Making Rituximab Directly Cytotoxic for Substantial Improvement in Therapeutic Efficacy

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Abstract

The humanised anti-CD20 antibody (Ab) rituximab (RTX) has significantly improved the prognosis of B cell non-Hodgkin's lymphomas (BNHL). However, major challenges remain: a) RTX is often used with toxic chemotherapy that not only causes serious side effects but may also compromise RTX activity and host antitumour immunity, predisposing patients to relapse; b) indolent low-grade BNHL remain largely incurable; c) a significant percentage of aggressive BNHL do not respond to RTX-based therapy; and d) a significant number of responders may eventually relapse in long-term follow-up. The data suggest that the limit in the efficacy may result from the inability of RTX to directly kill lymphoma cells. RTX primarily relies on indirect mechanisms to attack lymphoma cells, which include complement-dependent cytotoxicity, Ab-dependent cellular cytotoxicity, induction of apoptosis, and immune activation. These mechanisms could be readily compromised by various situations, such as chemotherapy. The new generation of anti-CD20 Ab have not been found to be directly cytotoxic. Cytotoxic radioactive isotope-conjugated anti-CD20 Ab appeared to be highly effective, but serious radiotoxicity prohibited their clinical application. Increasing Ab valency augments activity; a recent study has demonstrated drastic improvement in activity by non-covalently associating RTX with nanomaterial graphene oxide (GO). The multivalent Ab product RTX/GO is highly cytotoxic, capable of directly killing BNHL cells *in vitro* and rapidly eliminating established xenograft lymphoma *in vivo* in the absence of toxic chemo-agents. While further studies are needed to determine the mechanism of activity and clinical efficacy, the current data suggest a significant possibility that RTX/GO might constitute nontoxic but effective therapy for BNHL.

INTRODUCTION

The non-Hodgkin's lymphomas (NHL) are the most common haematological malignancies in adults, with approximately 85% of NHL of B cell origin expressing the specific B cell marker CD20. CD20 is a transmembrane protein of undefined function[1] and its expression is B cell maturation-regulated. The prototype therapeutic anti-CD20 antibody (Ab) is rituximab (RTX), a chimeric Ab composed of a human IgG1 heavy-chain constant region and murine Ig variable region that is specific for CD20. The first therapeutic monoclonal Ab (mAb) to receive U.S. Food and Drug Administration (FDA)

approval in 1997, RTX has been routinely used for treatment of almost all types of B cell NHL, whether indolent or aggressive. Addition of RTX to standard chemotherapy substantially enhances response to therapy and improves overall outcomes, which makes RTX therapy the most noticeable advance in lymphoma treatment over the past decades. Despite this remarkable success, major challenges remain: a) a significant percentage of patients with aggressive NHL are refractory to RTX-containing regimens;[2] b) the long-term survival of patients with aggressive lymphomas is still limited (with 10-year progression-free survival approximately 35% in high-risk patients;[3,4] c) most indolent lymphomas remain incurable;[5] and d) RTX relies heavily on chemotherapy to achieve optimal therapeutic outcome, but chemotherapy is often associated with toxic adverse effects, which may compromise RTX activity and antitumour immunity, as discussed below.[6] Continuous efforts have been made in an attempt to generate new anti-CD20 Ab with efficacy superior to RTX, but the new Ab do not seem to be fundamentally improving therapeutic outcome yet. The main reason for this appears to be that all anti-CD20 Ab are not directly cytotoxic to lymphoma cells but rely primarily on indirect effector mechanisms to attack lymphoma cells. In settings where the indirect effector mechanisms are absent, consumed, or compromised, such as in patients receiving toxic chemotherapy, the activity of anti-CD20 Ab can be disrupted.

Making anti-CD20 Ab directly cytotoxic to lymphoma cells may circumvent the dependence on indirect mechanisms and fundamentally enhance and sustain Ab activity. In this article, the mechanisms of RTX action and resistance, the features of newer generations of anti-CD20 mAb, and the therapeutic advantages of directly cytotoxic antibodies are reviewed.[7]

MECHANISMS OF RITUXIMAB ACTION AND RESISTANCE

The mechanism of RTX action is the subject of active study. While controversies remain, all the data demonstrate that RTX targets lymphoma cells through multiple mechanisms. Despite the multiple activities, the therapeutic capacity of RTX as a monotherapy appears rather limited.[8] The reason for this appears to be, as discussed below, that RTX targets lymphoma cells mostly via indirect mechanisms that could readily be compromised by various situations, including chemotherapy.

