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# Antigenic Modulation and Rituximab Resistance

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## Abstract

Several types of B cell lymphoma have been successfully treated with rituximab (RTX), and approval by the FDA for use of RTX in the treatment of rheumatoid arthritis has increased interest in targeting CD20 on B cells for other indications. Although large amounts of RTX can be infused into humans with no apparent dose-limiting toxicity, recent evidence suggests that the body's effector mechanisms, including complement-mediated cytotoxicity and NK cell-mediated killing, can be saturated or exhausted at high burdens of RTX-opsonized B cells. One of the consequences of this saturation phenomenon is that the opsonized B cells are instead processed by a different pathway mediated by FcγR on effector cells. In this alternative pathway both RTX and CD20 are removed ("shaved") from the B cells and are taken up by monocytes/macrophages. This process, formerly called antigenic modulation, appears to occur in several compartments in the body and may play a key role in the development of resistance to rituximab therapy.

## INTRODUCTION

The mechanisms of B cell resistance to rituximab (RTX)-mediated cytotoxicity, can, like much of immunology, be divided into innate and adaptive components. Innate components include expression of CD20 at levels below the thresholds required to trigger RTX-mediated cytotoxicity, and the activity and level of complement control proteins on malignant cells.[1–4] A patient's innate cytotoxic effector mechanisms will play critical roles, as demonstrated by differences in efficacy which correlate with polymorphisms in C1q and polymorphisms in and effector cell densities of FcγR.[5–9] In addition, as noted by Smith,[10] differences in resistance of cells in different compartments "could reflect differential access to antibody or to effector mechanisms," emphasizing the importance of tumor microenvironments.

Treatment with RTX does not destroy all malignant B cells, and over the long term (periods in excess of one year) surviving B cells appear to acquire an adaptive resistance to later RTX therapies,[11,12] although in many cases the malignant cells still express CD20. The surviving cells may take up residence in compartments in which they will be resistant to later attack by RTX, but there is no evidence for or against this possibility. Over the short term, that is, at the time of RTX treatment, there are several adaptive mechanisms by which targeted cells can escape RTX-mediated cytotoxicity. As noted below, deposition of C3 activation fragments on RTX-opsonized cells can block binding to FcγRIIIa on NK cells, inhibiting antibody-dependent cellular cytotoxicity (ADCC).[13] Although not yet formally proven for RTX, there is evidence that sublytic complement attack on nucleated cells induces Ca2+ fluxes, leading

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to protein synthesis and at least temporary upregulation of defenses against complement-dependent cytotoxicity (CDC).[14] RTX treatment may acutely exhaust effector mechanisms responsible for killing IgG-opsonized substrates. Under these conditions an alternative processing pathway which removes RTX/CD20 complexes from B cells predominates, thus insuring the resistance of CD20-negative cells to RTX therapy. Table I summarizes some of the mechanisms by which resistance to RTX develops and also presents potential therapeutic strategies to overcome this resistance. In this review we will focus our discussion on the loss of efficacy of RTX treatment due to blockade or saturation of host effector mechanisms.

## CYTOTOXIC MECHANISMS: VARIATIONS ON IMMUNE COMPLEX PROCESSING

Seven years ago our laboratory initiated a clinical study in chronic lymphocytic leukemia (CLL), to determine whether infusion of RTX promotes complement activation and deposition of C3 activation fragments on circulating CLL B cells.[15] We had demonstrated that in vitro binding of RTX to B cells in the presence of complement leads to deposition of C3 fragments on the cells; our findings in primates indicated that within minutes of RTX infusion, C3 fragments were deposited on RTX-opsonized cells.[3,16] In both cases, C3b/iC3b was co-localized with cell-bound RTX. In the clinical study, we found that after infusion of only 30 mg of RTX, C3 fragments were demonstrable on circulating B cells. However, an unexpected observation set the stage for future investigations: The number of circulating CLL B cells decreased considerably after infusion of 30 mg but then substantially increased after infusion of the remaining ~600 mg RTX (~ 5 hours later). Although the final concentration of RTX in the bloodstream (>100 ug/ml) was considerably higher than the concentration after infusion of the first 30 mg (<5 ug/ml), it appeared that the cytotoxic action of RTX had been saturated or exhausted. That is, B cells were not cleared from the bloodstream with a high level of efficacy. This finding has been quite durable (Table II); we observed this pattern in all of our studies of CLL patients receiving single agent RTX therapy in doses of ≥100 mg.[17] These observations dramatically emphasize the need for detailed analyses of the cytotoxic mechanisms of RTX in different compartments in the body and in the bloodstream in particular.

