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Variability in clinical and biological response to rituximab in autoimmune diseases: an opportunity for personalized therapy?

A better understanding of the variability in clinical response to B-cell-depletion therapy using rituximab in rheumatoid arthritis and systemic lupus erythematosus is important to optimize the use of this therapy and improve patient outcomes. To this end, we review current evidence on factors that affect pharmacokinetics and pharmacodynamics of rituximab, and the biological and clinical response to this drug. Also we briefly describe variability in B-cell depletion and reconstitution following rituximab treatment and summarize elements that have been shown to distinguish responders and nonresponders. Finally, we speculate on the prospects for exploiting the knowledge gained thus far in developing rituximab-based personalized therapy for rheumatoid arthritis and systemic lupus erythematosus.

Keywords: B-cell depletion • rheumatoid arthritis • rituximab • systemic lupus erythematosus

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B-cell-depletion therapy (BCDT) using rituximab is licensed for the treatment of refractory rheumatoid arthritis (RA) and antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis, and is also routinely used in clinical practice to treat a range of autoimmune diseases including systemic lupus erythematosus (SLE). The list of unlicensed indications where rituximab is used has been increasing over the past decade. Although there exists robust evidence on the safety and efficacy of rituximab in RA considerable variability has also been noted in biological and clinical response between patients. However, what underlies this variability remains poorly understood. A better understanding of the factors and mechanism/s that determine the clinical response to rituximab could be exploited to improve the overall effectiveness of BCDT strategies. To this end, this review focuses on describing factors and mechanisms that may explain the variability in biological and/or clinical response to rituximab in RA and SLE.

Variability in biological response to rituximab: what does it mean & why does it matter?

B cells involved in or driving the pathogenesis of autoimmune diseases are referred to as autoreactive B cells, but their identity by means of cell surface markers has not yet been clearly defined. Therefore, it is not possible to assess the effectiveness of rituximab in removing autoreactive B cells and total B-cell depletion is used as a surrogate marker.

It should be noted that even effective depletion as measured in peripheral blood does not always result in clinical response. There are practical difficulties in understanding how depletion in peripheral blood reflects depletion in solid tissues, which may be more relevant (see section 'Interindividual variability in depletion & clinical response'). To reconcile these observed discrepancies between effective depletion and clinical response we suggest that an inadequate clinical response despite effective depletion represents 'refractory disease' whereas simply not achieving effective depletion represents 'rituximab

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resistance'. Rituximab resistance may be related to insufficient dose or increased drug clearance, but also to resistance of autoreactive B-cell clones to depletion mechanisms induced by binding to rituximab. A failure to achieve clinical response despite effective depletion, 'refractory disease', may occur because the disease is not B-cell dependent for initiation and/or perpetuation. It is important to distinguish the two scenarios because it could be hypothesized that the group of patients who did not respond to rituximab but have achieved effective depletion and do not show any evidence of early return of B cells may not benefit from further B-cell-targeted therapies whereas at least some patients from the group with poor response who failed to achieve effective sustained depletion may benefit from alternative agents or treatment regimens that may improve B-cell depletion. Thus, it could be conceived that dissecting patient groups on the basis of effective sustained depletion, and relationship between depletion and clinical response, may help identify those likely or unlikely to respond to rituximab treatment and those who may benefit from alternative B-cell-depleting strategies (Figure 1).

However, distinguishing patients based on the criteria described above is riddled with practical problems. For example, the definition of B-cell depletion used in several studies thus far is 'arbitrary' and not based on evidence of correlation with clinical response. It is our view that at least in mechanistic or protocol-developing studies, when defining B-cell depletion highly sensitive flow cytometry (HSFC) should be used with a threshold set at <1 cell/µl because such a definition has been shown to correlate with clinical response in both RA and SLE [1–3] and therefore 'clinically meaningful'. Defining biological response based on changes in laboratory parameters is less complex than defining clinical response as indicated by changes in validated disease activity scores, often derived from composite objective and subjective measures, such as DAS 28 score and British Isles Lupus Assessment Group (BILAG), Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and SLE-responder index (SLE-RI) scores. Regardless of the mechanism of disease pathogenesis clinical response attributable to rituximab therapy could be defined as 'B-cell-dependent' activity. B-cell-dependent disease activity may be limited to a specific organ system or involve multiple organs. This is an important consideration when evaluating clinical response in characteristically multisystem autoimmune conditions such as SLE. Therefore, sev-