Complement-Dependent Cytotoxicity

RTX can activate the complement system by binding C1q to the Fc region of the Ab, generating a membrane attack complex to disrupt the plasma membrane and kill target cells. Components of the activated complement system can also act as opsonins by binding receptors on phagocytes and natural killer (NK) cells to activate Ab-dependent cell-mediated cytotoxicity (ADCC). While *in vitro* experiments have demonstrated complement-dependent cytotoxicity (CDC) in RTX activity,[9,10] controversies remain whether *in vivo* CDC is required, sufficient, or unnecessary to mediate therapeutic effects of RTX.[11,12] In patients with chronic lymphocytic leukaemia (CLL), RTX infusion results in rapid and profound depletion of complement components,[13] suggesting that complement depletion may contribute to observed RTX treatment failure.[14] Alternatively, CDC does not appear to be similarly active in follicular NHL, which is nodal, as opposed to CLL, which is predominantly bone marrow and peripheral blood-based. Genetic polymorphisms in the gene coding for C1q have been linked to variations in RTX efficacy,[15] and Ab-resistant cells surviving RTX therapy have been reported to express high levels of complement-regulatory proteins (mCRP), which inhibit complement action.[16-18] Therefore, the activity of RTX-induced CDC varies depending on patients, lymphoma type, and treatment duration, and can be compromised under various circumstances. CD20 downregulation is also reported as a mechanism of acquired RTX resistance. The primary mechanism for CD20 downregulation is believed to result from the shaving or stripping of CD20 from the cell surface by macrophages.[18] This phenomenon is particularly relevant during the binding of RTX to CD20, and it does not eliminate lymphoma cells because of absence or exhaustion of the host effector mechanisms such as CDC and ADCC. Making RTX directly cytotoxic might overcome this resistance mechanism.

Antibody-Dependent Cellular Cytotoxicity

RTX can also activate ADCC attack on CD20+ cells through Fcγ receptor-bearing effector cells, such as NK cells, granulocytes, and macrophages. Activation of ADCC by RTX has been established by *in vitro* experiments.[19] In murine models, depletion of normal B cells by RTX was reported to be dependent on FcγRI and FcγRIII; B cell depletion did not occur in FcγR-deficient mice, supporting an *in vivo* role for ADCC.[12] In humans, single nucleotide polymorphisms (SNP) in *FCGR3A* (low affinity immunoglobulin gamma Fc region receptor III-A) with substitution of either a valine (V) or phenylalanine (F) residue at position 158 of the FCγRIIIa receptor can substantially impact ADCC. Cells bearing Fc receptor homozygous for V (158V/V) have a higher *in vitro* affinity for IgG1 compared to cells with the 158V/F or 158F/F receptor,[20] showing higher response rates to RTX in NHL patients with the 158V/V receptor as compared to patients with 158V/F or 158F/F receptor.[21-23] The polymorphisms have no prognostic significance in patients treated with chemotherapy alone,[24] suggesting ADCC as an effector mechanism for anti-CD20 therapy. However, studies have not found an impact of the *FCGR3A* genotype on outcomes in B cell CLL treated with RTX,[25] suggesting that the clinical contribution of ADCC depends on the characteristics of the underlying malignant cells.

In addition to genetic variations and lymphoma types, other conditions affect ADCC. For instance, activated complement components, such as C3b, inhibit NK cell-mediated ADCC.[26] A common mechanism to disrupt ADCC is chemotherapy, which causes cytopenia with loss of effector cells of both myeloid and lymphoid lineages, including NK cells, granulocytes, macrophages, and T cells. Given that the FcγR-bearing cells are required for ADCC, loss of these effector cells in patients with cytopenia can be expected to halt RTX-mediated ADCC.