An overwhelming body of evidence indicates that RTX-mediated cytotoxicity must rely on the body's immune mechanisms.[1–3,9,15,18–23] These cytotoxic reactions are initiated by recognition of the RTX-CD20-B cell immune complex, either by FcγR on effector cells (NK cells and monocyte/macrophages) or by C1q, the first component of the classical pathway of complement. Recognition of IgG–opsonized substrate cells by FcγRIIIa on NK cells can promote ADCC.[18,24,25] IgG-opsonized cells can also bind to macrophages via several different FcγR, setting in motion cytotoxic pathways that lead to ADCC and phagocytosis.[19,26] The classic studies of Frank and Schreiber, which examined bloodstream clearance mechanisms mediated by the mononuclear phagocytic system (MPS), revealed that binding of a few thousand IgG molecules to a circulating cell such as an erythrocyte (E) leads to its rapid clearance due to recognition by FcγR on tissue macrophages in the liver (Kupffer cells) and spleen.[26,27] Studies of polymorphisms in CD16 (FcγRIIIa), first reported by Cartron et al. and confirmed by others[5–7], indicate that individuals homozygous for the higher affinity binding FcγRIIIa allotype (158 Valine) have considerably better responses to RTX therapy. This result underlines the importance of ADCC and phagocytosis in the RTX mechanism.

IgG-opsonized cells can bind C1q, promoting several consecutive steps in the classical complement activation pathway, including covalent deposition of C3 activation fragments on the cells, followed by CDC due to assembly and penetration of the cell membrane by numerous copies of the cytolytic membrane attack complex (MAC).[23] RTX can mediate CDC of B cells that express high levels of CD20. The results in certain mouse models,[28,29] as well as the report of Racila et al.,[8] which correlated the response of patients with follicular lymphoma to polymorphisms in C1q, all suggest that CDC may play a role in the therapeutic action of RTX. However, primary B cells from most CLL patients are refractory to RTX-mediated CDC due to relatively low levels of expression of CD20. Upon infusion of RTX, circulating CLL cells that are not lysed become heavily opsonized with C3 fragments;[3,17] these C3 fragments may promote clearance of the opsonized cells by the MPS, due to uptake mediated by complement receptors on fixed cells.[30,31] Conversely, several lines of evidence developed by Weiner and colleagues suggest that C3 fragments bound to RTX-opsonized B cells may actually interfere with NK cell-promoted ADCC due to C3b/iC3b-mediated blockade of access of NK cell CD16 to the Fc region of cell-bound RTX.[13,32] Thus, it will be important to determine the relative impact of C3 fragment deposition in targeting RTX-opsonized circulating cells to the MPS compared to the effect of complement activation on sessile, non-circulating cells in tumors.

These potential problems may be overcome with second generation anti-CD20 mAbs such as ofatumumab. Ofatumumab mediates CDC of B cells more effectively than RTX, most likely due to increased binding of C1q to opsonized cells.[33] Indeed, ofatumumab makes much *more efficient use of complement* than does RTX and can, in the presence of limited amounts of complement, mediate much higher levels of C3b deposition and CDC than does RTX in the presence of much larger amounts of complement.[33–35] Based on these observations, treatment of patients with high B cell burdens with rituximab is more likely to consume complement due to inefficient cell killing compared to treatment with comparable amounts of ofatumumab, and future clinical studies should allow for evaluation of this prediction.

