUCTION

New cancer therapies that are more efficacious and less toxic than the traditional modalities of chemotherapy and radiation are needed. The use of the immune system to combat cancer is an old idea, often credited to Paul Ehrlich and William Coley over 100 years ago, a time that predates our understanding of the cellular and molecular components of the immune system. It was the elucidation of mechanisms of immunity and the introduction of a theory of cancer immunosurveillance by Lewis Thomas and MacFarlane Burnet in the 1960s, however, that gave rise to the modern concept of using the adaptive immune system to recognize and eliminate tumor cells whereas sparing normal tissue. After decades of waxing and waning interest, the idea of immunotherapy has recently achieved widespread acceptance (1), in large part owing to the successful introduction within the last decade of antibody-based cancer therapies into the clinic. Having accumulated several years of experience with anticancer antibodies, researchers are now in a position evaluate these first examples of immunotherapeutic drugs, looking back to relate their structure, mechanisms of action, and target antigen characteristics to clinical efficacy *in vivo*. We can also look forward to the further evolution of antibodies away from agents of purely passive immunity toward vehicles for tumor targeting, potentially combining the best characteristics of immunotherapy, chemotherapy, and radiotherapy.

**Boom, Bust, Boom: The History of Antibody Therapeutics.** The successful realization of antibody-based cancer therapies has depended on three key developments: the ability to produce unlimited copies of a desired antibody molecule [i.e., monoclonal antibody (mAb)], the characterization of suitable tumor-specific antigens, and methods for making mAbs progressively more human in sequence.

As early as the 1960s, researchers were actively engaged in generating specific humoral responses to tumor cells, with dual goals of targeting tumors therapeutically and identifying common tumor markers (2, 3). Early results include the identification of carcinoembryonic antigen and α-fetoprotein as serum markers of cancer (4). However, polyclonal antisera showed only transient effects against tumors in case reports, with efficacy likely limited by low specific titers and the xenogenic nature of polyclonal antisera (5–7).

The invention of mAbs by Kohler and Millstein in 1975 made possible antitumor antibodies of improved titer and consistency. The technology also allowed the generation of panels of antitumor mAbs and the systematic identification of target antigens (8). Consequently, the 1980s saw a burst of interest in immunotherapeutic mAbs, with emphasis on identifying new tumor-specific antigens and mAbs effective in eliciting immune-mediated cytotoxicity on tumor cells (9). Several mAbs proceeded rapidly to early-stage clinical trials; among the first were the anti-Ep-CAM mAb edrecolomab for colon cancer (10), mAbs raised against patient-specific immunoglobulin idiotypes in B-cell lymphomas (11), anti-CD5/Leu-1 in T-cell disorders (12), and mAbs against melanoma antigens (13, 14). Initial results were encouraging and served to validate some antigens as suitable targets for immunotherapy [e.g., tumor regressions observed in 3 of 9 patients receiving edrecolomab for metastatic colon cancer (15), 6 of 11 patients

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**Requests for reprints:** Gary J. Schiller, Department of Medicine, David Geffen School of Medicine at University of California at Los Angeles, CHS 42-121, 10833 Le Conte Avenue, Los Angeles, CA 90095. Phone: 310-825-5513; Fax: 310-206-5511; E-mail: gschiller@mednet.ucla.edu.

receiving anti-idiotype mAbs for B-cell lymphomas (16), and 5 of 7 patients receiving anti-CD5 for T-cell lymphoma (17)]. Disappointing results with other mAbs served to identify undesirable antigen characteristics such as the presence of circulating antigen (18) or, more commonly, antigenic modulation in response to mAb treatment due to internalization of mAb-antigen complexes (19, 20).

Even in cases where mAbs produced transient clinical responses, a common observation in early trials of mouse mAbs was limited serum stability due to the generation of a human anti-mouse antibody response, rendering repeat dosing ineffective and more toxic (13, 16, 17). For example, responses of T-cell lymphomas to anti-CD5 lasted <4 months and were limited by the development of human anti-mouse antibody (17). It was also known that rodent constant regions were not as effective as human in inducing antibody-dependent cellular cytotoxicitiy (ADCC) or complement-dependent cytotoxicity (CDC) in human blood (21).