Induction of Apoptosis

RTX has very limited, if any, capacity to directly kill target cells in culture.[7] While studies have reported induction of a degree of apoptosis to some malignant B cell lines in culture, this often requires plate-coated RTX or the presence of a second Ab crosslink Ab or FcγR to crosslink with RTX.[27-29] Even with plate-immobilised RTX, the most vigorous form of crosslinking, the extent of cell death is limited, especially with cells of aggressive lymphomas, e.g., diffuse large B cell lymphoma (DLBCL) line SU-DHL-4.[29] While studies have reported CLL cell apoptosis in patients receiving RTX treatment, it is unclear whether indirect mechanisms are involved in the induction of apoptosis.[30] Discrepancies also remain regarding the mechanism of RTX-induced apoptosis. Some studies have suggested a central role of caspase in the induction of apoptosis,[30] while others have shown caspase-independent apoptosis.[31-33]

It is noteworthy that while brief exposure to RTX downregulates B cell lymphoma (BCL)-2 expression, sensitising cells to apoptosis and chemotherapy,[34] prolonged RTX exposure inhibits expression of the pro-apoptotic BCL-2 family proteins BAX and BAK, leading to resistance not only to apoptosis but also to multiple antineoplastic agents.[35,36] RTX is typically administered over a number of weeks in the induction therapy, followed by multiple cycles of therapy every 8 weeks for up to 12 doses. While the intensive therapy protocol might play an important role in improving the response and durability of RTX treatment, it may also contribute to induction of resistance, as indicated in the long-term follow-up studies.[3]

Immune Activation

In vitro experiments have shown that RTX facilitates the uptake and cross-presentation of apoptotic Daudi cell antigen to cytotoxic T lymphocytes (CTL).[37] Whether RTX therapy similarly promotes antigen presentation and activation of specific CTL *in vivo* in patients remains to be established. It is unclear whether antitumour immunity in patients is generally compromised as a result of the intensive chemotherapy administered along with RTX. Antitumour immunity is now known to play a critical role in the prognosis of cancers, including lymphomas.[38-40]

The Adverse Impact of Chemotherapy on Rituximab Activity

Despite the proposed multiple antilymphoma mechanisms of RTX, the therapeutic capacity of RTX as a monotherapy appears limited.[8] Patients treated with RTX alone showed very low response rates, especially for aggressive lymphoma, which is often fatal in months if untreated effectively. As a result, RTX has generally been used as adjuvant therapy in combination with chemotherapy consisting of multiple toxic chemotherapeutic agents for optimal therapeutic outcome, such as in CHOP (cyclophosphamide, doxorubicin [hydroxydaunomycin], vincristine [Oncovin®, Cellpharm GmbH, Hanover, Germany], and prednisolone), the most commonly used regimen.[8] While intensive CHOP substantially increases response rate to RTX, it is often associated with toxic side effects, including life-threatening cytopenia.[41] Cytopenia not only predisposes patients to high infection risk but can also disrupt RTX activity. For instance, ADCC might be compromised due to lack of effector cells. Induction of apoptosis might be impaired due to loss of FcγR-bearing cells that are required for RTX crosslinking. Lymphopenia can also seriously weaken immune activation function of RTX because of T cell deficiency.[6] Lymphopenia could have significant long-term adverse sequela because T cell recovery could be very slow, especially in adult patients, due to thymic involution.[42] A number of studies have demonstrated close relationships between lymphopenia and high incidence of relapse of lymphomas, including DLBCL,[6,43] implicating the role of antitumour immunity in preventing recurrence. Therefore, implementation of toxic chemotherapy to RTX therapy might not only compromise RTX's original activity but also the antitumour immunity of the hosts, predisposing patients to delayed lymphoma relapse. Novel strategies are needed to improve RTX efficacy in the absence of toxic chemotherapy.

