CD20-targeted therapy: a breakthrough in the treatment of non-Hodgkin’s lymphoma

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ABSTRACT

Targeting the CD20 antigen on B lymphocytes with the monoclonal antibody rituximab has greatly improved the outcome of patients with B-cell malignancies. Despite the success of rituximab, resistance occurs in about half of the patients, resulting in non-response to treatment or early relapse of the original disease. A better understanding of the mechanism of rituximab resistance has lead to the development of novel, improved anti-CD20 antibodies. This review describes the development of CD20-targeted therapy from its historical background towards the next generation of anti-CD20 monoclonal antibodies and explains new strategies to overcome resistance.

KEYWORDS

Anti-CD20-therapy, CD20, non-Hodgkin’s lymphoma, rituximab

INTRODUCTION

The goal of CD20-targeted therapy is to kill B lymphocytes by the use of monoclonal antibodies (MoAb) against the B-cell specific human CD20 molecule. Clinical success started by targeting non-Hodgkin’s lymphoma (NHL) with rituximab, a chimeric anti-CD20 MoAb. The use of rituximab as a single agent or as an addition to chemotherapy in NHL patients can be considered as one of the most successful and worldwide accepted forms of immunotherapy so far. However, despite its success, resistance occurs in about half of the NHL patients, resulting in non-response to treatment or early relapse of the original disease.

Rituximab eliminates CD20-positive cells mainly through three different mechanisms: complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and the induction of apoptosis. Resistance to rituximab can be lymphoma-related or host-related. The preference for one of these mechanisms depends on the patient-specific microenvironment of the lymphoma. Based on the physiology of these factors, novel anti-CD20 antibodies are being developed.

This article reviews the development of CD20 targeting from its historical background towards the next generation of anti-CD20 monoclonal antibodies and explains the new strategies to overcome resistance.

HUMAN CD20

Expression of the human CD20 molecule is restricted to B-cell precursors and mature B cells (figure 1). CD20

Figure 1. The human CD20 molecule

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expression is lost upon differentiation of the B cells towards plasma cells.\textsuperscript{1-3} As shown in figure 2, CD20 is expressed within key B-cell development stages that give rise to B-cell NHL and chronic lymphocytic leukaemia (CLL).

CD20 is an ideal target for antibody-mediated therapy because CD20 is not expressed in haematopoietic stem cell B cells, so that the B-cell haematopoesis and other cell lineages are not in danger. Moreover, CD20 is not expressed on plasma cells, which means that antibody therapy will not significantly decrease the immunoglobulin production against pathogens. Other advantages of targeting CD20 are that CD20 does not circulate in the plasma,\textsuperscript{4} is not shed from the cell surface\textsuperscript{5} and is not internalised\textsuperscript{6} after antibody binding. Although CD20 is the most frequently antibody-targeted antigen in general, its exact function is still unknown. Actually, the CD20 antigen was discovered through generation of the first anti-CD20 monoclonal antibody. Balb/c mice were immunised with Burkitt’s lymphoma cells and a new antibody was formed, called anti-B1, which recognised CD20.\textsuperscript{1} Still no natural ligand is known for CD20 and our current understanding of the function of the CD20 molecule comes from ligation with different antibodies to CD20.\textsuperscript{7-10} These experiments suggest that CD20 functions as a B-cell activating or proliferation molecule. Different antibodies have shown effects on B-cell proliferation, and some were able to block B-cell growth (reviewed in Deans et al.).\textsuperscript{7} In general, ligation of CD20 with most antibodies (type 1 anti-CD20 MoAb) leads to the formation of signalling platforms (lipid rafts) and eventually to calcium flux and activation of caspase-3.\textsuperscript{11} The formation of these signalling platforms and the downstream signalling cascade is probably in conjunction with the signalling potential of the B-cell receptor (BCR).\textsuperscript{12}

**Development of the Anti-CD20 Antibody Rituximab**

The first monoclonal antibody that recognised CD20, the murine anti-CD20 B1, was generated in 1980.\textsuperscript{1} Because of their potential in the treatment of B-cell disorders, in the years thereafter anti-CD20 antibodies were genetically engineered for clinical application. In 1997, rituximab (MabThera\textsuperscript{®}, Rituxan\textsuperscript{®}) was the first MoAb approved specifically for the treatment of patients with relapsed or refractory CD20-positive low-grade (follicular) non-Hodgkin’s lymphoma. Rituximab is a chimeric anti-CD20 antibody that is engineered as follows: the light and heavy chain variable regions from the murine 2B8 anti-CD20 antibody (IDEC-2B8), generated by immunising mice with a CD20-positive human lymphoma, are amplified by polymerase chain reaction and inserted into a cDNA mammalian chimeric antibody expression vector, which also contains the neomycin phosphotransferase gene (NEO). This vector is electroporated into Chinese hamster ovary (CHO) cells and under antibiotic pressure the cells stably secrete Ig levels.\textsuperscript{13} The resulting chimeric antibody is purified and consists of a human kappa constant region, a human IgG Fc portion (IgG1), and a murine variable region, recognising the human CD20 protein.\textsuperscript{13}