**Revitalization through Humanization.** To circumvent the problems associated with rodent antibodies, researchers substituted human sequences for the portions of the rodent mAbs outside the antigen binding region, a process we refer to generally as humanization. This was first done by making chimeras of rodent variable regions and human constant regions, as in the case of anti-Ep-CAM, anti-L7, and anti-CD20 (22–24). Subsequently, to generate the mAb alemtuzumab against the lymphocyte marker CD52, discontiguous hypervariable regions from a rat anti-CD52 molecule were cloned between the framework regions of human immunoglobulin (25), a process termed complementarity-determining region (CDR) grafting. (Some researchers limit the term "humanization" to refer to this specific method.) These and other humanized mAbs were shown more effective in inducing ADCC and CDC *in vitro* (23–27). As expected, they were also less immunogenic. CDR-grafted alemtuzumab (Campath), CDR-grafted anti-HER2 trastuzumab (Herceptin; ref. 28), and chimeric anti-CD20 rituximab (Rituxan; ref. 27) did not generally elicit immune responses in patients. For example, none of 355 patients in seven clinical studies receiving rituximab developed human anti-mouse antibody, and only three developed an anti-chimeric antibody response (29).

Subsequently, most therapeutic mAbs have been humanized, a task made easier by multiple *in vivo* and *in vitro* methods for humanization. Of particular note are methods for generating completely human mAbs: Phage display allows rapid *in vitro* screening of human immunoglobulin libraries for molecules with binding activity against target antigens (30), and mice expressing human immunoglobulin genes have been recently engineered to allow for *in vivo* generation of fully human mAbs (31, 32). Phage display allows for rapid multiplexing and is not limited by immunologic tolerance to conserved proteins, whereas humanized mice can be directly used with standard immunization protocols. Under development are additional techniques for screening of libraries of antibody fragments attached directly or indirectly to the encoding mRNAs, such as ribosome display and covalent protein-mRNA linking (33).

Whereas humanization may be preferable and in some cases necessary for a mAb to be clinically useful, actual comparisons of unhumanized versus humanized mAbs in clinical contexts are few. Humanization has apparently allowed for multiple dosing of humanized alemtuzumab, previously not possible with the rat molecule (34, 35). On the other hand, the pharmacokinetics of murine anti-Ep-CAM edrecolomab are not significantly affected by the development of human anti-mouse antibody, nor is there a relationship between clinical response and human anti-mouse antibody (36). The approved radioconjugated anti-CD20 mAbs 90Y-ibritumomab tiuexetan (Zevalin) and 131I-tositumomab (Bexxar) are fully mouse molecules as well, with mean half-lives after a single injection of 65 and 48 hours, respectively (37, 38), compared with 76 hours for chimeric rituximab (39). The half-life of rituximab does increase to 204 hours after four injections over 1 month, something unlikely to occur with fully mouse mAbs, but this is not a major concern for tositumomab or ibritumomab which are intended for one-time dosing.

**mAbs Reach the Clinic.** In 1997 and 1998, rituximab and trastuzumab were approved by the U.S. Food and Drug Administration for chemotherapy-relapsed/refractory non-Hodgkin lymphoma (NHL) and HER2-expressing breast cancer, respectively, becoming the first antibody therapeutics to achieve widespread clinical adoption. Since then, six additional mAbs have been approved (Table 1). These are anti-CD52 alemtuzumab for relapsed/refractory B-cell chronic lymphocytic leukemia, anti-CD33 gemtuzumab ozogamycin conjugated to calicheamicin (Mylotarg) for relapsed/refratory acute myeloid leukemia, the anti-CD20 radioisotope conjugates ibritumomab and tositumomab for relapsed/refractory NHL, anti-vascular endothelial growth factor (VEGF) bevacizumab (Avastin) for metastatic colon cancer in combination with chemotherapy, and anti-epidermal growth factor receptor (EGFR) cetuximab (Erbitux) for metastatic colon cancer. Clinical trials for other indications of these drugs are continuing. Most noticeably, rituximab has been shown to be effective for a variety of B-cell neoplasms beyond NHL (40). The results of clinical trials using these drugs, including clinical applications and toxicity profiles, have recently been extensively reviewed (41).

The approval of these antibodies (and their commercial success) provided impetus for a wave of development of more anticancer mAbs, and now there are >400 in clinical trials (42), including several additional anti-EGFR mAbs and mAbs for NHL (43). Meanwhile, some of the earliest mAbs to be tested in cancer have yet to find widespread clinical use. Follow-up studies have confirmed long-term benefits of humanized anti-idiotype mAbs, but adoption has been hindered by the need to customize mAbs for each patient (44). The first mAb proposed for solid tumors, edrecolomab, is still in clinical trials. A phase III trial in stage III colon cancer found edrecolomab as monotherapy to be inferior to chemotherapy, and edrecolomab combined with chemotherapy to be no better than chemotherapy alone (45). However, in a phase II trial of resected stage II colon cancer, edrecolomab monotherapy was shown to reduce 7-year mortality from 63% to 43% and recurrence rate from 68% to 52%, compared with no therapy (46). A phase III trial to confirm these findings is under way (47).

