

Rituximab: Mechanism of Action

George J. Weiner

Rituximab is a mainstay in the therapy for a broad variety of B-cell malignancies. Despite its undeniable therapeutic value, we still do not fully understand the mechanisms of action responsible for rituximab's anti-tumor effects. Direct signaling, complement-mediated cytotoxicity (CMC), and antibody-dependent cellular cytotoxicity (ADCC) all appear to play a role in rituximab efficacy. In vitro, animal model and clinical data addressing each of these mechanisms of action are reviewed, as are data speaking to the complexity of interactions between these mechanisms. Taken together, these data suggest different mechanisms are likely important in different scenarios. Study of the complex mechanisms of action that contribute to the clinical efficacy of rituximab have led to novel clinical trials including novel combinations, schedules, and generation of additional antibodies designed to have even greater effect. Such studies need to be accompanied by rigorous correlative analysis if we are to understand the importance of various mechanisms of action of rituximab and use that information to improve on what is already an indispensable component of therapy. *Semin Hematol* 47:115-123. © 2010 Elsevier Inc. All rights reserved.

Rituximab has become a mainstay in the therapy of a broad variety of B-cell malignancies. In some B-cell malignancies, rituximab alone can induce high response rates and long-term remissions,^{1,2} while in others, adding rituximab to chemotherapy enhances the rates of complete response, long-term remission, and cure.^{3,4} Despite its undeniable value as a component of therapy for B-cell malignancies, rituximab is not effective for all patients, and development of resistance to therapy is common. Understanding the mechanisms by which rituximab induces anti-tumor responses is central to our ability to improve on what is already a highly effective therapy.

We know that anti-cancer monoclonal antibodies (mAbs) can mediate anti-tumor effects by a variety of mechanisms, including signaling resulting in cell cycle arrest, direct induction of apoptosis, and sensitization to cytotoxic drugs, complement-mediated cytotoxicity (CMC) and antibody-dependent cellular cytotoxicity (ADCC). Ideally, we would study these, and any other, mechanisms of action of rituximab using experimental conditions that reflect clinical therapy. In reality this is more easily said than done. As a single agent, rituximab is usually administered weekly for 4 weeks. When used in combination with chemotherapy, it is often admin-

istered every 3 to 4 weeks. The pharmacokinetics of rituximab is similar to that seen with human IgG.⁵ Thus, whether given weekly or monthly, rituximab is present at therapeutic levels in the circulation of patients for months at a time. As an IgG, rituximab distributes in both the intravascular and extravascular compartments, and so should be present within involved lymph nodes with their complex architecture in an environment that includes not only malignant B cells but also stromal cells, benign lymphocytes, extracellular matrix, vasculature, proteins in the extravascular fluid, and a complex mixture of cytokines and chemokines.

In vitro studies allow for the rapid, rigorous, and focused evaluation of specific mechanisms of action. However, the conditions we have available in the research laboratory vary significantly from the real-world clinical environment. Studies of rituximab mechanisms of action often utilize tumor cell lines that have been selected based on their ability to grow rapidly in vitro, and sometimes their relative sensitivity to therapy. Effector cells, when present, are usually not syngeneic and often come from normal donors, not patients with malignancy. In vivo, lymphocyte behavior changes within seconds of cells being exposed to hypoxic conditions.⁶ It takes minutes to hours to harvest, wash, and otherwise manipulate peripheral blood cells for in vitro analysis. Obtaining malignant lymphocytes from lymph nodes involves much more drastic manipulation, and often they are cryopreserved and then thawed before analysis. These manipulations surely have effects on their response to therapy. Our best in vitro assays involve incubation times of minutes (analysis of direct sig-

Holden Comprehensive Cancer Center, University of Iowa, Iowa City, IA.
Address correspondence to George Weiner, MD, Holden Comprehensive
Cancer Center, University of Iowa, 200 Hawkins Dr, 5970 JPP, Iowa
City, IA 52242-1002. E-mail: george.weiner@uiowa.edu
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naling effects of rituximab) to hours (cytotoxicity assays), but never weeks or months—the time frame of clinical response to rituximab. Finally, *in vitro* assays usually focus on one mechanism. Most studies of CMC do not include immune effector cells, and studies of ADCC do not have functional complement, thereby preventing these analyses from informing us about interactions between mechanisms.