THE NEWER GENERATIONS OF ANTI-CD20 ANTIBODIES

There are several new-generation anti-CD20 mAb engineered to provide advantages over RTX, including ofatumumab, ocrelizumab, veltuzumab, ocaratuzumab, and ublituximab. These mAb induce potent CDC but relatively weak ADCC, similar to RTX, and are categorised as type 1 Ab. Ibritumomab tiuxetan and obinutuzumab (OBZ) are regarded as type 2 Ab because of stronger ADCC relative to CDC. Compartmentalisation and redistribution of CD20 into lipid rafts is believed to be the mechanism behind Type 1 Ab induction of intensive strong Fc clustering and complement activation.[44] The Type 2 Ab do not redistribute CD20 into lipid rafts.

Ofatumumab is at the most advanced stage of clinical development. Ofatumumab binds to a unique CD20 epitope, giving rise to a slow off-rate and a high capacity for complement activation. Ofatumumab-induced CDC in RTX-resistant CLL cells and lymphoma cell lines that express high levels of complement defence proteins and/or low levels of CD20.[45] In clinical trials, ofatumumab showed improved response rates and progression-free survival, but benefits are still limited. For treatment of follicular and DLBCL, chemotherapy is still required.[46,47] If enhancing CDC does not improve anti-CD20 mAb efficacy and RTX resistance is largely dictated by a failure of immune effector cell function, it would seem unlikely that these novel mAb will result in substantial improvements over RTX.

OBZ has a glycoengineered Fc region that includes nonfucosylated oligosaccharides that interact with FcR, particularly FcRIIIa, which enhances ADCC.[48] It has features of a Type 2 anti-CD20 mAb in evoking nonapoptotic programmed cell death in addition to not having a strong ability to translocate CD20 into lipid rafts for complement activation.[49] Studies reported superior tumour growth inhibition compared with RTX in lymphoma xenograft models,[50] and greater B cell depletion than RTX in nonhuman primates and hCD20 transgenic mice. OBZ is approved for CLL and follicular lymphoma. Despite reports of potent capacity to induce strong ADCC and nonapoptotic programmed cell death, OBZ relies on chemotherapy for optimal outcome. While OBZ-based immunochemotherapy and maintenance therapy resulted in longer progression-free survival than RTX-based therapy in follicular lymphoma, the improvement appeared limited.[51] The dependence on

chemotherapy might compromise OBZ-mediated ADCC. If enhancing ADCC does not improve anti-CD20 mAb efficacy because of chemotherapy-induced failure of effector cells, the importance of having cytotoxic anti-CD20 Ab capable of eliminating lymphoma cells in the absence of chemotherapy will be heightened.

Radioisotope-Labelled Anti-CD20 Antibodies

Radioisotope-labelled anti-CD20 Ab had the potential to be good examples of directly cytotoxic anti-CD20 Ab. Two such Ab have been produced: ^{90}Y-labelled ibritumomab tiuxetan and ^{131}I-labelled tositumomab.[52,53] Both Ab were FDA-approved for the treatment of relapsed or refractory follicular lymphoma or transformed B cell NHL. These Ab were highly effective even when used as monotherapy during the treatment of lymphomas refractory to salvage immunochemotherapy at relapse, demonstrating the unmatched potency of cytotoxic anti-CD20 Ab. However, neither Ab is in clinical application because of serious radiation-derived side effects, including bone marrow suppression, severe and prolonged cytopenia, and the sequelae of cytopenia.[54,55] Tositumomab and I^{131} tositumomab were discontinued by the manufacturer in February 2014 and are no longer available.

OTHER ANTI-CD20 MODIFICATION APPROACHES

Other anti-CD20 modifications have been comprehensively reviewed in recent literature.[56] Bispecific Ab, such as anti-CD20/CD3 or anti-CD20/NKGD2L, act through nonspecific activation of conventional or gamma/delta T cells initiating an attack on lymphoma cells. Toxin-combined anti-CD20 can directly attack,[57] but because of utilisation of only one CD20-specific single-chain, the avidity of the bispecific Ab for CD20 might be substantially diminished. Various approaches have been taken to generate hypervalent anti-CD20 Ab without using second crosslinking Ab, FcγR, or plate immobilisation. Tetravalent anti-CD20 Ab are generated by genetic engineering.[58] The dock-and-lock method has been used to produce hexavalent anti-CD20 antibodies.[59] More recently, a two-step method using pretargeting component (anti-CD20 Fab' conjugated with an oligonucleotide-1) and a subsequent crosslinking component (N-[2-hydroxypropyl] methacrylamide [HPMA] grafted with multiple complementary oligonucleotide-2) has also been reported to generate multivalent anti-CD20 Ab.[60] All these different forms of hypervalent anti-CD20 Ab demonstrate enhanced direct antilymphoma activity in culture in the absence of cross-linking antibodies as compared to the native Ab counterparts, which is manifested as enhanced antiproliferative and apoptosis-inducing activity. However, direct cytotoxicity is not reported. In animal experiments using severe combined immunodeficiency mice, both the dock-and-lock and two-step pretargeting components generated multivalent anti-CD20 Ab and inhibited lymphoma progression in the absence of chemotherapy.