**Monoclonal, but Multifunctional.** Being complex molecules capable of sequestration, leukocyte recruitment, complement fixation, and target cross-linking, it is not surprising that the effects of antibodies can be ascribed to multiple mechanisms. As with other drugs, some mechanisms were proposed based on preclinical research, but other mechanisms are only now coming

*Table 1* Food and Drug Administration–approved anticancer mAbs

| Antibody | Antigen | Indication | Species | Mechanisms of action | Brand name, distributor |
|---|---|---|---|---|---|
| Rituximab | CD20 | Relapsed/refractory NHL | Mouse-human chimera | ADCC, CDC | Rituxan, Biogen-IDEC (Cambridge, MA) |
| 90Y-ibritumomab tiuexetan | CD20 | Relapsed/refractory NHL | Mouse | Radiation | Zevalin, Biogen-IDEC |
| 131I-tositumomab | CD20 | Relapsed/refractory NHL | Mouse | Radiation | Bexxar, Corixa (Seattle, WA) |
| Gemtuzumab ozogamicin | CD33 | Relapsed/refractory acute myelogenous leukemia | Mouse-human chimera | Calicheamicin-mediated DNA damage | Mylotarg, Wyeth (Madison, NJ) |
| Alemtuzumab | CD52 | Relapsed/refractory B cell chronic lymphocytic leukemia | Rat-human chimera | ADCC, CDC | Campath, Millenium (Cambridge, MA) |
| Trastuzumab | HER2/ ErbB2 | HER2+ breast cancer | Mouse CDR-grafted | ADCC, receptor blockade | Herceptin, Genentech (South San Francisco, CA) |
| Bevacizumab | VEGF | Metastatic colon cancer | Mouse CDR-grafted | Ligand blockade | Avastin, Genentech |
| Cetuximab | EGFR | Metastatic colon cancer | Mouse-human chimera | Receptor blockade | Erbitux, Imclone (New York, NY) |

to light after several years of clinical use. Interestingly, mAbs seem to work through combinations of immunologic or non-immunologic mechanisms, depending on the particular molecule.

**Immunologic Mechanisms of Activity.** Originally, the most popular rationale for using mAbs in cancer was that mAbs would kill tumor cells though ADCC (48, 49). In ADCC, immunoglobulins complexed on a cell surface activate Fc receptors on host natural killer (NK) cells and monocytes, triggering a cytolytic response mediated by perforins, granzymes, induction of apoptosis through FasL, and oxidative mechanisms (50–53). Macrophages are also capable of phagocytosing antibody-opsonized cells (49, 54). Supporting the importance of ADCC for antitumor activity, relative antitumor activity of different mAbs in mouse models correlate with ADCC (55). Depletion studies suggested that NK cells and monocytes both contribute to tumor immunity conferred by certain mAbs (56). Finally, the antitumor activity of both rituximab and trastuzumab are negated in FcγR-deficient mice (57).

Complement activation is another proposed mechanism of mAb activity. In CDC, binding of C1q to Fc dimers results in production of iC3b and formation of the cytolytic membrane attack complex. iC3b also binds to complement receptor 3 on the surface of immune cells, enhancing FcγR-dependent ADCC (58). Human IgG1 and IgG3 are most efficient at complement activation, but not all IgG1 or IgG3 mAbs are able to activate complement (59–62), possibly because antigen density in certain cases may be too low to support the formation of Fc dimers (58). Whereas rituximab, trastuzumab, alemtuzumab, and edrecolomab activate complement *in vitro* (58), the importance of CDC to efficacy *in vivo* has only been established for rituximab. Complement products are observed following rituximab infusion in patients (63), and rituximab fails to protect C1q-deficient mice from lymphoma (64). Sensitivity of lymphoma subtypes to rituximab *in vivo* correlates with sensitivity to CDC *in vitro* (54), and may be mediated by tumor expression of membrane complement regulatory protein, which protects cells from CDC (58).