Animal models come one step closer to reflecting the clinical situation but also have significant limitations. Such models traditionally involve mice that have been inoculated with malignant cell lines. Resulting tumors differ from clinical lymphoma with respect to growth kinetics, phenotype, infiltrating benign cells, and heterogeneity. Many models utilize immune compromised animals as hosts, and xenografted human tumors. Furthermore, experimental conditions (tumor burden at time of therapy, dosing of therapy, etc) are usually selected with an eye towards enhancing our ability to detect a therapeutic response as opposed to understanding the mechanisms responsible for that therapeutic response. Each of these factors impacts on our ability to relate animal model results to clinical mechanisms of action.

Clinical trials and correlative assays are extremely valuable as they involve measurement of what is happening in patients. However, the vast majority of clinical trials are designed to assess efficacy of therapy, not understand mechanisms of action. This restricts our ability to manipulate conditions in a way that explores specific mechanisms. Correlative laboratory evaluation associated with clinical trials has proven to be extremely valuable, but even when informative, usually leads to a demonstration of a correlation rather than causation—they are more often hypothesis-generating than hypothesis-testing.

These limitations do not invalidate the significance of the information gained from such research but need to be taken into account when interpreting experimental results. This review will discuss what we know, and don't know, about the mechanisms of action of rituximab. It has been organized based on proposed mechanism of action, with a description of data available from *in vitro*, *in vivo* and clinical trials for each proposed mechanism. The primary mechanisms by which rituximab likely mediates its anti-tumor activity do not occur independently. The final sections of this review involve discussion of possible interactions between mechanisms, and how these interactions might impact on the efficacy, or resistance to, rituximab-based therapy.

SIGNALING-INDUCED CELL DEATH

In Vitro Studies

In the absence of immune effector mechanisms, rituximab can induce death of malignant B cell lines *in vitro*. The strength of this effect varies considerably

between target cell lines.⁷⁻¹⁰ Signaling mediated by cross-linking of CD20 appears to be related to functional reorganization of CD20 into lipid rafts. Changes that have been identified in response to rituximab *in vitro* include inhibition of p38 mitogen-activated protein kinase, nuclear factor- κ B (NF- κ B), extracellular signal-regulated kinase 1/2 (ERK 1/2), and AKT anti-apoptotic survival pathways. Development of *in vitro* resistance to rituximab is not associated with genetic changes in the CD20 molecule but has been found to be associated with downstream changes in signaling.¹¹ The selection of CD20-resistant clones in these studies was done in the absence of immune effector mechanisms (complement or cells capable of mediating ADCC); thus whether these changes also result in clinical resistance to rituximab remains unclear.

Detection of signaling changes in response to rituximab often requires cross-linking of the rituximab with secondary antibodies. The affinity of anti-rituximab antibodies for rituximab is much higher than the affinity of rituximab Fc for FcR, and there is considerable cross-linking because of the bivalent nature of the cross-linking IgG. This artificially enhances the strength of the signal. It is not clear whether cross-linking of rituximab serves to speed up and strengthen the physiologic effect of rituximab signaling so it can be measured in a short assay, or provides such a strong signal that nonphysiologic changes are induced that may not be relevant clinically.

Sample processing itself induces signaling changes, making the study of signaling in primary lymphoma samples extremely difficult. To avoid this predicament, most signaling assays involve use of cell lines that are very different from primary malignant cells. Chronic lymphocytic leukemia (CLL) cells require considerably less manipulation than do lymphoma cells harvested from nodes, and signaling in CLL cells in response to rituximab has been reported.¹²

In vitro synergy between rituximab and cytotoxic chemotherapy has been demonstrated.¹³ Clones grown to be resistant to rituximab *in vitro* also show resistance to cytotoxic chemotherapy suggesting common mechanisms may be at play.¹⁴ Vega and colleagues recently demonstrated that both intact rituximab and modified rituximab, which no longer fixes complement or mediates ADCC, inhibit cell survival/growth. The modified antibodies sensitized lymphoma cells to both cisplatin and Fas ligand-induced apoptosis.¹⁵ Evaluation of the effects of such combinations have been among the most valuable *in vitro* assays exploring rituximab mechanisms of action, and are particularly relevant given clinical evidence that such combinations are effective.