## SATURATION and/or EXHAUSTION

A key question is whether the body's effector mechanisms associated with immune complex processing, complement activation, phagocytosis and/or ADCC, can be saturated or exhausted, leading to persistence and proliferation of malignant B cells that would otherwise be eliminated. That is, the B cell burden may be so high that although large doses of RTX saturate CD20 on targeted B cells in different body compartments, the necessary effector mechanisms within these compartments are not adequate to effectively kill targeted cells.

Insight into this question may be drawn from studies in systemic lupus erythematosus (SLE). SLE is an autoimmune disease characterized by chronic proliferation of large quantities of immune complexes in the circulation and in tissues.[36–38] The kinetics of clearance from the circulation of a model particulate immune complex, $^{51}$Cr-labeled IgG-opsonized E, are reduced substantially in SLE, likely due to competition by circulating immune complexes.[36] These IgG-containing complexes can bind to FcγR on monocytes/macrophages, and then the immune complexes, along with FcγR, are internalized. This process appears to down-modulate expression of FcγRII and FcγRIII, compromising the activity of monocyte/macrophages in clearing IgG-opsonized particles.[38]

In another example of saturation and exhaustion, Bowles and Weiner reported that NK cell-mediated ADCC of RTX-opsonized cells comes at a price, in that CD16 (FcγRIIIa) on the NK cells is severely down-regulated.[39] The consequence of down-regulation is inhibition of killing of additional RTX-opsonized B cells until CD16 can be re-expressed. In vitro experiments reported by Bhat and Watzl suggest that one NK cell can kill at most 4 IgG-opsonized cells.[40] In vivo findings of Beredeja et al. indicate that after NK cell-mediated ADCC, re-expression of CD16 on the NK cell and restoration of full cytotoxic activity may require 24 hours or more.[41] Varchetta et al. reported that as a consequence of NK cell-mediated ADCC of trastuzumab-opsonized cells, CD16 on the NK cells is down-modulated, suggesting that NK cell-mediated ADCC of other targets may also be subject to saturation.[42] In the future, anti-CD20 mAbs with Fc regions engineered to maximize ADCC may provide increased efficacy and methods to increase NK cell cytotoxicity and/or numbers are under development.[43–47]

Treatment of immune thrombocytopenia (ITP) with IgG anti-E antibodies (anti-D) makes explicit use of saturation of effector mechanisms.[48] This treatment leads to modest decreases in the hematocrit due to clearance of IgG-opsonized E; however, the decoy IgG-E immune complexes saturate the clearance capacity of the MPS, sparing IgG-opsonized platelets and providing effective therapy for certain patients.[49] Part of this saturation phenomenon is likely due to down-modulation of FcγRIII. Song et al. examined a mouse model for this system and reported that as a result of targeting E by infusion of specific IgG antibodies, splenic FcγRIII was down-modulated.[50] It is likely that splenic FcγRIII was internalized coincident with phagocytosis of IgG-opsonized E.

The complement system can be exhausted as a consequence of RTX treatment. RTX-opsonized B cells at densities seen in CLL (100,000 B cells per ul) substantially depleted complement activity in serum.[15] Although the cells were saturated with bound RTX, CDC was reduced considerably compared to CDC seen for lower cell burdens, where complement activity was adequate. The first CLL patient we studied had a moderate burden of circulating B cells (< 20,000 per ul), yet RTX treatment led to massive depletion of complement immediately after the first infusion.[15] His complement titer was partially restored one week later, but after the second weekly RTX treatment (in a 4-week cycle) complement remained low for more than a month, suggesting that the RTX-B cell immune complexes, in several compartments in addition to the bloodstream, were continuing to activate and deplete complement. Complement titers were decreased in other CLL patients who received RTX therapy; the time for restoration of full complement activity after RTX treatment was variable, from days to weeks.[15] Therefore we suggested that fresh frozen plasma as a complement source may enhance RTX therapeutic activity,[15,51] and recent clinical studies, although not well controlled have provided some evidence in favor of this paradigm.[52] However, infusion of two units of fresh frozen plasma may not be adequate to provide substantial long-term restoration of complement titers, and additional studies will be required to determine if this approach has therapeutic utility.[52]