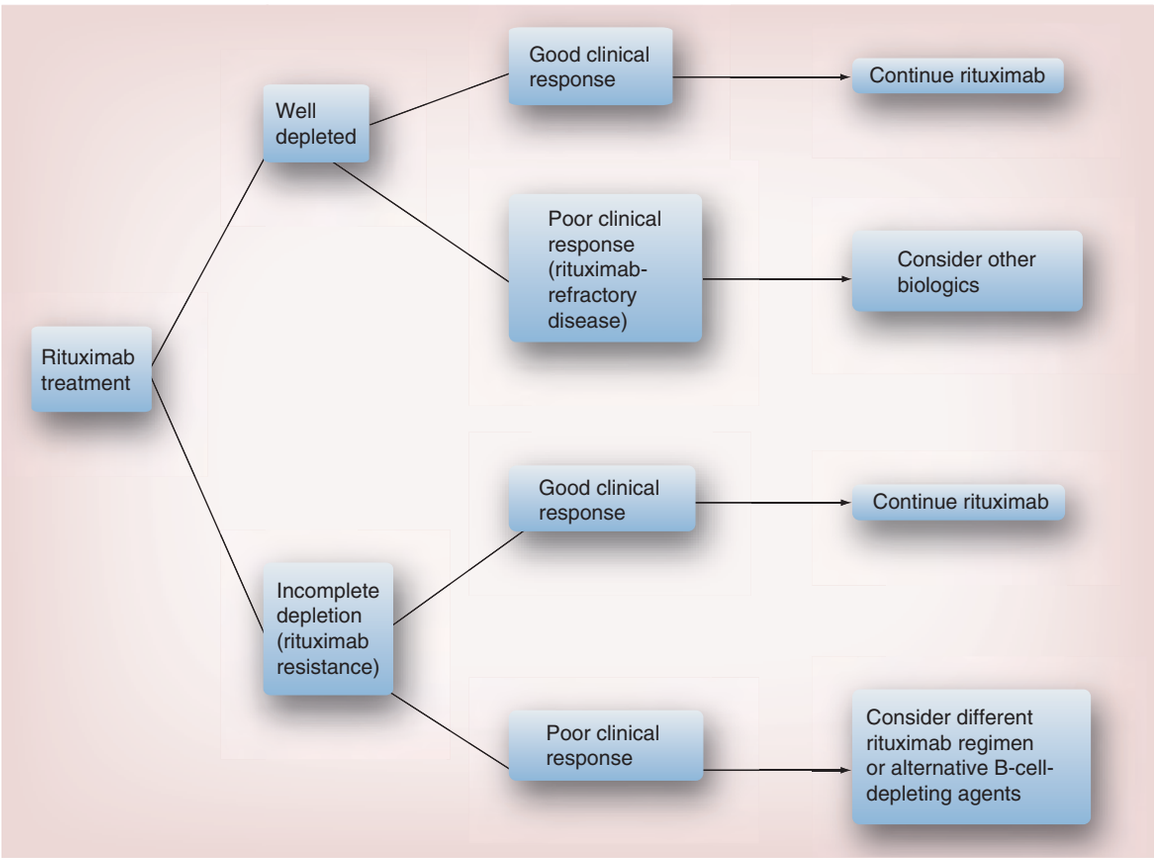

Figure 1. Proposed algorithm for defining rituximab resistance and refractory disease.

eral factors need to be taken into account when trying to understand the basis for the variability in clinical response to rituximab.

What can we learn from the experience of using rituximab for B-cell malignancies?

Rituximab (IDEC-C2B8) is a chimeric IgG1κ (variable CDR regions [mouse] and Fc constant portion [human]) monoclonal antibody (mAb) directed against CD20. It was first developed by IDEC Pharmaceuticals Corporation. CD20 was chosen as a therapeutic target because it was thought to: be exclusively expressed on B cells; not modulate or shed; and be expressed by more than 90% of B-cell malignancies such as non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL). The mechanisms of action of rituximab have been extensively studied in the context of B-cell malignancies. The knowledge thus gained is being exploited for the development of better therapies to improve patient outcomes.

Clinical response to rituximab: the effect of disease heterogeneity, rituximab pharmacokinetics & the host's immune system

In the initial Phase I, single dose-ranging (10, 50, 100, 250 and 500 mg/m^2) study of rituximab in NHL, peripheral blood B cells were specifically depleted and remained depleted for 1 to more than 3 months. Serum rituximab levels were variable and the serum half-life was 4.4 days for patients treated with doses of 100 mg/m^2 or higher. Tumor tissue examination 2 weeks after treatment showed rituximab bound to tumor cells in several of the cases, which suggests that not all rituximab-coated cells are deleted. Modest tumor responses were seen in seven of the nine patients treated with doses equal to or greater than 100 mg/m^2 [4]. Subsequently, the therapeutic regimen of 4 weekly infusions of 375 mg/m^2 was chosen following a Phase I, multiple-dose (125, 250 or 375 mg/m^2 each) study [5] and first used in a Phase II study of rituximab, in 37 patients with NHL, who had relapsed despite aggressive chemotherapy. Rituximab was licensed for use in refractory NHL, following the pivotal study by McLaughlin and colleagues [6] demonstrating that at least half patients with refractory low-grade or follicular lymphoma (FL) responded to rituximab monotherapy [6]. In this Phase II/III study of NHL, 166 patients with refractory NHL were treated with rituximab. The response rate was 48% after a median follow-up duration of 11.8 months. Responding patients were noted to have follicular histology, higher serum rituximab levels, and also lower tumor burdens. Fifteen of the 16 patients who did not deplete peripheral blood B cells to undetectable levels did not respond to treatment. Only one patient developed human antichimeric antibodies (HACA). Response rate in FL increased to 60% using, instead of four, eight consecutive weekly infusions of 375 mg/m^2 of rituximab [7]. Igarashi *et al.* reported that extranodal disease was also associated with poor response in FL [8].