A third immunologic mechanism that may come into play in specific cases is the generation of an idiotype network. Antibodies directed against the idiotype region of a mAb may carry an internal image of the antigen and thereby induce the formation of additional anti-anti-idiotype antibodies capable of antigen recognition (65). Although the first finding of an association between between anti-idiotype antibodies and clinical response, involving edrecolomab, has not been reproduced (36), similar associations have been observed in subsequent studies with other mAbs (66, 67).

**Nonimmunologic Mechanisms of Activity.** Other mechanisms of action are nonimmunologic and instead involve effects on signaling pathway activation. For example, the anti-VEGF mAb bevacizumab binds to VEGF and blocks its interaction with the VEGF receptor on endothelial cells, thereby preventing angiogenesis induced by tumor-secreted VEGF. Signal blockade rather than VEGF clearance is responsible, as bevacizumab is effective *in vitro* in the absence of immune cells (68), and the *in vivo* clearance of VEGF decreases rather than increases with bevacizumab (69).

The actions of mAbs that recognize cell surface receptors are more complex. The anti-EGFR mAb cetuximab blocks EGFR activation and induces its internalization (70, 71), resulting in inhibition of cell proliferation, decreased production of angiogenic factors (72), and increased sensitivity to chemotherapeutic agents and radiation (73). Trastuzumab, which targets the EGFR-related molecule HER2, shares similar mechanisms of action (74). However, whereas trastuzumab activity requires Fc receptor function, as mentioned (57), immune mechanisms do not seem to be required for cetuximab activity, as Fc-deleted cetuximab still inhibit tumor growth (75).

Using lymphoma cell lines, researchers have observed multiple biological responses to rituximab-induced CD20 cross-linking, including down-regulation of the anti-apoptotic molecule bcl-2 and the prosurvival cytokine interleukin (IL)-10 and sensitization to chemotherapy (76). However apoptosis induction is more apparent with B cell lines than with freshly isolated primary B lymphocytes (77). Furthermore, an antibody with rituximab variable regions and an IgG4 constant region, which lacks immune effector functions, is ineffective against B cells *in vivo* (78). This is consistent with the previously described observations that immune mechanisms are necessary for rituximab activity.

**Enhancing Activity Using Immunoconjugates.** Conjugated mAbs have additional mechanisms of action related to the conjugate. Immunoconjugates to radioisotopes have been extensively investigated since the 1960s, initially as tumor

detection and imaging agents and later as agents for delivering cytotoxic radiation to tumors (79). The two currently available radiolabeled mAbs, yttrium-90–labeled ibritumomab and iodine-131–labeled tositumomab, both target CD20, the same antigen recognized by rituximab, and show more clinical activity than rituximab. Ibritumomab is the murine mAb that was humanized to obtain rituximab (80), so direct comparisons between them are informative. In a trial comparing ibritumomab and rituximab in replased or refractory NHL, overall response rates were 80% for ibritumomab versus 56% for rituximab, with a median response duration of 6-month progression-free survival rate of 64% versus 47% (81). In the case of tositumomab, although comparisons against the unrelated mAb rituximab are more difficult to make, an overall response rate in relapsed/refractory NHL of 65% was observed, similar to ibritumomab (82). In previously untreated low-grade NHL, tositumomab induced an initial overall response rate of 97% (83), compared with 71% to 76% for rituximab in similar patients (84). Thus, even without the potential for multiple dosing or immune effector activation conferred by humanization, radiolabeled anti-CD20 mAbs are more effective than their unconjugated counterpart.

Chemotherapeutic drugs represent the other class of conjugates. Gemtuzumab ozogamicin is a chimeric anti-CD33 mAb conjugated to the DNA-cleaving agent calicheamicin approved for single-dose treatment of relapsed/refractory acute myelogenous leukemia (85). Binding to CD33 induces mAb internalization into lysosomes, where the calicheamicin is released. In mice treated with versions of gemtuzumab linked to caliceamicin via different linkers, *in vivo* antitumor efficacy was related to linker cleavage, implying that calicheamicin release is an important component of gemtuzumab activity (86, 87). Furthermore, the overall response rate of 30% observed with gemtuzumab ozogamicin (88) seems quite higher than the 6% observed with a humanized unconjugated anti-CD33 mAb (89). However, because efficacy is dependent on the calicheamicin component, tumor cells exhibiting P-glycoprotein–mediated multiple-drug resistance may be able to escape, necessitating possible coadministration of P-glycoprotein antagonists (90).