ANTIBODY–GRAPHENE OXIDE COMPLEX

More recent studies have reported drastic enhancement of RTX as well anti-HER2 Ab trastuzumab activity by non-covalently associating Ab with nanomaterial graphene oxide (GO).[7,61]

Noncovalent Binding Between Rituximab and Graphene Oxide Generates Stable Rituximab/Graphene Oxide Complex with Marked High Avidity for CD20

GO is the thinnest nanomaterial available. Composed of a single-atom-thick nanosheet, GO has recently attracted intense interest within research investigating drug delivery. GO is nontoxic at low concentrations, but can cause oxidative stress, rupture of liposomes, disrupt the integrity of bacterial cell membranes, and kill cancer stem cells at high concentrations. When mixed in low salt solution, RTX and GO form a stable complex at 37°C, which no longer dissociates in physiological solutions such as phosphate-buffered saline and serum.[7] Despite the fact that the RTX forms a complex with GO through a stochastic process, the random interaction between the two does not interfere with RTX reactivity with CD20. The RTX/GO

complex demonstrated substantially enhanced CD20-binding capacity as compared to free RTX. While the mechanism behind the substantial increase in binding capacity is not yet fully understood, the data suggest that RTX interacts with GO through the Fc region with the Fab region uninterrupted, and the multivalent nature of RTX/GO gives rise to high avidity.[7]

Rituximab/Graphene Oxide Complex is Directly Cytotoxic to Malignant B Cells

When Raji cells, which are known to be resistant to apoptosis, were cultured with RTX/GO they underwent rapid cell death, determined by trypan blue and microscopic cell counting, Cell Count Kit, LIVE/DEAD stain, and electron microscopy. Annexin V staining of RTX/GO-killed cells did not detect significant staining, and DNA electrophoresis did not identify apoptotic DNA fragmentation, indicating nonapoptotic cell death (necroptosis, as determined in unpublished experiments). In line with these findings, the pan-caspase inhibitor Z-VAD-FMK could not rescue RTX/GO-treated cells. In contrast, RTX/GO-killed cells show rapid loss of plasma membrane integrity.[7] Complement is not required for RTX/GO-mediated cytotoxicity, indicating RTX/GO directly kills lymphoma cells. Similarly, potent cytotoxicity was also demonstrated on other CD20+ cells, including Daudi (Burkitt lymphoma), SUDHL-4 and SUDHL-8/9 (DLBCL), primary lymphoma cells from a patient with CLL, as well as primary B lymphocytes from healthy donors. When the capacity of RTX/GO to activate complement was examined, RTX/GO had much weaker activity to activate complement as compared to free RTX. As complement activation is believed to be responsible for infusion-related side effects,[62] the weak complement activation function of RTX/GO might be beneficial. Therefore, association of RTX with GO confers potent, direct cytotoxicity to CD20+ lymphoma cells, which is not observed for any of the previously reported anti-CD20 Ab formulations.