Association of response with follicular histology was also described in another small early study where all seven responding patients had FL while the nonresponders included two with diffuse large-cell lymphoma (DLCL) and one with mantle cell lymphoma (MCL) [9]. Interestingly, serum rituximab levels increased incrementally with repeated infusions and were detectable in most patients at 3 months. Serum half-life of rituximab was estimated at 18 ± 15 (mean ± SD) days. Lower response rates in DLCL and MCL were also reported in another Phase II study of 54 patients with DLCL or MCL, which yielded response rates of 37 and 33%, respectively. The features that were more commonly noted in nonresponders were chemotherapy-refractory disease, MCL histology and tumor size > 5cm in diameter [10]. Response rates in small lymphocytic lymphoma (SLL)/CLL were also much lower [11,12]. A study of 33 patients with SLL/CLL treated with variable doses of rituximab (100 mg × 1, 250 mg/m^2, weekly infusions of 375 mg/m^2 for 4 weeks) showed that the overall response rate was 45% with only 3% achieving complete response [12]. O'Brien and colleagues reported that clinical response in CLL may be improved using a higher dose of rituximab [13]. In this study involving 40 patients with CLL, a dose-escalation regimen with a starting dose of 375 mg/m^2 and subsequently the dose was escalated from 500 to 2250 mg/m^2. Interestingly, a dose-dependent clinical response was noted with 22, 43 and 75% for dosing regimens 500–825, 1000–1500 and 2250 mg/m^2, respectively [13]. Further the duration of response was shorter than that seen for NHL [14]. Interestingly, even in patients with same histology, CLL, genomic microarray either alone or in combination with laboratory parameters such as IgVH mutation has been shown to accurately distinguish responders from nonresponders to rituximab [15].

Other studies explored the use of rituximab in combination with chemotherapy. In NHL, the response rates were impressive at 89–95% overall response rate and 56% complete response when rituximab was used in combination with cyclophosphamide, doxorubicin, vincristine and prednisolone (CHOP) chemotherapy [16,17]. Furthermore, the responses were sustained at long term with median follow-up period of 63 months achieving a 5-year progression-free survival of 82% [18]. The efficacy of a combination chemoimmunotherapy, rituximab-CHOP, for NHL was also confirmed in

larger studies [19]. In NHL, serum rituximab levels correlated with female gender (higher), tumor burden (bone marrow infiltration) and clinical response [20]. Pfreundschuh and colleagues have conducted a randomized trial comparing rituximab-CHOP versus CHOP in 824 patients with a follow-up duration of 6 years showing better overall survival in the group receiving rituximab at 74% when compared with 56% for the group without rituximab [21]. Griffin *et al.* have published an excellent critical overview of the use of rituximab in NHL in RCT settings [22].

Thus, taken together, it appears that the clinical response to rituximab in B-cell malignancies was influenced by: the histological type, extra-nodal disease and the tumor burden; rituximab dose used; persistent high serum levels of rituximab; better response to prior chemotherapy; and rituximab used in combination with chemotherapy. However, the mechanisms underlying variability between individuals in clinical response within each disease category and factors influencing serum rituximab levels remained elusive. Therefore, several groups focused on understanding the mechanisms of action of rituximab (see secion 'So, what factors determine resistance to depletion with rituximab?').

B-cell depletion in autoimmune diseases

The rationale for using rituximab in B-cell malignancies was straightforward with response reflecting how well tumor cells were killed and/or their proliferation prevented. By contrast, the rationale for using rituximab in autoimmune diseases was initially based on the presence of pathogenic autoantibodies, in particular IgM isotope, which is more dependent on formation of new plasma cells. Therefore, removal of B cells would interrupt the formation of new plasma cells. An alternative, more complex hypothesis such as the 'self-perpetuating B-cell hypothesis' was the main rationale for using B-cell depletion in RA [23]. In autoimmune diseases, variability in clinical response to rituximab will necessarily reflect a combination of how well B cells are depleted and the exact role and importance of different B-cell subsets and their products in disease pathogenesis.

The safety and efficacy of rituximab in RA is now well established following the pivotal randomized controlled trial (RCT) of 161 patients with active RA refractory to methotrexate. At least 41% of patients receiving rituximab achieved 50% improvement in the American College of Rheumatology (ACR) responses when compared with 13% for methotrexate alone [24]. Subsequently, rituximab was licensed for the treatment of refractory RA and to date several thousands of patients with RA worldwide have been treated. The robust safety record of rituximab in RA, which is comparable to that of other biologics used to treat RA such as anti-TNF antagonists, has reinforced BCDT as an important therapeutic strategy. Therefore, the focus now is on optimizing the use of rituximab for RA. An important starting step to achieve this is to understand the variability in biological and clinical response to rituximab. Below, we shall briefly review selected early studies that described variability in biological and/or clinical response and focus on identifying factors that may have influenced the observed variability.

Interindividual variability in depletion & clinical response

Variability in biological and/or clinical response to rituximab as assessed by changes in several clinical and laboratory parameters have been reported in studies (Table 1).

Variability in peripheral & tissue B-cell depletion & clinical response

The first pilot study investigating the effectiveness of BCDT based on rituximab included five patients with refractory seropositive RA [32]. The treatment regimen used a combination of rituximab, cyclophosphamide and oral steroids. All five patients responded to treatment but even in this small number there was variability in the extent of improvement noted and duration of response. The study was then extended to 22 patients with refractory RA, treated with various doses of rituximab, with or without cyclophosphamide or oral corticosteroids [33]. The results of this study suggested that a minimum dose of rituximab was required (600 mg/m^2). Two patients seronegative for rheumatoid factor (RF) did not respond but had received a lower dose of rituximab. Again, variability was noted in the extent of the clinical response and its duration. Relapses were preceded by or coincided with B-cell repopulation of the peripheral blood even after repeated treatments. The time to relapse from repopulation of B cells varied from 0 to 17 months [26].