## EXHAUSTION CAN RESULT IN INCREASED RESISTANCE: SHAVING

High levels of RTX-opsonized B cells can saturate effector mechanisms that would otherwise promote RTX-mediated cytotoxicity. Under these conditions the RTX/CD20 complexes on B cells will be subject to an alternative processing pathway, the “shaving reaction.”[25,53] In this process RTX and CD20 are removed from opsonized cells in a reaction mediated by FcγR on acceptor cells, but the cells are not killed! This phenomenon was originally described in other systems as antigenic modulation: loss of expression of surface antigens after binding of antibody.[54,55] Loss of the target of infused mAb T101 (CD5) on circulating malignant T cells was reported by Bertram et al. more than 20 years ago.[54] The shaving reaction resembles trogocytosis (from the Greek, to gnaw or nibble), in which formation of an immunological synapse leads to removal and internalization of ligands associated with donor cells by cognate receptors on acceptor cells. Trogocytosis has been documented for B cells, T cells, neutrophils and NK cells.[56]

By definition, macrophages are “big eaters” and might not be expected to engage in trogocytosis. However, macrophages can make use of FcγR, especially FcγRI, to endocytose small IgG-opsonized substrates by taking them into the cell without surrounding them.[57–59] Therefore macrophages and other cells that express FcγR may be capable of either wholesale phagocytosis and/or ADCC (most likely mediated by FcγRII and FcγRIII[38]), or trogocytosis, in which the target cell-bound RTX (the ligand for FcγR) and CD20 are removed and internalized by the acceptor monocyte/macrophage but the target cell is left alive and intact.[56] Our investigations of low-dose RTX for treatment of CLL (see below) suggest that phagocytosis and shaving of RTX-opsonized B cells occur simultaneously. That is, some RTX-opsonized cells that enter the liver are effectively bound to Kupffer cells and phagocytosed.

However, other RTX-opsonized cells may pass through the gauntlet of Kupffer cells and escape with some loss of bound RTX and CD20. We have recently obtained additional evidence that macrophages can mediate either shaving or phagocytosis. After RTX-opsonized CD20-positive mouse 38C13 B cells[29] were incubated with adherent mouse peritoneal macrophages, the cells were separated and examined. Macrophages had either phagocytosed entire cells, or had trogocytosed small amounts of RTX and plasma membrane from the B cells. Moreover, the recovered B cells had lost CD20 (Lindorfer and Taylor, unpublished observations).

The effector cell phenotype may influence the outcome of an encounter with a target cell. THP-1 cells and freshly isolated monocytes promote shaving of RTX-opsonized B cells, but do not mediate ADCC or phagocytosis.[53] On the other hand, NK cells promote ADCC of RTX-opsonized cells, and also take up RTX and CD20 from the cells.[25] This process will lead to down-regulation of NK cell CD16, thus suppressing/exhausting its ability to execute ADCC.[39] Neutrophils, which express several FcγR, also mediate shaving of RTX-opsonized cells, but in the absence of complement activation and/or other mediators, they do not kill the cells.[25]

It is an oversimplification to assign all phagocytosis/ADCC to FcγRII and FcγRIII, and all trogocytosis to FcγRI. However, we suggest this provides a reasonable paradigm to explain the *transition* from clearance and destruction of RTX-opsonized cells to shaving that occurs when CLL patients with high burdens of circulating cells are treated with standard doses of RTX (Table II). The large burden of B cells cleared from the circulation ($\sim 2 \times 10^{11}$) after infusion of only 30 mg of RTX will likely saturate the phagocytic capacity of liver Kupffer cells ($\sim 2 \times 10^{10}$) as well as the ADCC capacity of circulating NK cells ($\sim 1 \times 10^{9}$) and in many cases complement activity will be reduced.[9,15,60] After RTX-opsonized B cells are cleared, CLL B cells will re-equilibrate into the circulation from other compartments,[61] and be opsonized by RTX. However, based on the SLE immune complex model[38] as well as the ITP model,[48,50] FcγRII and FcγRIII on tissue macrophages and FcγRIII on NK cells will be substantially reduced, but FcγRI on macrophages and monocytes will be available to promote shaving. The result is that cells which enter the circulation after the first round of clearance will not only escape ADCC and phagocytosis by the MPS, but these cells will also have CD20 removed by the shaving reaction. This adaptive resistance may allow them to escape targeting by RTX and proliferate.

