Review

Mechanisms of Resistance to Rituximab Used for the Treatment of Autoimmune Blistering Diseases

Liliana Gabriela Popa , Ioana Dumitras , Calin Giurcaneanu , Ovidiu Berghi , Diana Sabina Radaschin , Iolanda Vivisenco * , Marius Nicolae Popescu , Cristina Beiu

Posted Date: 8 August 2024

doi: 10.20944/preprints202408.0419.v1

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Carol Davila University of Medicine and Pharmacy
* Correspondence: oberghi@yahoo.com

Abstract: Autoimmune blistering diseases represent a group of chronic severe, disabling, potentially fatal disorders of the skin and/or mucous membranes primarily mediated by pathogenic auto-antibodies. Despite their rarity, these diseases are associated with significant morbidity and mortality, a profound negative impact on the patient's quality of life and impose a considerable economic burden. Rituximab, an anti CD-20 monoclonal antibody represents the first line of therapy for pemphigus, regardless of its severity as it ensures high rates of rapid, long-lasting complete remission. Nevertheless, disease recurrence is the rule, all patients requiring maintenance therapy with rituximab sooner or later. While innate resistance to rituximab in pemphigus patients is exceptional, acquired resistance is frequent and may develop even in patients with initial complete response to rituximab, representing a real challenge for physicians. We discuss the various resistance mechanisms and their complex interplay, as well as the numerous therapeutic alternatives that may be used to circumvent rituximab resistance. As no therapeutic measure is universally efficient, individualization of rituximab treatment regimen and tailored adjuvant therapies in refractory pemphigus cases are mandatory.

Keywords: autoimmune blistering diseases; pemphigus; rituximab; mechanism of action; resistance

Introduction

Autoimmune blistering diseases represent a group of chronic severe, disabling, potentially fatal disorders of the skin and/or mucous membranes primarily mediated by pathogenic auto-antibodies. They encompass pemphigus, bullous pemphigoid (BP), mucous membrane pemphigoid (MMP), linear IgA dermatosis (LABD), dermatitis herpetiformis (DH) and epidermolysis bullosa acquisita (EBA). Pemphigus is characterized by intraepidermal acantholysis triggered by autoantibodies that target desmogleins (Dsg 1 and 3 in pemphigus vulgaris and Dsg 1 in pemphigus foliaceus), which are crucial for epithelial cell adhesion. On the other hand, the pemphigoid type and IgA mediated bullous diseases are characterized by subepidermal blistering induced by autoantibodies directed against molecules of the basement membrane zone, while EBA is caused by anti - type VII collagen antibodies that impair the adhesion of the basement membrane to the dermis.

Reported incidence rates for pemfigus vulgaris (PV), the most common form of pemphigus are 0.1-5/100.000/year [1]. The onset of the disease usually takes place during the fifth and sixth decade of life [2]. While BP has an estimated incidence of 4-22 cases/million/year, MMP is even rarer, with an incidence of approximately 2 cases/million/year. BP and MMP generally affect the elderly, occurring after the age of 60 [3]. The incidence of DH is estimated at 0.4-3.5 cases/100.000/year [4]. LABD and EBA are very uncommon disorders, with an incidence of 0.5-2.3 cases/million/year [5] and 0.26 cases/million/year, respectively [6].

Despite their rarity, these diseases are associated with significant morbidity and mortality and have a profound negative impact on the patient's quality of life. They also impose a considerable economic burden as they usually require life-long treatment and freuent and thorough monitoring due to the high risk of complications generated by both the disease and its aggressive treatment. Even

 doi:10.20944/preprints202408.0419.v1

with the adjunct of modern therapies, pemphigus still carries a mortality rate of 5-10%, a substantial proportion of which may be attributed to treatment complications [7].

The mainstay of therapy has long been represented by systemic corticosteroids (CS) associated with steroid sparing agents, especially conventional immunosuppresants. Although these treatments brought about a major drop in mortality, the adverse effects of long term administration of high doses of CS and immunosuppressive agents are redoubtable. Moreover, severe, refractory forms of autoimmune blistering diseases are not uncommon and warrant alternative treatment strategies, like plasmapheresis or high dose intravenous immunoglobulins. Fortunately, in the last decades the use of antiCD20 monoclonal antibodies in patients with severe, unresponsive autoimmune blistering diseases proved highly effective, leading to the recommendation of rituximab as first line treatment not only for moderate or severe, but also for mild cases of pemphigus by the EADV pemphigus treatment guidelines [8].