B-cell depletion in the bone marrow was also variable. In a small number of patients with RA, at 3–4 months after treatment, there was variability in the degree of B-cell depletion and in the proportion of CD19$^+$ cells and of different subpopulations in the bone marrow [29]. The extent of depletion correlated with clinical response with greater depletion associated with better clinical response. Several groups have reported on the variability in B-cell depletion achieved in the synovium [34–37]. Although as yet there is no conclusive link between nondepleted B cells contributing to poor response in RA, it is worth noting that such a phenomenon has been implicated in chronic active

Table 1. Variability in biological and clinical response to rituximab.

Variability in parameters	Comments	Condition	Ref.
Degree of BCD and development of HACAs	The degree of depletion did not depend on the dose of rituximab used and the development of HACAs was associated with lower rituximab levels	SLE (n = 17)	[25]
Time to clinical relapse from B-cell repopulation varied from 0 to 17 months	Variability in depletion was noted between patients despite the same dose of rituximab	RA	[26]
The decrease of anti-dsDNA antibody levels from baseline	The extent of decrease in anti-dsDNA antibody levels was greater in responders	SLE (n = 16)	[27]
Duration of peripheral BCD (3–8 months)	One patient remained depleted at 4 years	SLE (n = 24)	[28]
Bone marrow B-cell lineage cells at 3 months after RTX	Variability was noted in the proportion of pro- and pre-B cells and there was a trend towards better response in patients with better depletion	RA (n = 6)	[29]
Degree of depletion	BCD (<5 cells) correlated with clinical response and inversely with HACAs	SLE (n = 18)	[30]
HACAs	No correlation between *FcgRIIIa* polymorphism and clinical response	SLE (n = 18)	[30]
Response to immunization with pneumovax	HACAs were higher in 3 patients treated with low-dose RTX (100 mg)	SLE (n = 18)	[30]
7 months after Rx with RTX	70% response (SLEDAI improved >2)	SLE (n = 18)	[30]
Degree of peripheral BCD	BCD defined as <5 cells/µl	SLE (n = 65)	[31]

BCD: B-cell depletion; HACA: Human antichimeric antibody; RA: Rheumatoid arthritis; RTX: Rituximab; SLE: Systemic lupus erythematosus; SLEDAI: Systemic lupus erythematosus disease activity index.

antibody-mediated graft rejection (renal transplant) [38] and in Sjogren's syndrome [39], which was attributed to local production of BAFF thought to protect against rituximab-induced apoptosis. Further exploration of the area is out of remit of the current review.

The initial pilot open study to investigate the safety and efficacy of rituximab in six patients with refractory SLE [40] at University College London (UCL) employed two 500-mg doses of rituximab and two 750-mg doses of intravenous cyclophosphamide plus prednisolone 30 mg or 60 mg for 5 days. Similar to RA, clinical relapse occurred either at the time or after B-cell repopulation, but there was considerable variability between patients in duration of B-cell depletion and interval between B-cell repopulation and clinical flare. Prolonged periods of B-cell depletion (CD19 count <10 cells/µl for >12months) was noted in 15 patients with one patient not having repopulated for 7.5 years after treatment with rituximab and one patient who did not deplete [41]. We found that in 63 patients with SLE under follow up at UCL, all but five patients of whom received 2 doses of 1 g rituximab a week apart, the duration of depletion varied such that following the first cycle of treatment, four patients (6%) did not deplete (with B-cell counts above 0.005×10^9/l at 1 month and no subsequent decrease). B cells repopulated at 2 months in five (7%), at 3 months in seven (11%) and by 6 months in 34 (54%) [31]. In an early Phase I/II study involving 17 patients with refractory SLE, Looney and colleagues, using either a single dose of 100 mg/m^2 (low dose) or a single dose of 375 mg/m^2 (intermediate dose) or 4 doses (1 week apart) of 375 mg/m^2 (high dose), also noted a considerable variability in the degree of B-cell depletion; with some patients in the low-dose group achieving adequate B-cell depletion while some patients in the high-dose group did not deplete well [25]. Albert and colleagues also noted variability in B-cell depletion with seven of 24 patients achieving incomplete depletion and the degree of depletion correlated with clinical response [30]. In the EXPLORER study, B-cell depletion (CD19 count <10 cells/µl) was not achieved in 9.5% of patients [42]. Thus, there occurs a clear variability in the degree and duration of B-cell depletion between patients with RA and SLE regardless of the dose of rituximab used. Our clinical experience suggests that SLE patients tend to deplete less well. However, data are lacking on whether there is a clear differ-

ence in the degree and duration of depletion between RA and SLE. Moreover, comparing rituximab-induced depletion in RA and SLE would be limited by confounding factors including background/concomitant therapies and rituximab regimen.