**Action Mechanisms of Rituximab**

Upon ligation of CD20, rituximab triggers different effector mechanisms. Many in vitro and in vivo studies have been conducted to explore the most important one. In vitro, it is well established that there are three main modes of action of rituximab: 1) induction of apoptosis 2) CDC and 3) ADCC, as described below (figure 3).
Data concerning the mechanism of the apoptotic effect of rituximab are conflicting. Different groups obtained different results, even if they used similar target cell lines.\textsuperscript{7,14,15} It has been suggested that one of the late apoptotic pathways, caspase-3, is activated.\textsuperscript{16} However, others documented that the apoptotic pathways are caspase or Fas ligand/Fas death pathway and mitochondria independent, and do not require lipid raft formation.\textsuperscript{14,17} Hyper-crosslinking of rituximab, either by a secondary antibody or by Fc bearing effector cells, generally increased the apoptosis.\textsuperscript{7} An important observation is that within a treated cell population not all cells uniformly undergo apoptosis. This is the current focus of many groups that study rituximab resistance.

Rituximab and CDC

The Fc portion of rituximab is able to trigger the classic complement system, resulting in CDC. In \textit{vitro}, C1q is bound efficiently by rituximab.\textsuperscript{11,18} and simple CDC assays demonstrate that complement activation induces cell kill.\textsuperscript{9,19-20} Rituximab-induced CDC has a variable degree of efficiency, which has been associated with expression of complement regulatory proteins (CRP) CD55 and CD59.\textsuperscript{15,20,21} Whereas CD20 expression level has been suggested to be an important predictor of clinical CDC efficiency, several studies show contradictory results and no clear evidence for this relationship.\textsuperscript{9,21-23}

Rituximab and ADCC

ADCC is mediated by effector cells expressing FcγRI (CD64), FcγRII (CD32) or FcγRIII (CD16). Effector cells, such as NK cells, granulocytes or macrophages, are able to recognize the Fc portion of rituximab, and kill the ligated cells by phagocytosis or the release of cytotoxic granules.\textsuperscript{7,15-24} For ADCC, it has been demonstrated that the efficacy depends on polymorphisms of the effector cells.\textsuperscript{25,26}
was FcyR dependent for a panel of murine anti-CD20 MoAb and rituximab. Other groups demonstrated that complement was responsible for CD20-positive tumour clearance by rituximab. However, there is no agreement in the literature about the dominance of one particular in vivo effector mechanism. Also, some evidence concerning the mechanism of rituximab has been obtained in patients. One of the infusion-related side effects of rituximab is the complement consumption after administration, indirectly confirming CDC. On the other hand, clinical responses have been correlated to polymorphisms in the FcγRIIIA gene, indirectly confirming ADCC. In addition, a significant direct effect of rituximab cell kill by activating caspase-3 was demonstrated in vivo in patients with chronic lymphocytic leukaemia (CLL).

**Clinical Application of Rituximab**

The first phase I trial in humans with rituximab as a single agent was conducted in 1993 for patients with relapsed low-grade B-cell lymphoma. Within five single-agent trials, no severe toxicities were found and only infusion-related adverse events occurred within the first hours, in particular after the first infusion. The most common side effects were chills, fever, nausea, fatigue, headache and angio-oedema. Several phase II and III trials studied the optimal schedules and dosing with or without chemotherapy, biologicals, and radiotherapy. After approval in the USA in 1997 and in Europe in 1998, rituximab was included in the standard treatment of NHL. Rituximab works very efficiently in combination with chemotherapy. For diffuse large B-cell lymphoma (DLBCL), follicular lymphoma and mantle cell lymphoma, inclusion of rituximab in standard chemotherapy regimens significantly improved patients outcome with or without pretreatment and is accepted as a standard first-line therapy for CD20-positive lymphomas. Moreover, if patients with low-grade lymphoma respond to single-agent rituximab therapy, progression-free survival and overall survival are substantially prolonged with scheduled maintenance treatment. In patients who achieved complete or partial remission after the combination of chemotherapy and rituximab, maintenance with rituximab increased the overall and progression-free survival. In addition, rituximab maintenance in patients treated after standard chemotherapy significantly increased the three-year progression-free survival from 33 to 68%. The therapeutic effect of rituximab, through the depletion of B cells, has also proven to be successful for patients with B-cell related autoimmune diseases.