Mechanism of Action of Rituximab in Autoimmune Blistering Diseases

Rituximab is a chimeric monoclonal antibody composed of human IgG1 immunoglobulin constant regions and murine variable regions that target CD20, a transmembranary protein structurally similar to the β subunit of the high affinity immunoglobulin E receptor (FcεR) I important for the differentiation, growth, and activation of B lymphocytes [9]. Originally intented for the treatment of B-cell haematologic malignancies, it has proven useful in numerous autoimmune diseases given its complex mechanism of action.

The major effect of rituximab is the rapid and long lasting depletion of CD20+ B cells, specifically peripheral mature B cells, as well as bone marrow immature B cells, autoantigen activated marginal zone and follicular B cells. This is achieved principally, but not exclusively by antibody-dependent cellular cytotoxicity (ADCC). Direct induction of apoptosis, antibody-mediated phagocytosis and complement-dependent cytotoxicity (CDC) contribute to CD20+ B cells death [10]. Autoreactive memory B-cells (MBCs) are particularly affected by rituximab, rendering its prompt and sustained therapeutic effect in autoantibody-mediated autoimmune conditions [11]. Inherently, the treatment does not impact CD20- proB cells, leading to the repopulation of peripheric blood with B cells in 6–12 months [12]. Long lived plasma cells (LLPCs), which are CD20- are also spared, explaining the insignificant variation of total serum antibody levels and anti-infectious antibodies levels following treatment with rituximab [12]. Upon B-cell repopulation, apart from the markedly increased naive/MBC ratio, the expansion of circulating regulatory B-cells (Bregs) occurs. Most of these Bregs are transitional interleukin 10 (IL-10) producing B cells, able to decrease the antigen presenting potential of dendritic cells, as well as the CD4+ T cells responses [13]. This leads to the maintenance of immune tolerance and of disease remission [14]. While the population of IL-10 producing B cells increases following treatment with rituximab or IVIg, this does not occur following corticotherapy and treatment with conventional immunosuppressive agents [15].

Several other mechanisms confer rituximab its currative valences. Although it does not influence the total number and function of peripheral CD4+ and CD8+ T cells [16, 17], rituximab induces a swift and prolonged decline in autoreactive CD4+ T cells owing to the loss of the stimulation exerted by autoreactive B cells, probably acting as antigen-presenting cells [18]. Another possible explanation is the depletion of CD20+ T helper (Th) 17 cells, as it was demonstrated in patients with rheumatoid arthritis [19]. The number of autoreactive T cells correlates with the serum levels of autoantibodies and with the clinical activity of the disease [17, 18]. Moreover, circulatory regulatory T cells (Treg) numbers are reduced in PV patients, contributing to the overactivity of autoreactive B cells [20, 21]. Unlike other autoimmune diseases, in PV they decrease even more after rituximab administration, only to be detected in higher numbers in lesional skin [22], suggesting an increased Tregs skin homing meant to contain the cutaneous autoimmune process [23]. This is also a rituximab-specific response that aids in the control of the disease.

In addition, rituximab exerts other specific effects, as it leads to a substantial decrease in the number of autoreactive T follicular helper (Tfh) cells in the peripheral blood, although the circulating Tfh cells total number is not modified. The drop in autoreactive Tfh cells numbers is associated with

 doi:10.20944/preprints202408.0419.v1

a considerable decrease of serum IL-21 levels. The latter correlates with B-cell depletion given the critical role of IL-21 in B-cell maturation, particularly MBC generation [24]. On the other hand, as demonstrated by Baumjohann et al., to maintain their phenotype, Tfh cells require constant antigenic stimulation by B cells. Therefore, the impact is bidirectional, as the depletion of autoreactive B cells hampers autoreactive Tfh cells development and maintenance [25].

B cells repopulate the peripheral blood 6–12 months after the administration of rituximab [12]. However, these are naïve B cells. B cell maturation is hindered for a much longer period of time, delaying the reappearance of MBCs [26]. The naive/MBC ratio is markedly increased, explaining the lack of increase in serum autoantibodies levels after B-cell repopulation [12]. Furthermore, the renewed B cells display rearrangement of their Ig repertoire, which converts from oligoclonal into polyclonal [13]. Nevertheless, persistently high autoantibodies levels have been reported in 16–40% of patients with complete remission of the disease [27, 28]. These are most likely non-pathogenic antibodies, targetting different epitopes [13].