HSFC is a useful tool in predicting response

In many studies of rituximab in RA and SLE B-cell depletion has been arbitrarily defined as peripheral $CD19^+$ cells <5–10 cells/µl [28,41–46]. However, based on this definition there was no consistent correlation between the degree of depletion and clinical response. The thresholds used in these studies for defining B-cell depletion may not be sensitive enough. For example, using HSFC (where 500,000 lymphocytes are counted instead of the usual 2000–20,000) it was shown that, in both RA and SLE, the degree of B-cell depletion correlates with clinical response. A more stringent definition of B-cell depletion as peripheral $CD19^+$ cell count as <1 cell/µl using HSFC is predictive of clinical response to rituximab in both RA and SLE [1,2]. In RA, clinical response to rituximab was shown to depend on the degree of depletion, regardless of the dose of rituximab used. Important findings of these studies show that peripheral B-cell depletion was incomplete in 18% of patients with RA [47] and 54% of patients with SLE [2]. The majority of cells circulating during the depletion period are plasmablasts/plasma cells and memory B cells [2,44,48]. In addition, the presence of circulating plasmablasts at 6 weeks after rituximab was shown to correlate with clinical response in both RA and SLE. All nonresponders were found to have detectable plasmablasts at 6 weeks after rituximab. Thus, a B-cell depletion threshold of < 1cell/µl appears to be a good biomarker that predicts response to rituximab. Nevertheless, some patients may not respond despite achieving complete depletion, as defined by HSFC [1].

Variability in reconstitution of total B cells, B-cell phenotypes & clinical relapse

As discussed earlier, in RA the time to relapse from repopulation of B cells varied in the initial UCL cohort from 0 to 17 months [26]. Repopulation of the peripheral blood after rituximab occurs predominantly with naive mature and transitional B cells similar to after bone marrow transplantation [44]. Patients with prolonged clinical response showed delayed reconstitution of peripheral blood $CD27^+$ memory B cells, in some cases for years, and shorter responses seemed to be associated with repopulation with more memory B cells in RA [44,49–50] and in SLE [2,50]. It is not known whether this represents incomplete depletion of memory B-cell clones or increased maturation. In SLE prolonged response was associated with seronegativity for autoantibodies against extractable nuclear antigens (ENAs; RNP, Sm, Ro and La) [43].

Furthermore, it has been suggested that patients with SLE grouped based on the baseline levels of dsDNA antibodies demonstrate different kinetics of B-cell repopulation, with variability in time taken to clinical relapse following treatment with rituximab [51]. It was shown that patients with high dsDNA antibodies tended to flare earlier and had a greater frequency of plasmablasts whereas those with low dsDNA antibodies had a greater frequency of double-negative (IgD^-CD27^-) cells at the time of relapse, but not in remission.

Variability in changes in serum autoantibodies & correlation with clinical response

Although, it has been shown that seropositive RA patients respond better than seronegative RA patients [52] it is unclear whether clinical response correlates with the extent of decrease in autoantibody serum levels. Cambridge and colleagues noted in the initial study that in all patients with RA treated with rituximab, serum IgA, IgG and IgM levels decreased, and to below normal range for IgG in three patients and IgM in eight patients. Clinical response correlated with a significant decrease in the levels of autoantibodies, RF and anti-CCP antibodies [26]. In a study of 16 patients with SLE treated with rituximab at UCL, Cambridge and colleagues noted a variability in the extent of decrease in serum levels of anti-dsDNA both in responding and nonresponding patients at mean ± SD of 42 ± 36% and 60 ± 40% of baseline, respectively. An extension of the study to 50 patients with SLE also demonstrated a significant variability in biological response in terms of serum complement levels and anti-dsDNA antibodies [41]. Interestingly, in SLE, antibodies against ENAs did not decrease following rituximab treatment in contrast with anti-dsDNA [53,54] and were associated with poor response and/or early relapse [43].

Variability in the development of HACAs

The development of HACAs may contribute to lack of clinical response, particularly in SLE. However, in RA, it it does not seem to significantly influence the clinical response to treatment with rituximab. Nevertheless, patients with autoimmune diseases are more likely to develop HACAs when compared with patients with lymphoma. In the early Phase I/II study of rituximab in SLE HACAs were documented in six of 17 patients with SLE, who had African ancestry, higher disease activity and received low-dose rituximab [25]. As discussed earlier, the development of HACAs was <1% in B-cell malignancies whereas

HACAs were noted in 5% of patients with RA in the Phase II trial [24] and in 26% of patients with SLE in the EXPLORER trial [42].

Variability in rituximab pharmacokinetics

Variability in rituximab pharmacokinetics has been well documented in B-cell malignancies and RA. Early studies in NHL employing the same treatment regimen found remarkable variability in serum half-life and levels of rituximab achieved (see section 'What can we learn from the experience of using rituximab for B-cell malignancies?').

In RA, the relationship between rituximab levels and its effects as measured by peripheral B-cell count is also variable. Others and we have reported, despite the same dosing regimen employed, a remarkable variability in serum rituximab levels between patients with RA and SLE [55]. In RA, there is no evidence for correlation between rituximab levels and clinical response. A Phase II study involving 161 patients randomized to receive either methotrexate alone, rituximab + methotrexate or rituximab + cyclophosphamide or rituximab alone were compared. Serum rituximab levels (area under the curve) did not differ between the groups and peripheral $CD19^+$ B-cell levels did not correlate with clinical response as all patients achieved B-cell depletion (low sensitivity, <10 cells/μl) at 2 weeks [56]. The mean terminal half-life of rituximab in this study was 19–22 days. In a preliminary analysis of 102 patients from the same Phase II study cohort two parameters including body surface area and male gender influence rituximab pharmacokinetics to a minor extent [57]. Also, in RA, serum rituximab levels did not correlate with synovial B-cell depletion or clinical response [34]. We showed that at both 1 and 3 months after rituximab therapy, serum rituximab levels were highly variable in patients with RA and SLE. Furthermore, serum rituximab levels achieved in RA were >9-fold greater than that achieved in SLE at both 1 and 3 months [55]. A plausible explanation for this finding may lie in the fact that the serum half-life of IgG is lower in SLE when compared with RA; 8 and 14 days, respectively [58]. However, the underlying reasons for the interindividual variability in rituximab levels with in each disease category remain elusive.