Overall, *in vitro* studies suggest rituximab-induced signaling can contribute to the anti-tumor effect of therapy and can synergize with cytotoxic therapy.

Animal Model Studies

Animal models carry the potential advantage of allowing for prolonged exposure of malignant cells to rituximab in an environment where the cells are growing in a physiologic environment. Synergy has been observed in animal models of rituximab and chemotherapy^{16,17} and these studies have helped provide impetus for clinical evaluation of such combinations; however, they add little to our understanding of rituximab mechanisms of action as either single agents or in combination with chemotherapy.

Clinical Studies

Indirect evidence suggests that anti-CD20 therapy can result in regression of B-cell lymphoma in a manner that is not dependent on robust immune participation. The earliest such evidence comes from the first studies of radiolabeled anti-CD20 therapy. Subjects were preloaded with cold (unradiolabeled) antibody to improve biodistribution of the radiolabeled antibody. Unexpectedly, some subjects demonstrated shrinkage of lymphoma after the cold antibody but before the radiolabeled antibody was given.¹⁸ The cold antibody used in this study was a murine IgG2a (B1), demonstrating that even a murine IgG antibody, with its relatively poor ability to interact with human Fc receptors, can have anti-tumor activity.

Rituximab injected directly into the cerebrospinal fluid in patients with central nervous system (CNS) lymphoma has been reported to have local anti-lymphoma effects.¹⁹ The lack of significant levels of complement or cells capable of mediating ADCC in the CNS would suggest a direct effect of rituximab on the malignant cells. On the other hand, a small study suggests the therapeutic effect of CNS administration of rituximab can be augmented by concomitant injection of serum into the CNS as a source of complement.²⁰

The overall concept that rituximab will enhance the efficacy of chemotherapy may seem counterintuitive if one assumes that the mechanism of action of mAb therapy is based on activation of immune effector mechanisms. However, clinical data in a variety of B-cell malignancies provide strong evidence that rituximab and chemotherapy can work well together.^{3,4,21,22} One potential explanation for the success of these regimens that include immune effectors is that chemotherapy enhances the sensitivity of malignant B cells to immune-mediated lysis. However, Fc γ RIIA (CD16) and Fc γ RIIA (CD32) polymorphisms in follicular lymphoma patients treated with rituximab plus cyclophosphamide, vincristine, doxorubicin, and prednisone (CHOP) chemotherapy did not predict clinical outcome.²³ These negative data provide circumstantial support for the hypothesis that direct signaling either induces apoptosis in chemosensitized cells or sensitizes the malignant cells to cytotoxic therapy when rituximab is given along with chemotherapy.

Signaling-Induced Cell Death—Conclusions

Taken together, there is indirect but convincing evidence extending from in vitro analyses through clinical trial correlative studies that signaling effects of rituximab on B cells independent of additional immune effector mechanisms can contribute to the anti-tumor activity of rituximab. The strongest such evidence is the clear demonstration that the combination of rituximab and cytotoxic chemotherapy is effective in a variety of B-cell malignancies. Indeed, many investigators are evaluating novel approaches to combining rituximab with cytotoxic chemotherapy in vitro, in animal models and in clinical trials based on the synergistic effects of rituximab signaling and cytotoxic therapy.

COMPLEMENT-DEPENDENT CYTOTOXICITY

In Vitro Studies

Several studies have demonstrated in vitro that rituximab is highly efficient at mediating CMC of various B-cell lines, as well as fresh malignant B-cell samples. The expression of complement inhibitory molecules (CD55 and CD59) on malignant B cells correlates with the extent of in vitro lysis.^{24–29} Follicular lymphoma is more sensitive to rituximab clinically, and follicular lymphoma cells are more effectively lysed by complement in vitro when compared to cells from subjects with large cell lymphoma or mantle cell lymphoma.³⁰ These studies generally use serum as a source of complement, which speaks to the importance of CMC in the circulation, but tells us little about whether complement plays a role in the anti-tumor activity of rituximab within lymph nodes or at other extravascular sites. To begin assessing this issue, we recently evaluated whether CMC is observed when rituximab is added to transudative pleural fluid or ascites that served as surrogates for extravascular fluid. Although the concentration of various components of the complement cascade was lower in these fluids than in plasma, there was enough complement in these fluids to mediate CMC³¹ and to suggest that CMC could be contributing to the anti-tumor activity of rituximab in the extravascular, as well as intravascular, compartments.