Examples are rheumatoid arthritis, autoimmune thrombocytopenic purpura, inflammatory skin diseases and pemphigus, systemic lupus erythematosus and other forms of vasculitis, diabetes, neurological diseases such as chronic inflammatory demyelinating polyneuropathy (CIDP) and multiple sclerosis and chronic graft versus host disease after allogeneic stem cell transplantation.

**Rituximab Resistance**

However, despite the success story, resistance of lymphoma B cells towards rituximab is observed in about half of the patients in the course of prolonged treatment. The precise mechanism of resistance to rituximab is unknown. Resistance may be tumour-related or host-related. Tumour-related resistance could be the lower number of CD20 molecules per cell, the increased expression of complement regulatory proteins or expression of antiapoptotic genes. Host-related resistance is determined by polymorphisms in the FcγRIIIA gene effector cells. The cellular microenvironment probably contributes to the dominant effector and resistance mechanism of rituximab. There is a difference in the extent of B cell depletion in peripheral blood, lymph nodes and spleen. Also, within the lymph node there is a differential susceptibility of different B-cell subsets to MoAb treatment. In a human-CD20 transgenic mouse model, Gong and colleagues demonstrated that circulating B cells are depleted mainly through the macrophages of the reticulo-endothelial system, while B cells within the marginal zone compartment in lymph nodes depend on CDC rather than FcγR-mediated depletion. In fact, marginal zone B cells that are trafficking from the marginal zone to the vasculature make them susceptible for depletion with MoAbs. B cells residing in the lymphoid tissues depend on the vasculature for accessibility of effector cells. In addition, in some lymph node compartments (germinal centres) B cells receive additional survival signals. Exposure to these signals makes these cells less sensitive to anti-CD20. The significance of the microenvironment in rituximab-induced cell death is also indirectly observed by differential responses to rituximab therapy in different subtypes of CD20-positive lymphomas (which have unique microenvironments), and is furthermore supported by the observation that molecular remissions in the blood and bone marrow induced by rituximab can occur in the setting of progressive nodal disease. More knowledge on and/or manipulation of the microenvironment may lead to developing a means to decrease or overcome rituximab resistance.
Several attempts have been made to improve rituximab efficacy and thereby to overcome resistance. For example, down-regulation of the antiapoptotic bcl-2 gene by antisense oligonucleotides may enhance the apoptotic effect of rituximab. Other attempts were made to improve ADCC by immunostimulatory molecules such as IL-2, IL12, IL15 or CpG sequences or improving CDC by down-regulation of complement regulatory proteins, but with limited success.

More promising is the next generation of monoclonal anti-CD20 antibodies (figure 4). In recent years, different murine, humanised and completely human anti-CD20 MoAbs have been developed (for nomenclature see table 1). These antibodies may bind to a different epitope or induce a specific mechanism of action. Another way to classify these antibodies is the ability to translocate CD20 into the lipid rafts. Anti-CD20 antibodies are either type I or type II (see also table 2). Type I antibodies relocate CD20 molecules into lipid microdomains, which can act as signalling platforms. These antibodies are potent CDC inducers. Rituximab belongs to the type I antibodies. Type II antibodies do not redistribute CD20 into signalling platforms and do not induce CDC. However, type II antibodies promote strong homotypic adhesion and have a strong induction of direct cell death.

Table 1. Nomenclature of therapeutic monoclonal antibodies

<table>
<thead>
<tr>
<th>Suffix to generic name</th>
<th>Origin</th>
</tr>
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<tbody>
<tr>
<td>-omab</td>
<td>Murine</td>
</tr>
<tr>
<td>-amab</td>
<td>Rat</td>
</tr>
<tr>
<td>-emab</td>
<td>Hamster</td>
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<tr>
<td>-imab</td>
<td>Primate</td>
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<tr>
<td>-ximab</td>
<td>Chimeric</td>
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<tr>
<td>-zumab</td>
<td>Humanised</td>
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<tr>
<td>-umab</td>
<td>Human</td>
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Table 3 gives an overview of new anti-CD20 MoAbs in comparison with rituximab. They are summarised below.