Other factors: ethnicity

In a subgroup analysis in the LUNAR study of patients with lupus nephritis, Hispanic and African–American patients showed a favorable response to rituximab. In fact, in this subgroup of patients none required cyclophosphamide rescue therapy when compared with eight patients in the group that did not receive rituximab for the treatment of lupus nephritis [46]. The African–American/Hispanic patients with non-renal SLE also achieved a better clinical response, as reported in the EXPLORER study, with 13.8 versus 9.4% and 20 versus 6.3% major and partial clinical response with rituximab when compared with placebo [42]. There is no evidence of difference in clinical response to rituximab in patients with RA of different ethnicities.

Thus, several important factors are associated with poor clinical response (Table 2) and relapse following treatment with rituximab (Table 3).

So, what factors determine resistance to depletion with rituximab?

The exact mechanisms of depletion of B cells by rituximab and of resistance have been extensively studied in B-cell malignancies. by contrast, there remains a gap in our knowledge of the possible mechanisms of resistance to depletion by rituximab in the context of autoimmune diseases.

B cells from lupus-prone mice transgenic for human CD20 have greater resistance to depletion [67], which may, at least in part, be due to an acquired defect in Ig-dependent phagocytosis [68]. Murine studies suggest that B-cell depletion is dependent on both the cellular characteristics as well as the microenvironment [69]. Data on B-cell resistance to rituximab in patients with RA and SLE are limited. We have recently presented our preliminary results, which suggest that, *in vitro*, B cells from patients with RA and SLE are more resistant to depletion with rituximab when compared with B cells from healthy controls and that B-cell depletion may be improved by using newer anti-CD20 agents [70]. However, the mechanisms underlying the resistance to depletion remain elusive. Despite several differences in B-cell biology between autoimmune and malignant B cells and the microenvironment they survive in important lessons could be learned from the bulk of evidence from studies in B-cell malignancies.

Anti-CD20 mAbs evoke three main mechanisms of cell death: complement-dependent cell death (CDC); antibody-dependent cellular cytotoxicity (ADCC); and direct cell death (DCD) [71]. Factors that could influence the outcome of triggering of these cytotoxic mechanisms include the target antigen CD20, expression of complement defense proteins involved in CDC and Fc receptors that may determine the efficiency of ADCC.

Does the level of expression of the target antigen, CD20, matter?

CD20 expression of CLL cells is characteristically low [72] and can be higher in the circulating CLL cells when compared with CLL cells found in the bone marrow or

Table 2. Factors associated with poor response to rituximab in autoimmune disease.

Factor associated with poor response	Comments	Condition	Ref.
Good response correlated with a drop in CRP and autoantibodies	Reduction in autoantibodies would suggest that rituximab interupts the formation of autoantibody antibody secreting cells	RA	[26]
BM B-cell lineage cells at 3 months after RTX	Variability was noted in the proportion of pro- and pre-B cells and there was a trend towards better response in patients with better depletion	RA	[29]
Anti-ENA-positive patients were more likely to flare	Baseline autoantibody profiling may help predict response to rituximab in SLE	SLE	[43]
Incomplete depletion	Patients with incomplete B-cell depletion (<1 cell/μl) included all nonresponders	RA (n = 80)	[2]
Depletion of memory B cells in PB and BM (n = 8) at 3 months	Reduction of $CD19^+HLADR^+$ activated B cells. Insignificant reduction in BM B cells	RA (n = 11)	[59]
Nonresponders had a higher frequency of IgD^+CD27^+ MCs at the time of relapse	B-cell repopulation with a higher frequency of IgD^+CD27^+ MCs was associated with early relapse (no differences in subsets at baseline between Rs and NRs)	RA	[49]
BAFF levels (>1011 pg/ml), RF-negative and lymphocyte count >1875/μl		RA	[60]
FcRIIIa F158V polymorphism	High-affinity polymorphism associated with good response	RA (n = 177); and RA (n = 111)	[61,62]
FcRIIIa F158V polymorphism	High-affinity polymorphism associated with good response	SLE	[63]
No IFN type 1 signature	Genome-wide microarray in a small prospective RA cohort suggests IFN type 1 signature as a marker of nonresponse	RA (n = 14)	[64]
TTTT *BLyS* promoter haplotype in seropositive patients	The frequency of the promoter haplotype was comparable in both seropositive and seronegative patients, but good response to RTX was seen only in seropositive patients with the promoter haplotype. BLyS levels were not of predictive significance	RA (n = 152)	[65]
$IgJ^{hi}FCRL5^{lo}$, a combination biomarker of plasmablasts was associated with poor response	Pooled samples from RCTs	RA	[66]