The serum level of B cell-activating factor (BAFF), a key factor for the maturation of B cells is high while MBCs are absent in the peripheral blood and greatly decreases upon B-cell recovery, signaling the risk of recurrence [29]. Rituximab specifically decreases BAFF-R mRNA in non-autoreactive, as well as in reemerging autoreactive B cells, an effect that contributes to sustained remissions despite B cell repopulation [30].

Disease Relapse after Rituximab Administration

Disease relapse occurs in virtually all patients with autoimmune blistering diseases after a variable period of time, usually 6 to 24 months following administration of rituximab [31,32]. This is due to several interconnected processes.

Autoreactive MBCs persist in the spleen and lymph nodes of pemphigus patients given the intense survival signals in these areas [11]. This incomplete MBC depletion does not always lead to early relapse as these cells may remain dormant even for decades in lymphoid organs. Persistence of autoreactive CD4+ Th cells is also possible.

The other main mechanism underlying disease recurrence is the emergence of new lineages of antidesmoglein B cells. Autoantibody-producing LLPCs may also induce disease recurrence [12].

Hence, all patients need maintenance treatment eventually, yet the optimal regimen is to be established. Current EADV guidelines for the management of pemphigus recommend maintenance treatment with rituximab administered 6 months after the initial cycle in doses ranging from 500mg to 1g in patients who achieved complete remission, especially patients with severe PV at initial presentation and/or patients with high anti-Dsg antibodies levels at month 3 and a full cycle (two infusions of 1g two weeks apart) in patients without complete remission. Thereafter, administration of a dose of 500mg of rituximab every 6 months is recommended. [8] Several experts, however, argue that additional doses of rituximab should only be administered in patients with incomplete response or disease recurrence. We also embrace this concept and comply with this practice as the majority of our patients presented long-lasting complete remission in the absence of any specific treatment after rituximab administration, ranging from 18 months to 13 years. We find the frequent administration of rituximab, a profoundly immunosuppressive therapy, in patients with sustained complete remission is unnecessary and unjustified and that maintenance therapy should be individualized. Ideally, subsequent administrations of rituximab should be prompted by the detection of biomarkers signaling an imminent recurrence. Such biomarkers are under investigation and include circulating CD19+ B-cells counts, a slower B-cell depletion and early B-cell repopulation 28], CD4+ T cells counts, pathogenic anti-Dsg autoantibodies serum levels, BAFF serum level, as well as several genetic markers predicting response to rituximab [31,33,34].

Mechanisms of Resistance to Rituximab and Strategies to Overpass It

Innate resistance to rituximab in pemphigus patients is exceptional and is usually due to a low CD20 expression or an accelerated drug clearance [9]. Acquired resistance is, on the other hand, frequent and may develop even in patients with initial complete response to rituximab. Apart from

 doi:10.20944/preprints202408.0419.v1

the evading mechanisms already discussed, a series of additional phenomena may be involved in the development of rituximab resistance, such as CD20 downregulation, especially on MBCs [35], impaired rituximab – CD20 binding due to the release of human anti-chimeric antibodies (HACAs) to rituximab [36], or CD20 alterations like CD20 alternative transcript (D393–CD20) [37] or lipid raft signaling biochemical changes [38]. Certain comorbidities and polymorphisms of FcR may also impact the immunological effects of rituximab [38].

Resistance to rituximab may be caused by the interference with its mechanisms of action.

CD46, CD59 and CD55 are membranary proteins that inhibit complement activation and the formation of the membrane attack complex. B cells that overexpress these complement activation regulators are resistant to rituximab as they are not susceptible to CDC. This might represent a result of selective pressure due to previous exposure to rituximab [38]. The purine analog fludarabine seems to act sinergically with rituximab, an effect explained by its ability to decrease CD55 activity [39]. Thus, it is a useful adjuvant therapy in such cases of resistance to rituximab. However, as the role of complement activation regulators in the protection of normal cells against CDC is crucial, aggressive downregulation of these proteins would be unquestionably detrimental.

Rituximab induced CDC may also be hindered by the consumption of complement proteins. Klepfish et al. proved that coadministration of fresh frozen plasma with rituximab is successful in counteracting this deficiency and restauring the latter's efficacy [40].

Nevertheless, there is a very fine line between benefit and harm with such interferences. As previously discussed, rituximab exerts its distructive effect on B cells mainly through ADCC. The ability of natural killer (NK) cells to perform ADCC is impeded by C3b, therefore complement depletion favors this process [41]. Furthermore, upregulation of human leukocyte antigen (HLA) I on B cells renders them resistant to NK cells mediated ADCC [42].