Therefore, we initiated a pilot clinical trial in CLL to evaluate the effects of much lower, more frequent (thrice-weekly) doses of RTX on the biology of circulating malignant B cells.[17] The testable hypothesis was that at RTX doses of ~35 mg (20 mg/m$^2$), the plasma concentration of RTX would be quite low (< 5 ug/ml) after the first round of clearance of opsonized cells. As additional CLL cells entered the circulation from other compartments, these B cells would not be opsonized by RTX, and cell-associated CD20 would be preserved. It would then be possible to target circulating cells with an additional low RTX dose two days later, when the body's effector systems, previously saturated after the first RTX dose, would have recovered and could then mediate additional clearance of opsonized B cells. In five out of six patients who received the 20 mg/m$^2$ dose, CD20 was largely preserved and in 4 of 6 patients additional RTX infusions continued to promote clearance of malignant cells. This trial tested mechanisms leading to loss of CD20 on CLL B cells, and the low dose paradigm may not be adequate for targeting B cells that do not re-equilibrate into the bloodstream.[17] However, these results, taken in context with our findings in CLL patients who received RTX doses >100 mg (60 mg/m$^2$), provide compelling evidence that the body's effector mechanisms responsible for clearing RTX-opsonized cells can be saturated, thus sparing a population of RTX-opsonized cells. This phenomenon evolves into a perfect storm: CD20 will be removed from RTX-opsonized cells, allowing them to proliferate under conditions which support continued shaving of re-expressed CD20. On the basis of these considerations it is not surprising that single agent RTX therapy at standard doses has modest efficacy in CLL.[1] Finally, in collaboration with A. Wiestner and his colleagues, we have found that subcutaneous delivery of lower, more frequent doses of RTX has the potential to be an effective and convenient paradigm for CLL treatment.[62]

## SHAVING OUTSIDE THE BLOODSTREAM

The clinical success of RTX in non-Hodgkins lymphoma is well-documented, suggesting that RTX therapy is more effective at killing B cells in tissues than in the bloodstream.[9,10] However, approximately 50% of patients treated with single agent RTX do not respond, and responding patients often suffer relapses one to two years later. It is therefore important to ask how effective are the body's effector mechanisms in tissue compartments, and, can these mechanisms also be saturated or exhausted?

It is certainly reasonable that CD20-negative B cell lymphomas can develop long after RTX therapy, likely due to outgrowth/mutation of a population of CD20-negative cells.[63] However, several immunohistochemical investigations have described CD20-negative B cells (positive for CD79a, CD19, and other B cell markers) that appeared within a few months or even days after completion of RTX infusions.[63–71] Under these conditions, RTX was likely present in the circulation and tissues. These studies made use of reagents that would reveal CD20 even in the presence of bound RTX, thus precluding simple steric blockade as an explanation of the findings. In many of these reports, B cells lacking CD20 were demonstrable in tumors of patients with B cell lymphomas after RTX treatment.

RTX therapy is also used for other indications including rheumatoid arthritis (RA) or to eliminate B cells before kidney transplants.[70,71] In these investigations B cells were cleared from the bloodstream for long periods. However, in the RA study, B cells lacking CD20 were demonstrable in the bone marrow and synovium. In the transplant study, splenectomies were performed 3–13 days after RTX infusion. B cells that were CD79a-positive but CD20-negative were found in the spleens of patients who received RTX infusions ≥35 mg/m$^2$. The results of these two investigations suggest that although bloodstream clearance was effective due to the large capacity of the MPS to clear "normal" levels of opsonized B cells, cytotoxic mechanisms within tissue compartments responsible for eliminating normal B cells may have been exhausted. We suggest that a substantial fraction of the B cells in tissues had lost CD20 due to the shaving reaction. In principle it should be possible to test this hypothesis by sterile culture of isolated B cells for a few days in the absence of RTX, to allow for CD20 to be re-expressed. Examination of the levels of CD16 on monocyte/macrophages and NK cells within tissue compartments in which shaving is suspected could be quite informative. If CD16 were down-regulated, this would be consistent with the overall working hypothesis we have presented.