BM: Bone marrow; ENA: Extractable nuclear antigens; MC: Memory cell; NR: Nonresponder; PB: Peripheral blood; R: Responder; RA: Rheumatoid arthritis; RCT: Randomized controlled trial; RF: Rheumatoid factor; RTX: Rituximab; SLE: Systemic lupus erythematosus.

lymph nodes [73]. The expression of CD20 was lowest in B-cell CLL; higher in FL, MCL and splenic lymphoma; and was highest in hairy cell leukemia. Using specific antimouse Ig antibodies it was shown that, in patients with CLL, rituximab treatment was associated with downmodulation of CD20. Consequently, rituximab was not bound to cell surface [74]. It was suggested that soluble CD20, found circulating in CLL, may also bind to rituximab and reduce its efficacy [75] with further influence by cytogenetic abnormalities such as *del(17)(p13.1)* [76]. Therefore, CD20 expression may, at least in part, explain the discrepancy in clinical response between the histological types. However, variation in CD20 expression by means of altered expression after treatment with rituximab [77] or due to genetic variances is thought to be rare and therefore unlikely to explain more frequent variability in clinical response noted between individuals. Data are lacking on the expression of CD20 in different B-cell phenotypes in autoimmune diseases.

CDC

In vitro studies using cells obtained from patients with CLL suggested that complement-mediated cytotoxic effects of rituximab correlate with expression of CD20 and blocking of complement defense proteins CD55 and CD59 [78]. However, CDC has not been shown to be the predominant cytotoxic mechanism *in vivo*. Rituximab led to significant depletion of B cells even in C1q-deficient mice and also it was shown that rituximab with K322A mutation of the Fc portion despite lacking the ability to bind complement or activate CDC still achieved significant depletion [79]. The expression of complement defense proteins has not shown to predict clinical response in FL [80]. Jones *et al.* showed that, *in vitro*, in the presence of sera from patients with seropositive RA, CDC induced by rituximab was inhibited [81]. However, whether such a phenomenon occurs *in vivo*, and if so, what effect this would have on the final degree of depletion and on clinical response remains to be determined.

Direct cell death

In the absence of activation of CDC or ADCC, DCD would be a direct mechanism of deleting B cells. However, the relative importance of this mechanism alone *in vivo* remains unclear. *In vitro* studies using tumor cell lines suggest a role for the cytokine IL-10, the levels of which correlated with Bcl-2 expression and resistance to rituximab-induced apoptosis. The mechanisms of inhibition of IL-10 synthesis are purported to occur through rituximab-mediated inhibition of p38 MAPK and STAT3 [82,83]. Following incubation with rituximab the secretion of IL-10 was inhibited, which would be expected to increase sensitivity to apoptosis, a plausible mechanism that may explain improved response rates noted with rituximab used in combination with CHOP [84].

Antibody-dependent cell cytotoxicity

ADCC is considered the most important mechanism of cell death *in vivo* in both FL and autoimmune diseases, RA and SLE. In FL, the high-affinity *FcγRIIIa* 158V polymorphism and FcγRIIa 131H were shown to be associated with better clinical response [85,86]. By contrast, polymorphisms of activatory Fc receptors (*FcγRIIIa* and *FcγRIIa*) do not predict response to rituximab in CLL [87], which suggests that the most important effector mechanism may vary between diseases. An outstanding study by Lim *et al.* suggests that rituximab modulates CD20, a process regulated by the inhibitory FcvRIIb on B cells. Briefly, the variability in clinical response to rituximab both at an individual level and histological type of lymphoma was shown to correlate inversely with B-cell expression of FcγRIIb [88]. Subsequently, although genetic polymorphisms in the inhibitory *FcγRIIb* 232I/T was found not to predict clinical outcome in FL [89], it was shown that the target tumor expression of the inhibitory FcγRIIb was predictive of response in FL with significantly better responses seen in patients with tumor targets expressing low levels of FcγRIIb [90]. Thus, Fc receptor polymorphisms and tumor expression of the inhibitory Fc receptors are important factors influencing clinical response to rituximab in FL.

Interestingly, SLE patients from the early small Phase I/II study were assessed for the genotypes of *FcRIIa* and *FcRIIIa* showing that clinical response and B-cell depletion were better in those with high affinity *FcRIIIa* 158VV genotype. This finding is similar to that noted in NHL, as discussed earlier, and implicates the importance of ADCC as an important mechanism evoked by rituximab *in vivo* in patients with SLE [63]. By contrast, another study reported a trend towards less good depletion was noted in SLE patients with the high-affinity *FcRIIIa* genotype [30]. In RA, a retrospective study found that the high-affinity *FcRIIIa* 158V variant

Table 3. Parameters associated with clinical relapse.

Factor associated with early clinical relapse	Comments	Condition	Ref.
Relapse was preceded by B-cell repopulation and an increase in levels of autoantibodies	These findings suggest that relapse occurs after B-cell repopulation	RA (n = 22)	[26]
Higher numbers of IgD^+CD27^+ memory B cells at repopulation	Repopulation occurred mainly with naive cells whereas relapse was associated with memory B cells	RA (n = 24)	[44]
Higher numbers of IgD^+CD27^+ memory B cells at baseline and at the time of repopulation	Variability in the frequency of IgD^+CD27^+ memory B cells at baseline	RA (n = 17)	[49]
Anti-dsDNA antibody levels and B-cell numbers, B-cell phenotype were predictive factors for relapse	Relapse was associated with B cell markers and anti-ds DNA antibody levels	SLE (n = 67)	[51]

RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus.

was associated with better clinical response [61], however, another study did not find such an association [91]. These studies suggest that ADCC may be the primary mechanism of depletion *in vivo* also in RA and SLE.