Animal Model Studies

A number of in vivo tumor models suggest the anti-tumor activity of rituximab is dependent, at least in part, on complement.^{32,33} Depletion of cellular effectors in these models had no effect on the therapeutic response to rituximab, while depletion of complement through use of cobra venom factor abolished the therapeutic response. However, these models involved use of rituximab (a chimeric humanized IgG) in mice, which would require interaction between a murine target cell, human IgG, and murine complement cell if

CMC were to take place. In addition, the target cells express low levels of complement inhibitor molecules. Complement activation was recently found to play a role in antibody-induced infusion toxicity in both a rodent model and non-human primates. Use of antibodies modified to have a reduced ability to fix complement induced fewer infusion reactions. In the rodent model, this reduction in infusion reaction had little effect on anti-tumor activity.³⁴ These data support the contention that complement fixation contributes to infusion reactions, although they also confirm that infusion reactions are not good predictors of a therapeutic response to antibody.

Clinical Studies

Clinical observations demonstrate complement is activated during treatment with rituximab in some but not all patients³⁵ and that complement activation correlates with infusional toxicity.³⁶ CLL cells that remain after rituximab treatment have been found to have a higher surface expression of the complement inhibitor CD59 when compared to pretherapy expression of this marker.^{37,38} This suggests selective elimination or trafficking out of the circulation of CLL cells expressing lower amounts of CD59 that might be more sensitive to CMC. However, no correlation has been found between expression of complement inhibitors and clinical response to rituximab treatment.²⁹ Thus, complement consumption mediated by rituximab may not translate into anti-tumor efficacy.

CMC—Conclusions

CMC can clearly result in rapid cell death of rituximab-coated target cells, and is a prime mechanism of action for antibody therapy in some animal models. There are considerable data suggesting CMC occurs in patients after rituximab therapy, and that complement fixation plays a role in infusion reactions. What is less clear is the degree to which CMC contributes to the clinical anti-tumor response to therapy, and whether it is active against cells that are outside the intravascular compartment. Overall, the evidence that CMC contributes to the clinical efficacy of rituximab has been seen as strong enough to encourage the clinical development of next generation anti-CD20 antibodies with an enhanced ability to fix complement,³⁹ and infusion of plasma during rituximab treatment to replenish complement.⁴⁰ Whether such approaches will be clinically superior to rituximab therapy remains to be seen.

ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY

In Vitro Studies

mAbs can induce ADCC mediated by a variety of effector cells, including natural killer (NK) cells, gran-

ulocytes, and macrophages.^{41,42} These processes require that the Fc of the antibody bound to the target cell bind to Fc γ receptor (Fc γ Rs) on the effector cells triggering immune cell activation and death of the target cell.⁴³ Rituximab can induce ADCC of human lymphoma cell lines by human peripheral blood mononuclear cells.²⁴ However, in vitro detection of ADCC with rituximab and other antibodies often involves very high, nonphysiologic, effector:target cell ratios, often in the 25–50:1 range. Immune effector cells are often pre-activated with cytokines such as interleukin-2 prior to the ADCC assay. It is not clear whether these manipulations serve to speed up ADCC so it can be measured in a 4-hour time frame, or provide for killing via a mechanism that is not relevant clinically.

Most research exploring ADCC and rituximab has focused on interactions of rituximab with CD16 on NK cells; however, other Fc receptors, including CD32,²⁵ CD64,⁴⁴ and CD89,⁴⁵ may also contribute to anti-CD20-mediated ADCC. Expression of these receptors, and ADCC mediated by cells expressing them, is enhanced by the presence of a number of different cytokines including interferon gamma (IFN γ), which can be produced by activated NK cells. Thus, it is conceivable that CD16 plays a role in activating NK cells locally, and that the resulting cytokines produced by NK cells enhance ADCC mediated by other receptors and other effector cells.