**Human Antibody (Type I)**

Ofatumumab

Ofatumumab is a completely human anti-CD20 antibody. Ofatumumab, a type I MoAb, is generated in human immunoglobulin transgenic mice. Compared with rituximab, it binds a different epitope on the CD20 molecule and has a slower off rate. Ofatumumab binds the small 7-mer loop of the human CD20 molecule, which is in a closer proximity to the cell membrane than the binding site of rituximab, which binds the larger 44-mer loop. This is probably the most important reason why ofatumumab is more potent than rituximab in inducing complement.

First clinical data with ofatumumab showed safe application and responses to therapy in

**Table 2. Differences between type I and II anti-CD20 monoclonal antibodies**

<table>
<thead>
<tr>
<th>Type I MoAbs</th>
<th>Type II MoAbs</th>
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<tbody>
<tr>
<td>Localise CD20 to lipid rafts</td>
<td>Do not localise CD20 to lipid rafts</td>
</tr>
<tr>
<td>High CDC</td>
<td>Low CDC</td>
</tr>
<tr>
<td>ADCC activity</td>
<td>ADCC activity</td>
</tr>
<tr>
<td>Full number of binding sites/B-cell</td>
<td>Half number of binding sites/B-cell</td>
</tr>
<tr>
<td>Weak homotypic aggregation</td>
<td>Strong homotypic aggregation</td>
</tr>
<tr>
<td>Weak direct cell death induction</td>
<td>Strong direct cell death induction</td>
</tr>
<tr>
<td>Examples: Rituximab, Ocrelizumab</td>
<td>Examples: GA101, B1 (Tositumomab)</td>
</tr>
<tr>
<td>Ofatumumab, Veltuzumab</td>
<td></td>
</tr>
<tr>
<td>AME-133, PRO131921</td>
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</tbody>
</table>

**Figure 4. Development of monoclonal antibodies recognising CD20**

Murine anti-CD20 mAbs are generated by immunisation of mice with CD20-positive cells. In chimeric antibodies, the 30% that is of murine origin is the variable region that recognises the CD20 antigen. The variable regions are cloned into a chimeric antibody expression vector, resulting in an antibody which contains the constant κ region and the IgG1-Fc region of human origin. For humanised antibodies, also with cloning techniques, the variable region is modified to be more human. Humanised antibodies contain complementary-determining regions of murine origin, which recognise the CD20 antigen. Only 10% of the antibody is of murine origin. Human anti-CD20 mAbs are derived from human immunoglobulin transgenic mice. The latter antibodies are likely to be non-immunogenic in men.
rituximab-resistant patients. Clinical responses to ofatumumab in a phase I/II trial are promising. In this trial in patients with follicular lymphoma, previously treated with rituximab, clinical responses with ofatumumab were up to 63% with a median time to progression of 32.8 months. Ofatumumab is currently being used in different phase III trials.

**Humanised Antibodies (Type I)**

**Ocrelizumab (PRO70769 or rhuH27)**

Ocrelizumab is derived from the murine 2H7 anti-CD20 antibody and humanised with recombinant techniques. Ocrelizumab is a type I MoAb and has an IgG1 isotype. Compared with rituximab, ocrelizumab binds a different, but overlapping epitope on the large extracellular part of CD20 and shows a two to fivefold increased ADCC and three to fivefold decreased CDC, which might lessen infusion-related reactions. In a phase I/II study, ocrelizumab was administered to rituximab-pretreated patients with relapsed/refractory follicular NHL. Ocrelizumab was well tolerated and showed a response rate of 36%. In cynomolgus monkeys ocrelizumab was shown to have the same B-cell depleting capability as rituximab. In the ACTION study group, ocrelizumab in combination with methotrexate was studied in a phase I/II trial in the treatment of RA. Over a 72-week follow-up ocrelizumab appeared to be safe with minimal immunogenicity and longer duration of the B-cell depletion. Currently, ocrelizumab is undergoing phase III clinical trials for RA and lupus nephritis, and phase II trials for multiple sclerosis. Modification of ocrelizumab resulted in an antibody with improved binding to FcγRIIa and possibly a better ADCC. This version of ocrelizumab, called PRO131921, is studied in a phase I/II trial in the treatment of relapsed or refractory CLL and indolent NHL.