ADCC is also hindered by a defectuous link between rituximab and its target and by conformational changes of the CD20/rituximab complex [38]. In some patients, FcR polymorphisms may be the cause of rituximab failure to induce ADCC. Alteration of the lipid rafts of B cells membranes in patients recieving statins was demonstrated to induce in vitro resistance to ADCC [43]. Some authors also point to vitamin D deficiency as potentially involved in rituximab-mediated ADCC resistance [44]. Administration of rituximab in association with granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), interferon-gamma (IFN-γ), IL-2, IL-12, and IL-15 may help surpass ADCC resistance by increasing the function of NK cells [38]. Most studies focused on the effects of G-CSF and GM-CSF, which upregulate the expression of neutrophil adhesion molecules [45] and IL-2, which also augments ADCC [46]. The potential of CpG oligonucleotides, bromohydrin pyrophosphate, and Toll-like-receptor 9 (TLR-9) agonists to enhance ADCC is currently investigated [47-49].

Last, but not least, after several courses of rituximab, B cells may become resistant to rituximab - induced apoptosis, mainly due to the excessive activation of the nuclear factor kappa B pathway, known to markedly stimulate cellular antiapoptotic mechanisms. This triggers overexpression of the anti-apoptotic proteins belonging to the Bcl-2 family, whereas Bax and Bak, the pro-apoptotic members of the Bcl-2 family proteins are substantially down-regulated, leading to resistance not only to rituximab, but also to chemotherapy [50]. Studies of combination therapy with rituximab and Bcl-2 inhibitors like oblimersen have shown encouraging results in follicular NHL patients, achieving response rates of 60%, even in rituximab - resistant cases [51]. Several therapeutic agents, such as temsirolimus, bortezomib, and histone deacetylase inhibitors also sensitize lymphoma cells to rituximab in vitro [52, 53]. Moreover, in lymphoma patients, resistance to apoptosis can be overcome by combined treatment with rituximab and cytotoxic agents [38]. Researchers are currently exploring other techniques that increase CD20 expression in order to restore B cells susceptibility to rituximab - induced apoptosis.

Apart from the development of resistance to rituximab induced CDC, ADCC and direct apoptosis, other factors may impede the treatment's beneficial effect. Among these, the most important is represented by the altered expression of CD20 on the surface of B cells, which may be caused by a variety of processes, from lipid raft reorganization and perturbed signaling, CD20

 doi:10.20944/preprints202408.0419.v1

internalization upon repeated administration of rituximab, and CD20 gene deletion mutations affecting the C-terminal region and impairing antibody binding [38]. Shimizu et al. explored the epigenetic changes linked to decreased CD20 expression and demonstrated the ability of valproic acid and romidepsin, both acting as histone deacetylase inhibitors to increase CD20 expression in vitro and subsequently amplify rituximab induced CDC [53].

Another interesting resistance mechanism is the removal from the B-cell surface of CD20-rituximab complexes upon recognition by FcR on macrophages and monocytes. However, this so called "shaving" of CD20-rituximab complexes can be avoided by the addition of intravenous immune globulines (IVIG) to the therapeutic management [54].

As higher serum concentrations of rituximab were detected in responders vs non-responders [38], it is only intuitive that higher doses or more frequent administrations could be beneficial in non-responders. While rituximab's highly variable pharmacokinetics most probably plays a key role in rituximab resistance, the minimum effective rituximab serum concentration is unclear. Although some authors support the use of higher doses of rituximab (e.g., 1g/m^2) in non-responders [55], no large studies have addressed this issue so far.

Interestingly, intralesonal rituximab is a valuable therapeutic option even in patients refractory to intravenous rituximab. Vinaj et al. reported marked improvement of the oral lesions in 3 PV patients refractory to conventional immunosuppressants and intravenous rituximab [56]. Yuan et al. detected the accumulation of Dsg-3 and Dsg-1 specific B-cells within pemphigus cutaneous lesions and postulated that in pemphigus, the skin behaves as a tertiary lymphoid organ, with infiltrating auto-reactive B-cells releasing anti-Dsg autoantibodies due to an intense cross-talk between B cells and IL-21- and IL-17A-producing CD4+ T cells. Thus the lesions resistant to intravenous rituximab treatment may remain susceptible to the intralesional administration of the drug [57].