Animal Model Studies

As outlined above, CMC is the central mechanism of action in some animal models of rituximab therapy. In others, CMC appears to play a limited role and ADCC is most important.⁴⁶ Perhaps the strongest evidence for the importance of ADCC in animal models comes from the work of Clynes and colleagues, who found that antibody was effective in wild-type mice but not in mice lacking the common Fc γ R chain.⁴⁷ Furthermore, the anti-tumor effect of mAb therapy was enhanced in mice lacking Fc γ RIIb, which is an inhibitory receptor,⁴⁸ providing additional evidence that the interactions between the antibody and FcR is central to determining the efficacy of therapy.

Clinical Studies

The most convincing evidence that ADCC is mechanistically involved in clinical response to rituximab therapy comes from correlative studies demonstrating an association between polymorphisms on CD16 (also known as Fc γ RIIIa) and clinical response to single-agent rituximab. CD16 homozygous for valine at 158 (VV) has a higher affinity for IgG1 than does CD16 with phenylalanine at that position (VF or FF).⁴⁹ Patients with follicular lymphoma with the VV genotype have a better clinical response to rituximab than patients with the VF or FF genotype.^{50,51} The same polymorphism is

also predictive of rituximab response in patients with Waldenström's macroglobulinemia.⁵² Follicular lymphoma patients homozygous for histidine at 131 (HH) on Fc γ RIIa also have an improved response to rituximab.⁵¹ These data highlight the importance of Fc-Fc γ R interactions in the anti-tumor effects of rituximab and suggest that ADCC is a major mechanism of action. They also point towards NK cells, the principal cells that express CD16, as being key contributors to the anti-tumor activity of mAbs. However, not all data indicate these polymorphisms are clinically relevant. CD16 polymorphisms did not predict clinical response to rituximab in CLL patients⁵³ or follicular lymphoma patients treated with rituximab plus CHOP.²³

This strong, if indirect, evidence that the interaction between rituximab Fc and CD16 is central to the mechanisms of action of rituximab has led to the development and clinical evaluation of novel strategies for enhancing target cell lysis by NK cells, including the addition to rituximab of immunostimulatory agents designed to activate NK cells,^{54,55} and development of anti-CD20 antibodies with stronger affinity for CD16.^{43,56} Thus far, none of these strategies has been unequivocally successful, but evaluation is ongoing.

ADCC—Conclusions

In vitro, animal model and correlative clinical studies suggest that interaction of antibody Fc with CD16 contributes to the clinical anti-tumor activity of single-agent rituximab. The most obvious interpretation of these findings is that NK cells are mediating ADCC of rituximab-coated target cells. However, there are alternative explanations. For example, NK cells are activated and produce IFN γ when they come in contact with rituximab-coated target cells.⁵⁷ This IFN γ could have direct anti-tumor effects on the malignant cells, or could be activating other immune effector cells that could contribute to ADCC. Indeed, IFN γ is known to upregulate the high-affinity Fc γ receptor (CD64) on granulocytes.⁵⁸ Whether the contribution of NK cells is direct or indirect will be difficult to discern. However, clinical trials comparing rituximab to next generation anti-CD20 antibodies that have an enhanced ability to bind to Fc Fc γ R and activate NK cells will help us understand the clinical importance of ADCC in the anti-tumor activity of rituximab.

INTERACTING MECHANISMS

The discussion above addresses the major mechanisms of action of rituximab independently. In fact, results of a number of studies point to more than one mechanism playing a role sensitivity or resistance to therapy including an increase in complement inhibitory molecules, decreased expression of CD20, and enhanced expression of anti-apoptotic molecules.^{59,60}

There are also extensive interactions—both synergistic and antagonistic—between these mechanisms of action. A mechanism that may contribute to the anti-tumor activity of rituximab in one clinical scenario may actually inhibit the therapeutic response in another. Different mechanisms may contribute in different ways at different sites and times within an individual patient.