**Veltuzumab (ha20, immU-106)**

Veltuzumab is a type I, humanised IgG1 MoAb generated by using the same human framework as epratuzumab (humanised anti-CD22). The complementary determining regions (CDR) were taken from the parental murine A20. Compared with rituximab there is a single amino acid difference in CDR3-VH. For this reason, veltuzumab has a slower off rate and improved in vivo activity. In vitro, the three main mechanisms of action are similar to rituximab. The first clinical studies have shown favourable safety and efficacy results in NHL patients with lower doses and less administrations of antibody. Overall response rate in rituximab-pretreated patient with refractory or relapsed NHL was 44%. In a phase I/II study, subcutaneous administration of veltuzumab in NHL and CLL is being studied and also a phase I study is ongoing for the treatment of autoimmune thrombocytopenic purpura.

**AME-133 (LY2469298)**

The production of this antibody is based on the fact that there is a strong correlation between FcγRIII (CD16)
polymorphisms and MoAb efficacy.\textsuperscript{73,76} AME-133 is a type II, humanised IgG\textsubscript{1} MoAb. It consists of a human germline framework region in which CDRs were inserted. CDRs were synthesised using a mutagenesis procedure by targeted insertion of synthetic oligonucleotide pools and their selection is based on enhanced MoAb affinity for CD20. In addition, the Fc region was also modified by targeting the constant region with synthetic oligonucleotides. This resulted in an antibody with enhanced affinity for human Fc\textgamma RIII and with an enhanced ADCC activity as compared with rituximab. The clinical efficacy of AME-133 is currently being studied in a phase I/II trial for the treatment of NHL. No clinical data are available yet.

**MURINE ANTIBODY (TYPE II)**

**Tositumomab**

Tositumomab (B1) is a murine IgG\textsubscript{2a} lambda MoAb. Ionising radiation therapy with covalently linked Iodine-131 to tositumomab is successfully used for the treatment of patients with follicular and transformed NHL who failed or relapsed from prior rituximab treatment and standard chemotherapy.\textsuperscript{77} Without the conjugation of an ionising agent, tositumomab also has a direct toxic effect. In vitro data show that tositumomab is far more efficient in inducing apoptosis and murine models show that tositumomab can prolong the survival of mice injected with Daudi lymphoma cells, in the absence of complement.\textsuperscript{78} In addition, preclinical studies demonstrate that tositumomab is more efficient in depleting B cells than rituximab.\textsuperscript{78} In patients, the direct effect of tositumomab alone is not clear. It is administered often as a pre-dose before the isotope-labelled tositumomab. This pre-dose was shown to exert a tumour-reducing effect, but on the contrary slowed down the effect of tositumomab linked with iodine-131.\textsuperscript{77} These results suggest the need for humanised B1-like antibodies for CDC-independent treatment of B-cell malignancies.

**HUMANISED ANTIBODY (TYPE II)**

**GA-101 (RO5072759).**

GA-101 is a humanised type II anti-CD20 MoAb. GA-101 is generated by grafting CDR sequences of the B-ly1 anti-CD20 MoAb on framework regions of fully human IgG1-kappa germline sequences. Different elbow hinge sequences in the variable region were optimised for optimal induction of apoptosis. In addition, the Fc region has been glycoengineered, which results in a 50-fold higher affinity to human Fc\gamma RIII receptors.\textsuperscript{79} In cynomolgus monkeys, GA101 was shown to have a superior efficacy for B-cell depletion in the tissues as compared with rituximab.\textsuperscript{80} Currently ongoing phase I and II clinical studies will demonstrate the efficacy of GA-101 and its unique property to enhance ADCC and apoptosis of B cells. The first clinical data in a rituximab-pretreated patient population showed a favourable toxicity profile and an overall response rate of 38%.\textsuperscript{81}

**DISCUSSION**

Although CD20-targeted therapy with rituximab has greatly enhanced the outcome of patients with B-cell malignancies, resistance to rituximab is still a major problem, resulting in non-response and early relapse of disease (figure 4). Second- and third-generation anti-CD20 MoAbs have been developed to overcome resistance to rituximab. To assess the additional value of new antibodies, two approaches are recognised, i.e. to show superior efficacy if compared head-to-head with rituximab or to yield significant responses in rituximab-refractory NHL patients. Resistance is determined by a complex combination of the three mechanisms of action of rituximab (CDC, ADCC and apoptosis) and a patient-specific microenvironment of the lymphoma. B-cell depletion studies in monkeys and mice have also demonstrated that distinct subtypes of B cells in the lymph nodes exert different mechanisms of cell-specific resistance.\textsuperscript{18,59} Therefore, the combination of each patient and each lymphoma subtype may have its unique mechanism of resistance. Understanding all these factors that contribute to resistance may eventually lead to an individual-patient-based anti-CD20 therapy.

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