An alternative approach in rituximab resistant cases is the use of second and third generation humanized or completely human anti-CD20 monoclonal antibodies. These possess a series of advantages over rituximab, including significantly lower immunogenicity and superior eficacy due to higher affinity for FcR, enhanced ADCC (ocrelizumab, GA-101), enhanced ADCC and direct apoptosis (obinutuzumab), greater binding avidity, improved CDC, and a slower dissociation rate (veltuzumab), more potent CDC due to the stronger binding to CD20 at a situs more proximal to the cell membrane and a slower dissociation rate, as well as resistance to the effects of complement-regulatory molecules (ofatumumab) [38,58-60]. As evidence of their ability to surmount rituximab resistance mechanisms stems from a limited number of case reports, further studies are needed to assess their efficiency in resistant PV cases.

BAFF targeting therapies such as belimumab and atacicept have also been successfully used in PV patients resistant to rituximab [61].

Treatment with ibrutinib is another appealing strategy in rituximab resistant PV patients. The bruton kinase (BTK) inhibitor has proven highly efficient not only in patients with B-cell lymphomas, but also in pemphigus. BTK is principally expressed on B-cells, except plasma cells and its activation leads to stimulation of p38MAPK, NFkB and MEK/ERK pathways, resulting in B-cell proliferation and maturation, autoantibody production and Ig class switching [61-63], which implicitly favors Tfh cells differentiation. Furthermore, topical p38MAPK inhibitors may prove useful and safe adjuvants in refractory PV patients [61].

Janus kinases (JAK) 1 and 3 inhibitors (tofacinib) may also be used as adjuvants in refractory PV, even as a topical treatment as they suppress the activation of Dsg-specific Tfh cells and their influence on B cells phenotype and function [61,64].

STAT 3 inhibitors (rapamicin, mTOR inhibitors such as sirolimus, or Stat3 inhibitor XVIII) were shown to be effective in PV in animal studies by increasing Dsg3 expression [65,66].

Effects of anti CD19+ monoclonal antibodies (inebilizumab), which also act on plasma cells are studied in PV given that the production of anti-Dsg autoantibodies by LLPCs represents one of the mechanisms of resistance to rituximab. CD19-directed CAR-T-cell therapy and Dsg3-CAAR T-are also studied in PV, with very promising preliminary results [67,68].

 doi:10.20944/preprints202408.0419.v1

Considering the significant decrease of Dsg-specific Tregs observed in PV patients, the benefit of autologous polyclonal Tregs infusion in PV and PF patients is under investigation in a phase 1 open-label multicenter trial (NCT03239470) [69].

Conclusion

The therapeutic landscape of autoimmune bullous diseases has dramatically changed with the introduction of anti-CD20 monoclonal antibodies. Nowadays, rituximab represents the first line of therapy for pemphigus, regardless of its severity as it ensures high rates of rapid, long-lasting complete remission, often in the absence of corticotherapy and conventional immunosuppressive therapy, while maintaining a favorable safety profile. Nevertheless, disease recurrence is the rule, all patients requiring maintenance therapy with rituximab sooner or later. In order to avoid unnecessary suplimentary doses, inherent side effects and additional costs, there is an urgent need for the identification and validation of biomarkers predictive of imminent pemphigus recurrence that would allow the implementation of optimal, personalized therapeutic regimens.

Resistance to rituximab of PV patients represents a great challenge for the physician. Despite the numerous interesting therapeutic alternatives that offer theoretical promise of circumventing rituximab resistance, clinical studies are warranted in order to prove their potential. The large array of resistance mechanisms and their complex interplay suggest there is no universally efficient therapeutic measure. Individualization of rituximab treatment regimen and tailored adjuvant therapies in refractory cases are mandatory.

Author Contributions: Popa Liliana Gabriela: writing – original draft preparation, review & editing; Dumitras Ioana: writing – original draft preparation, review & editing; Giurcaneanu Calin: conceptualization, methodology, supervision; Berghi Ovidiu: resources, data collection; Radaschin Diana: resources, data collection; Vivisenco Iolanda: conceptualization, methodology; Beiu Cristina: writing – original draft preparation, review & editing, supervision. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Funding: This research was funded by the ”Carol Davila” University of Medicine and Pharmacy, Bucharest, Romania, Publish not Perish Grants.

Conflicts of Interest: none to declare.

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 doi:10.20944/preprints202408.0419.v1