An excellent example is complement. The influence of the complement system on the immune response goes beyond traditional CMC. Complement can impact on B-cell activation directly⁶¹ and C3 has been shown to have a direct inhibitory effect on NK cell function.⁶² Clearance of apoptotic bodies by complement may impede development of an active immune response.^{63,64} This may help explain our apparently paradoxical finding of a correlation between polymorphisms in the C1q component of complement (A *v* G at position 276) and duration of response to single-agent rituximab.⁶⁵ In this study, subjects with a polymorphism associated with decreased protein expression had a better outcome. More specifically, we found prolonged remission among subjects that were carriers of the A allele, which is associated with lower C1q levels and less biologic C1q activity, when compared to homozygous G subjects who have more biologically active C1q.

The presence of immune complexes on the surface of cells does not always lead to CMC. Taylor and colleagues have demonstrated that monocytes and macrophages may “shave” rituximab-complement complexes off the surface of CLL cells,^{66,67} resulting in viable malignant cells that no longer have surface antigen on their surface. Whether this phenomenon is limited to situations such as CLL where opsonized cells circulate through the liver and spleen, or also takes place in the lymph nodes, remains to be seen.

We recently reported that NK cell activation and ADCC induced by rituximab-coated target cells are inhibited by the C3b component of complement.⁶⁸ The epitope to which C3b binds to IgG Fc is very close to the epitope on the IgG Fc that binds to CD16. Thus, complement fixation may actually impede rituximab binding to CD16 and ADCC. We have also found that depletion of complement can actually enhance the efficacy of antibody therapy in a murine model.³¹

Complement depletion, systemically or locally, could result in loss of CMC, or alternatively loss of the inhibitory effect of complement on other mechanisms.⁶⁶ Taken together, CMC can result in anti-tumor activity in some situations, but this may be offset by shaving of the immune complex from the surface of viable malignant cells, or inhibition of ADCC through C3b blockade of the interaction between rituximab and NK cells. Thus, complement may be a positive contributor to therapy in some circumstances while having negative effects in others (Figure 1).

The dynamics of interaction between such mechanisms are extremely complex given the differences in microenvironment, saturation of receptors and possi-