## Selectivity: Balancing Efficacy and Toxicity

**Direct Relationship between Specificity and Selectivity.** The basis of mAb selectivity for tumor is fundamentally different than that of traditional modalities. With chemotherapy or radiotherapy, selectivity derives from the relative ability of normal cells to tolerate toxicity, regardless of how specific a drug is for its target molecule. This type of selectivity still applies in the case of function-blocking antibodies, such as anti-HER2 and anti-EGFR. However, for all mAbs, a large degree of selectivity is directly related to binding specificity of the antibody for a tumor, which in turn is the product of antigen specificity for the tumor and mAb specificity for the antigen. Sometimes mAbs are selected for these qualities sequentially (e.g., HER2 or EGFR overexpression in tumors was characterized), then specific mAbs are generated. Other times, both types of specificity are selected in combination (e.g., edrecolomab was isolated in a panel of anti-colon cancer mAbs and confirmed to preferentially stain tumor tissue before its target molecule, Ep-CAM, was identified; ref. 91). Antigens need not be completely absent from normal tissue; in certain cases, their relative overexpression in tumors is sufficient to confer a high degree of specificity of mAb binding (92). Furthermore, tumors may be more accessible to mAbs than normal tissue, due to the enhanced permeability and retention effect in many tumors (93).

However, antibody and antigen specificity is not the whole story. In the case of function-blocking mAbs, additional contributions to selectivity derive from differences in susceptibility of normal and tumor cells to pathway inhibition. EGFR is overexpressed in 60% to 75% of solid tumors (94) and HER2 in 20% to 30% breast cancer (74, 95), but they are both also expressed at lower levels by many if not most normal cells. However, toxicity effects are generally mild with trastuzumab and cetuximab. Selectivity of action derives from the increased dependence of receptor-overexpressing tumor cells on receptor activity for survival (96), which is expected given receptor activation is a causal event in the progression to cancer for many of these tumors.

An interesting example of toxicity likely caused by the confluence of all these factors in normal tissue is trastuzumab-induced cardiotoxicity. Trastuzumab cardiotoxicity is almost entirely confined to patients with a history of chemotherapy with anthracyclines, and anthracycline-stressed cardiomyocytes may be especially dependent on HER2 signaling for survival (97). Cardiomyocytes may unfortunately share with tumor cells multiple components of selectivity: expression of HER2, access to trastuzumab, and dependence on HER2 activity for survival.

Antigens need not be entirely tumor specific as long as their expression is confined to tissues whose functions are not critical, at least temporarily. This is the case for all the approved mAbs for hematologic tumors. CD20 is expressed on all B lymphocytes, CD52 on B and T lymphocytes, and the CD33 on the entire myelomonocytic lineage. Thus, as an inherent part of their therapeutic mechanisms, the anti-CD20 mAbs cause B lymphopenia, alemtuzumab causes generalized lymphopenia, and gemtuzumab causes neutropenia and thrombocytopenia. Resulting adverse effects generally correlate in severity with the distribution of the target antigen. Effects of rituximab are mild; grade 3 or 4 lymphopenia occurs in 37% of patients with a mean duration of 14 days (98). Grade 1 or 2 infections occur in 21% of patients and can be treated with appropriate antibiotics as needed. Only 2% of patients experience grade 3 or 4 infections. In contrast, alemtuzumab-induced lymphopenia affects both B and T cells and is long-lasting, with duration greater than a year (99). Without prophylactic antibiotics, the vast majority of patients, 86% in one study, develop opportunistic infections (99). Finally gemtuzumab, as expected, causes myelotoxicity, with grade 3 or 4 anemia, neutropenia, and thrombocytopenia in 52%, 98%, and 99% of patients, respectively (100). Mean duration of neutropenia is 40 days, and the incidence of grade 3 or 4 infections and bleeding are 30% and 13%, respectively.

Although the *sine qua non* of mAbs may be their specific binding to antigens, this property may not always be necessary for clinical efficacy. For example, there is no correlation between CD33 expression and efficacy of the anti-CD33 calicheamicin conjugate gemtuzumab (101). Gemtuzumab may instead show leukemia-selective cytotoxicity due to the enhanced endocytotic and proliferative activities of leukemic cells. Enhanced endocytosis may

lead to increased gemtuzumab-calicheamicin uptake, and higher rates of proliferation may increase susceptibility to calicheamicin, as observed with the related compound daunorubicin (101).