The Mechanism of Rituximab/Graphene Oxide Complex-Mediated Cytotoxicity

It remains incompletely understood how RTX/GO kills target cells. The results show that the highest valence of RTX in RTX/GO gives rise to the strongest cytotoxicity.[7] As CD20 crosslinking causes CD20 capping, and reorganisation of the actin network is required for capping,[63] the actin polymerisation inhibitor, latrunculin B (LatB), was tested in a RTX/GO-cytotoxicity assay. LatB completely abrogated RTX/GO-induced cell death, indicating that the actin network is involved in the cytotoxicity.[7] Anti-major histocompatibility complex Class I (HLA-A/B/C) and Class II (HLA-DR) mAb W6/32 and L243 were also used to generate mAb/GO complexes. HLA-DR is a lipid raft-associated protein whereas HLA-A/B/C are located outside of lipid rafts.[64] W6/32/GO induced limited Raji cell death; on the other hand, L243/GO killed the target cells as extensively as RTX/GO.[7] These results suggest that lipid raft-associated proteins are involved in RTX/GO-mediated activity. Given the previous report that the actin network plays a role in controlling location and function of lipid raft proteins, the protective activity of LatB might result from interruption of relocation of lipid-raft proteins. Calcium (Ca) influx is required for RTX/GO-mediated killing as Ca^{2+} chelator alleviates the cytotoxicity. RTX/GO also induces strong reactive oxygen species in the target cells and blocking reactive oxygen species production prevents the cell death (unpublished data). All the results indicate that potent cytotoxicity of RTX/GO results from a combined action of CD20-crosslinking-induced intracellular activity and biological activity of GO.

Rituximab/Graphene Oxide Complex Rapidly Eliminates Established Burkitt Lymphoma *in Vivo*

The therapeutic potential of RTX/GO *in vivo* was studied in immunodeficient NODrag$^{ko}\gamma^{ko}$ (NRG) mice that were intravenously transplanted (Burkitt lymphoma) with Raji cells. As NRG mice are deficient in T, B, and NK cells along with a defective complement system and macrophage activity,[65] they constitute an ideal animal model for the evaluation of RTX/GO therapeutic activity in the absence of host effector mechanisms such as CDC and ADCC. To determine whether RTX/GO could eliminate established lymphoma, the treatment was not initiated until the 8th day of Raji cell transplantation, when extensive lymphoma infiltrates were identified in the bone marrow

and liver.[7] The mice were treated intravenously every 2–3 days for a total of four treatments. When analysed 3 days after the last treatment, extensive lymphoma infiltrates were identified in the bone marrow of the PBS, GO, and RTX-treated animals, but not in RTX/GO-treated mice. The Raji cell counts in the bone marrow of GO and RTX-treated mice were similar to that of PBS-treated mice. Infiltrating lymphoma was identified in all the PBS, GO, and RTX-treated mice but not in any of the RTX/GO-treated mice. No pathological abnormalities nor any evidence of morbidity was identified in the RTX/GO-treated mice.[7] The *in vivo* results demonstrate that RTX/GO has the capacity to diffuse out of the blood circulation, penetrate through the tissue to reach target cells, and rapidly eliminate established lymphomas in the absence of host effector mechanisms.

DISCUSSION AND CONCLUSION

The findings with RTX/GO demonstrate a new strategy to substantially improve therapeutic activity of RTX. While CD20-crosslinking-induced cell death has been reported to be apoptotic, RTX/GO kills by necroptosis. Necroptosis might provide a therapeutic advantage because faulty apoptotic pathways often contribute to therapy resistance. The serum half-life of RTX/GO may be shortened compared to that of free RTX, according to previous reports;[66,67] however, an extended serum half-life may not be necessary for the RTX/GO therapeutic effect, as RTX/GO appears to eliminate lymphoma rapidly. Elimination of established lymphoma in the NRG hosts is unique to RTX/GO therapy. The results suggest effectiveness of RTX/GO therapy in patients with compromised host effector mechanisms. More importantly, the independence on toxic chemo-agents of RTX/GO therapy implicates protection of the host immune cells that are critical in antitumour immunity and maintenance of durable remission. Clearly, much is yet to be understood regarding RTX/GO, including the mechanism of cytotoxicity, pharmacological kinetics, side effects, and clinical efficacy. In a more recent study, GO-associated anti-HER2 Ab complex, trastuzumab/GO, demonstrated the ability to directly kill *HER2^{lo}* cancer cells,[61] similar to RTX/GO. Therefore, the data taken together indicate that noncovalent association of antitumour Ab with GO might constitute a highly effective methodology to enhance efficacy.

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