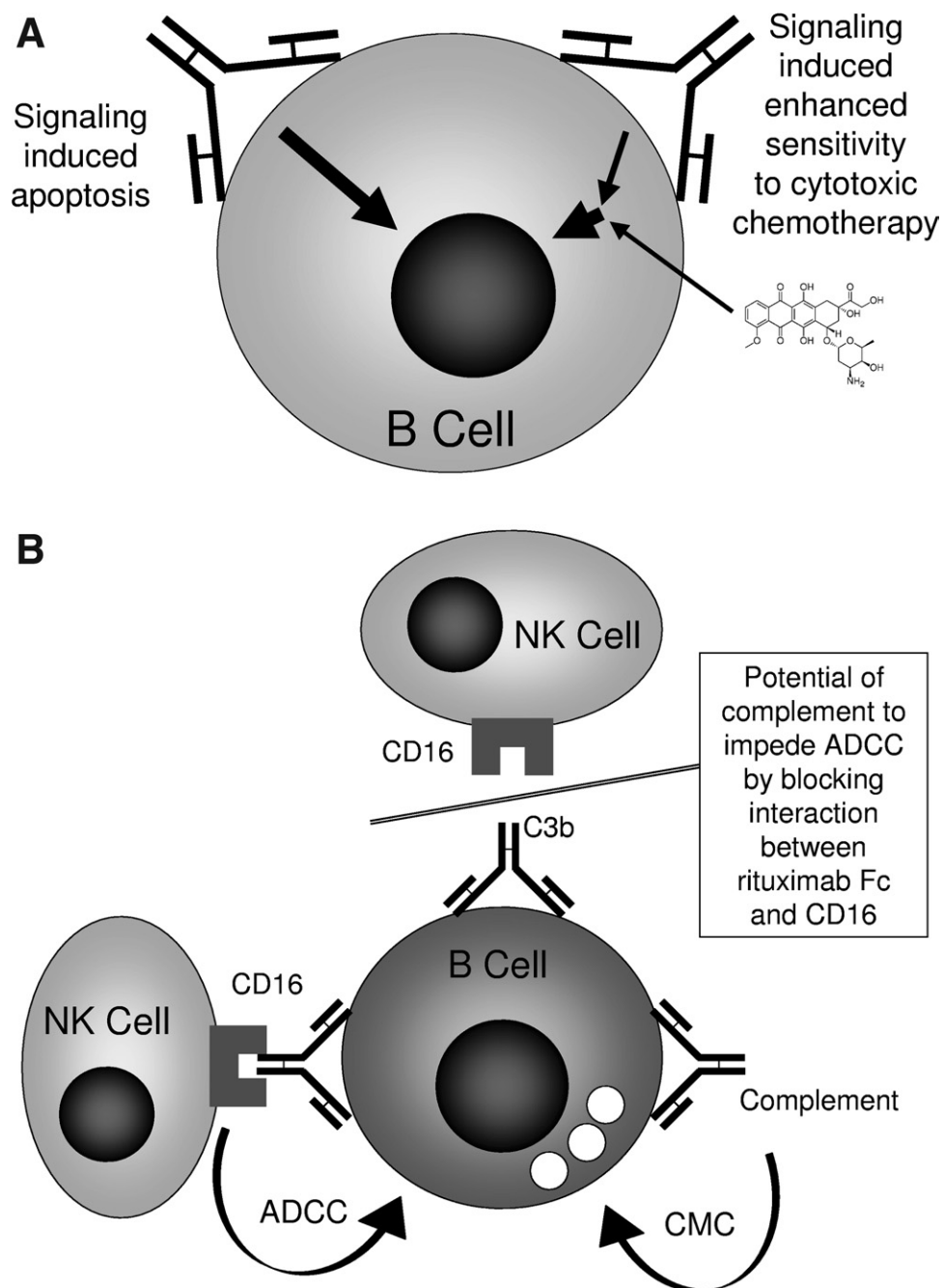


Figure 1. (A) Mechanisms of action related to direct rituximab-induced signaling. (B) Mechanisms of action related to rituximab-induced CMC and ADCC with potential that these two mechanisms can be antagonistic.

ble exhaustion of proteins or cells vital for effective target cell destruction. This could well explain many of the apparently conflicting results discussed above. Rituximab concentration, immune effector cell infiltration, FcR saturation, and complement depletion in the local microenvironment could all impact on whether a given mechanism is active. Mechanisms that are important in one setting may be less important in another, even in the same patient at different times or locations. This review has focused on the mechanisms of action of rituximab. There is growing evidence that not all

anti-CD20 mAbs are alike.⁶⁹ Class I mAbs, including rituximab, and class II anti-CD20 mAbs, such as tositumomab, vary in how they cross link CD20. When compared to class I anti-CD20, class II anti-CD20 appear to result in less movement of CD20 into lipid rafts, increased signaling-induced cell death, decreased CMC, and possibly increased ADCC.⁷⁰ Thus, findings related to the relative importance of rituximab mechanisms of action and how they interact may not be applicable to all anti-CD20 mAbs. Whether class I or class II mAbs are superior clinically remains to be determined.

CONCLUSION

The data presented here suggest that rituximab-mediated signaling, CMC, and ADCC all contribute to rituximab's anti-tumor activity. Given this complexity, where do we go from here? We are unable to say "This is it!" with respect to a single mechanism being central to clinical response to rituximab. However, the ongoing evaluation of mechanisms of action has led to new rituximab-based combinations, novel schedules, and the design of the next generation of antibodies discussed at length elsewhere in this issue of *Seminars in Hematology*. Preclinical validation of novel strategies is being followed by clinical evaluation of the most promising approaches. Clinical trials based on these data include evaluation of antibodies with an enhanced ability to signal, mediate CMC, and mediate ADCC. Such studies need to be accompanied by rigorous correlative analysis, and will continue to be central to our ability to understand the importance of various mechanisms of action of rituximab in different settings. This knowledge can be used to improve on what is already an indispensable approach to therapy. The results related to an enhanced understanding of mechanisms of action could well be applicable beyond rituximab therapy of B-cell malignancies and impact on treatment of other cancers and benign disorders.

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