**Other Causes of Toxicity.** The selectivity that results from these multiple specificity factors has resulted in low toxicity compared with chemotherapy or radiation, a major advantage of mAbs. For example, toxicity is not related to cellular proliferation, and so patients are spared systemic side effects. Besides the antigen-dependent causes of toxicity discussed above, however, mAbs do have common elements of toxicity.

Infusion-related events are common to mAb administration and are believed to be mediated by cytokine release (102). Symptoms include fevers, chills/rigor, hypotension, and dyspnea, occur within hours of infusion, and usually respond to antihistamines and supportive treatments such as albuterol and i.v. fluids. However, in rare cases, roughly 0.5% in one study of rituximab (98), they can be fatal. Development of immunity can cause anaphylactic reactions with later administrations of mAbs, which are managed by discontinuation of therapy, antihistamines, and supportive treatments.

Conjugation to radioisotopes or chemotherapeutic compounds considerably broadens the toxicity profile of mAbs, with clinical dosing limited by systemic toxicity. Ibritumomab and tositumomab exhibit myelotoxicity, which, consistent with the greater radiation penetration of yttrium-90, is more common with ibritumomab than tositumomab. Ibritumomab causes grade 4 neutropenia in 32% and thrombocytopenia in 9% of patients, whereas the rates are 17% and 3% for tositumomab (103). In both cases, onset occurs in 4 to 9 weeks and recovery within a month afterwards. The calicheamicin conjugate gemtuzumab shows less extramedullary toxicity when compared historically with systemic chemotherapy, but can cause hepatotoxicity, leading to hyperbilirubinemia in 23% and veno-occlusive disease in 1% to 5% of patients (104). Hepatotoxicity is believed to reflect nonspecific endocytosis of antibody and conjugate in hepatocytes rather than antigen-mediated effects (101).

Finally, antibodies share with chemotherapy the possibility of inducing tumor lysis syndrome depending on tumor load. For gemtuzumab, leukoreduction with hydroxyurea or leukapheresis is recommended to reduce starting white cell levels to below 30,000/μL (85).

**One Leg Good, Two Legs Better: Combination Therapy with mAbs.** Much recent ongoing clinical research on approved mAbs has been aimed at improving results by combining mAbs with chemotherapy or radiation. The rationale behind this has been 2-fold. First, as mAbs show relatively low toxicity, combination therapy may not increase toxicity significantly beyond chemotherapy or radiation alone. Second, in the case of function-blocking mAbs, extrapolating from *in vitro* evidence, mAbs may increase susceptibility of tumor cells to the effects of chemotherapy or radiation. Data thus far have been supported both hypotheses.

Much effort is currently being devoted to improving chemotherapy results in untreated NHL with rituximab. In the largest trial to examine this question, rituximab plus cyclophosphamide-doxorubicin-vincristine-prednisone chemotherapy was more active than cyclophosphamide-doxorubicin-vincristine-prednisone alone in high-grade diffuse large B-cell lymphoma (overall responses, 82% and 69%) with no additional toxicity (105). In smaller trials, rituximab plus chemotherapy was superior to either alone in low-grade follicular NHL, mantle-cell lymphoma, and B cell chronic lymphocytic leukemia (106). Rituximab can also be used in combination with autologous peripheral stem cell transplantation, where it is believed to purge stem cells of tumor cells (106). The benefit to be gained from combining radioconjugates with chemotherapy can be expected to be less, due to the higher efficacy and toxicity of radioconjugates as monotherapy. Nevertheless, a trial of low-grade NHL with tositumomab plus multi-agent chemotherapy as initial therapy found a 2-year progression-free survival rate of 81%, higher than typical rates with either alone (107). Therefore, across different subtypes of B-cell lymphoma, mAb plus chemotherapy seems superior to either agent alone, with little or no additional toxicity.

*In vitro* evidence suggests that inhibition of HER2 and EGFR signaling by trastuzumab and cetuximab sensitizes tumor cells to chemotherapy and radiotherapy (108, 109). A recent large randomized trial of trastuzumab plus chemotherapy (paclitaxel or an anthracycline and cyclophosphamide) in previously untreated metastatic HER2-positive breast cancer confirmed a superior response rate (42% versus 16%) and longer progression-free and overall survival compared with chemotherapy alone (110). By comparison, trastuzumab alone typically shows response rates of about 15% (111). Trials of trastuzumab combined with other chemotherapeutic agents are still ongoing (112). Cetuximab plus irinotecan in irinotecan-refractory metastatic colon cancer induced response rates of 23%, versus 11% for cetuximab alone (113). The fact that irinotecan plus cetuximab showed activity on tumors refractory to irinotecan alone could be explained by cetuximab increasing tumor susceptibility to irinotecan (113). Cetuximab plus chemotherapy was also superior to chemotherapy alone in small trials of non–small cell lung cancer and head and neck cancer (114, 115), but final interpretation awaits the results of testing cetuximab alone. Trials comparing cetuximab plus radiation to radiation alone are ongoing.