## CONCLUSIONS and FUTURE DIRECTIONS

As we have noted in the introduction, there is increasing evidence that long after patients with B cell lymphomas are successfully treated with RTX, they suffer relapses and in many cases their disease is refractory to additional RTX treatments that include chemotherapy, although their B cells do express CD20.[11,12] The reason(s) for this apparent adaptive RTX resistance remain unknown, and it is not unreasonable to speculate that one or more of the short-term mechanisms that allow cells to chronically escape RTX-mediated cytotoxicity may play key roles in this phenomenon. The elucidation of these long-term resistance "pathways" constitutes a significant challenge not only for anti-CD20 therapies, but also likely with respect to other mAb-based therapies in the treatment of cancer. Many different approaches for abrogating resistance to immunotherapy are currently under investigation (Table I).

Our discovery of the shaving reaction (or perhaps rediscovery of antigenic modulation) was based on clinical observations in CLL. Additional clinical studies as well as in vitro experiments have provided important new insights into how RTX-opsonized B cells are either killed by or escape the body's immune effector mechanisms. RTX is now used in combination with a variety of chemotherapeutic regimens, with a higher level of clinical efficacy, and therefore mechanisms of resistance will change. However, it is likely that many of the lessons learned in analyzing innate and adaptive mechanisms of resistance of targeted cells to RTX will be applicable toward understanding the action of other immunotherapeutic mAbs.[9]

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## Table I

Mechanisms of Resistance to Rituximab Therapy and Potential Therapeutic Strategies to Overcome Resistance

| Mechanism | Potential Therapeutic Strategies | Ref |
|---|---|---|
| • **Loss of epitope** | | |
| Permanent loss of CD20 (i.e. outgrowth of a CD20-negative clone) | Target alternative epitope (e.g. CD52) | 11,64 |
| Temporary mAb-mediated loss of CD20 (i.e. Shaving) | Lower, more frequent doses of mAb; IVIG or other agents to block FcRγI | 15,17,62,72 |
| • **Reduced ADCC** | | |
| Exhaustion of ADCC | Lower, more frequent doses of mAb; increase activity or number of NK cells | 39–41,46,47 |
| Blockade of ADCC by deposited C3 activation fragments | Engineer mAbs, or adjust the dose in order to maximize ADCC while minimizing C3 fragment deposition | 13,32 |
| Polymorphisms in effector molecules (i.e. FcγR) | Screen polymorphisms prior to therapy; engineer Fc region of mAbs to maximize ADCC for all patients | 5–7,43–45 |
| • **Reduced CDC** | | |
| Increased surface expression of complement control proteins | Block the action of CD55 or CD59 | 73 |
| Exhaustion of CDC (i.e. temporary depletion of complement components) | Infusion of fresh frozen plasma; lower, more frequent doses of mAb. | 15,17,52 |
| Sublytic C attack leading to increased resistance to CDC | mAb designed to maximize C activation and thereby minimize sublytic C attack | 14,33,35 |
| • **Tumor microenvironment** | | |
| Protective factors in tumor microenvironment | Mobilize malignant cells to periphery; attack stromal factors | 74–76 |

## Table II

Key Measurements on CLL Blood Samples, Before, During and After RTX Infusion

| | **Before treatment** | **After 30 mg** | **After 375 mg/m$^2$** |
|---|---|---|---|
| Normalized lymphocyte count | High[a] 100% | Low 20% | Intermediate 60% |
| Plasma RTX concentration | 0 | 1–5 ug/ml | >100 ug/ml |
| CH50 | Normal | Normal | Depleted (10–90%) |
| CD20/cell, normalized | 100 | 2–10 | 2–10 |
| C3 fragments deposited | No | Yes | Yes |

[a] Pretreatments levels varied from 5,000–100,000 lymphocytes/uL.[15,17]