**Future Directions.** Ongoing research with mAb therapeutics are aimed at improving the efficacy of mAbs, whereas expanding their range of applications. Recent findings in pharmacogenetics are raising the possibility of predicting patient responses prior to mAb therapy. Antibodies against immune cell receptors are being tested as immunomodulatory agents to further augment immune responses when combined with other immunotherapeutics. Finally, a variety of conjugates are being developed to allow mAbs to serve as vehicles for tumor targeting of radioisotopes and chemotherapeutic drugs.

**Defining the Target with Pharmacogenetics.** Recent researches have uncovered genetic influences on patient responsiveness to mAb therapy. The 158V polymorphism in the gene for the Fc receptor FcγRIIIa, which enhances receptor binding affinity to IgG1, was associated with improved responses to rituximab as first-line therapy for follicular NHL, with 90% of patients homozygous for 158V showing objective responses at 12 months, compared with 51% of patients homozygous for the more common 158F allele (116). It may eventually be useful to determine FcγRIIIa genotype when deciding among chemotherapy, rituximab, or combination therapy for NHL. These findings also confirm the

importance of NK cell-mediated ADCC as a mechanism of rituximab efficacy *in vivo*.

In addition to the effects of host genotype, the unique genotype of the tumor may also influence tumor susceptibility to mAbs. It is well known that response to trastuzumab in metastatic breast cancer correlates with HER2 expression (117). Given its similar mechanism of action, we might also expect cetuximab efficacy to be correlated with tumor EGFR activity. However EGFR is often activated in tumors not by overexpression, but by mutation (118). Indeed tumor inhibition by a small-molecule EGFR antagonist is not always correlated with EGFR expression (119) but is dramatically associated with mutations in the receptor's cytoplasmic kinase domain that result in enhanced signal transduction (120–122). Thus, understanding of EGFR activation in tumors at the single nucleotide level may be helpful in predicting clinical response. These findings raise the possibility that genetic analysis may be useful in patient evaluation for therapy with anti-EGFR mAbs such as cetuximab as well.

**Boosting mAb Efficacy with Immunomodulation.** Given the central role of immune effector functions in the action of some mAbs, methods to boost immune effector function during mAb therapy might be beneficial. In mouse models of lymphoma, treatment with IL-2 and rituximab increased survival versus either agent alone (123, 124). In several phase I trials of stage III to IV NHL with various histologies, addition of IL-2 to the rituximab treatment regimen correlated with increased NK and NKT cell levels and enhanced rituximab-mediated ADCC by patient leukocytes (124–126). Adverse effects were similar to those observed with rituximab and IL-2 separately, and IL-2 did not increase the severity or incidence of infusion-related events. In one combination protocol, 8 of 13 patients showed clinical responses that correlated temporally with ADCC activity (125). This protocol is being further investigated in a phase II trial.

Effects of immunomodulation on trastuzumab therapy have been investigated using IL-12. In a small phase I trial, IL-12 enhanced patient NK-mediated ADCC activity on trastuzumab-coated tumor cells and cytokine release (127). In this case, clinical responses correlated with cytokine release but not ADCC activity. In a phase I trial of IL-2 and trastuzumab, IL-2 was also capable of enhancing NK-mediated ADCC on trastuzumab-coated cells, but also with no correlation to clinical response (128).

**Antibodies as Immunomodulatory Adjuncts for Other Immunotherapies.** Antibodies that can activate immune system functions may themselves be effective adjuncts in immunotherapy with cancer vaccines. The best studied of immunomodulatory mAbs are those targetting CTLA-4, a T cell-specific inhibitory receptor molecule. In a trial of nine patients previously vaccinated with irradiated melanoma or ovarian carcinoma cells, tumor necrosis was observed in all patients following administration of a CTLA-4-blocking antibody (129). Not surprisingly given the immunosuppressive function of CTLA-4, CTLA-4 blockade resulted in the development of autoantibodies; however, other than a mild erythematous rash no clinical evidence of autoimmune disease was observed. Among other immunomodulatory mAbs in preclinical development are anti-CD25 mAbs for the depletion of inhibitory T cells and anti-4-1BB mAbs for the stimulation of T and NK cells (130). In the latter case, mAbs that can cross-link and activate the costimulatory molecule 4-1BB/CD137 have been characterized. This approach is an interesting example of using mAbs to activate rather than inhibit signal transduction from cell surface receptors and is especially appropriate since the natural 4-1BB ligand is a transmembrane protein and so cannot be directly given.

**Novel Immunoconjugates: Increasing Efficacy, Decreasing Toxicity.** An exciting area of research involves the conjugation of mAbs with novel radionuclides or chemotherapeutic drugs in order to improve efficacy (131). Conjugates of trastuzumab to $\alpha$ emitters are currently undergoing preclinical evaluation (132). Alpha emitters are cytotoxic over shorter ranges than the $\beta$ emitter 90-yttrium or the $\beta$ and $\gamma$ emitter 131-iodine, and so could have less toxicity whereas maintaining effectiveness in micrometastatic disease. A promising novel chemical conjugate is the potent antitumor drug geldanamycin. In one mouse study, trastuzumab-geldanamycin induced tumor regression in 69% of mice versus 7% with trastuzumab alone (133). Like calicheamicin, geldanamycin toxicity prohibits its systemic administration. Geldanamycin requires internalization for activity, and the effectiveness of geldanamycin-mAb conjugates was correlated with the ability of the mAb to mediate internalization (134). Cells lacking the target antigen but sensitive to free geldanamycin were not inhibited by geldanamycin-mAb conjugates *in vitro*, demonstrating specificity of action. How geldanamycin conjugates compare with calicheamicin conjugates in terms of efficacy and toxicity *in vivo* remains to be determined.

Because current immunoconjugates are limited by systemic toxicity, modifications to reduce systemic effects are being investigated. Liver uptake of circulating immunoconjugates is a major source of toxicity, and so one approach is to create linkers that are cleavable by hepatocyte lysosomal proteases in order to accelerate removal of the conjugate and clearance from the liver. Radionuclide immunoconjugates with cathepsin-cleavable linkers showed reduced radioactivity in the liver by 31% to 68% in mice and in patients compared with noncleavable linkers (135, 136).

Another interesting strategy is to add temporal specificity to the spatial specificity of mAbs. One approach is to use mAb-enzyme conjugates to activate systemically given prodrugs specifically at tumor sites (137). In mice, the combination of a carcinoembryonic antigen-glucuronidase conjugate in conjunction with a doxorubicin-glucuronide prodrug was shown to result in intratumor doxorubicin concentrations 4 to 12 times higher and extratumor concentrations five times lower than achievable with free doxorubicin at the maximally tolerated dose (138). Alternatively, mAb-prodrug conjugates can be targeted to tumors, where they are activated by endogenously expressed enzymes (139) or by external stimuli such as light (140). A final possibility is the use of bivalent mAbs with one site binding a tumor antigen and another binding a drug. In an initial pretargeting step, the slow process of antibody binding to tumor can be allowed to progress to completion in the absence of drug. A small diffusible cytotoxic drug can then be given and captured at the tumor site in a shorter second step (141).

These examples illustrate the potential of immunoconjugates to confer utility on drugs and radionuclides that otherwise would be considered too toxic to be used in patients. Immunoconjugation can be seen as both a strategy for improving

the specificity of chemotherapy or radiation and for improving the efficacy of immunotherapy, with the goal of combining the best characteristics of these different modalities.

## CONCLUSION

The history of the development of antibody therapeutics of cancer has been an exciting one, alternating between times of rapid progress and unbridled enthusiasm on one hand and disappointing setbacks and deep pessimism on the other hand. The successful introduction of anticancer antibodies into the clinic within the last decade, however, has firmly established mAb as effective and important components of cancer treatment. The result since has been a productive period of research in which the mechanisms of mAb efficacy have been elucidated, the clinical utility of mAbs expanded, and the technology of mAb making steadily improved. A wave of antibody therapeutics is now making its way through clinical testing, and promises to expand the applications of mAbs to additional tumor types in the next few years. However, as shown by response rates that far short of 100% in most cases, mAbs are still far from being "magic bullets" against cancer. It will be interesting to see the extent to which combinations of mAbs and chemotherapy or radiation can improve clinical responses, and how the new generation of mAb technologies now under development in the laboratory further improve the efficacy and toxicity profiles of mAb therapies.

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