Alternative resistance mechanisms

An alternative proposed mechanism of resistance is 'shaving of rituximab–CD20 complexes', whereby, probably due to effector cell exhaustion, rituximab treatment, instead of triggering the effector cell phagocytosis or cytotoxic degranulation to cause target cell death, leads to 'trogocytosis' of RTX–CD20 complexes. It has been suggested that using low-dose more frequent regimens (fractionated subcutaneous rituximab thrice weekly) may minimize trogocytosis and preserve CD20 on the cell surface [92,93]. Further studies are warranted to evaluate the relevance of this phenomenon in real clinic situations and outside the CLL context with cells with lower expression of rituximab.

Also, it has been suggested that the type 1 interferon pathway may be activated in rituximab nonresponders in RA. An *in vitro* study of the effects of rituximab on B-cell lines transfected with certain viruses suggested that rituximab may promote the production of the proinflammatory cytokine type 1 interferon by B cells, a plausible mechanism of resistance to rituximab in SLE [94]. However, the EXPLORER study did not find a significant association between type 1 interferon expression and changes in serum complement or anti-dsDNA antibody levels to rituximab [54]. In RA, the expression of a cluster of type 1 interferon genes including *LY6E*, *HERC5*, *IFI44L*, *ISG15*, *MxA*, *MxB*, *EPSTI1* and *RSAD2* in peripheral blood cells distinguished responders and nonresponders [64]. However, it should be noted that rituximab-refractory disease may be due to incomplete depletion of B cells, persistence of CD20-B lineage cells such as plasmablasts and plasma cells, repopulation of nondepleted B cells, changes in subgroups of T cells or simply because the disease pathogenesis is independent of B cells. Disease activity may be B-cell dependent, particularly in SLE, where some but not all clinical manifestations may improve with rituximab. For example, the pool of plasma cells may be only partially affected by transient interruption of the 'feed-in' from B cells following their depletion and, therefore, plasma cells, as they are not directly affected by rituximab, continue to secrete DNA antibodies. The authors believe that B cells and autoantibodies drive disease in SLE and seropositive RA patients. We believe that poor depletion and long-lived plasma cells contributes to poor response. Our current research is also based on this hypothesis.

Biomarkers that predict response prior to treatment would be ideal, however, heterogeneity of patient characteristics and disease characteristics would be expected to limit the prospects. Therefore, biomarkers that accurately predict the course of response following treatment may serve to improve our understanding of disease pathogenesis and mechanisms of response and resistance to rituximab. For example, HSFC, B-cell-phenotype profile, serum BAFF levels, Fc receptor genotype, HACA development and immune architecture at tissue level.

In vitro experiments suggested that statins may impair rituximab effects by inducing conformational changes in CD20 in lymphoma cell lines [95]. However, the results of two studies in patients with RA treated with rituximab and receiving concomitant statins or not revealed conflicting effects. The Dutch Rheumatoid Arthritis Monitoring (DREAM) registry reported that patients receiving statins experienced nearly 2 months less effective period of rituximab benefit when compared with those not receiving statins [96] whereas another study, involving comparable number of patients, did not find a difference in clinical response between patients receiving statins or not [97]. Thus, *in vitro* findings may not translate into clinical effects; perhaps a key factor may be due to the activation of multiple effector pathways *in vivo* (as discussed earlier), which may be difficult to accurately study *in vitro.*

Thus, three important factors influence the variability in clinical response to rituximab: first, disease or condition being treated; second, host-related factors; and third, the biological agent, rituximab.

Conclusion

In summary, the precise mechanisms influencing variability of individual responses remain poorly understood. Similar to clinical studies in patients with B-cell malignancies, host-related factors such as Fc receptor polymorphisms and disease-specific factors including heterogeneity, target cell expression of CD20 and FcγRIIb may independently impact on interindividual variability in biological and clinical response to rituximab. Identification of the key effector pathways of response or resistance would provide new insights into developing patient tailored B-cell-targeted therapies for the treatment of autoimmune diseases.

Future perspective

Future B-cell-depletion strategies include employment of different treatment regimens such as using rituximab more frequently administered via the subcutaneous route, its use in combination or sequentially with other biologics such as BLYS/APRIL-targeting drugs such as belimumab, anti-TNF agents and tocilizumab, an anti-IL-6 receptor antibody. Newer anti-CD20 antibodies have been developed to increase their efficacy. Such modifications include antibodies such as 2F2 (ofatu-

mumab), which binds to a different epitope than that of rituximab and has remarkable slower off-rate and enhanced CDC [98] and has been shown to be effective in methotrexate-refractory RA [62]. Radiolabeled mAb conjugates such as ibritumomab (yttrium-90 labeled anti-CD20) and toxin-conjugated mAbs, some approved for B-cell malignancies, may prove to be too aggressive for autoimmune disease. Alternatively, second-generation anti-CD20 antibodies such as glyco-engineered GA101 or obinutuzumab, which is currently in Phase III studies for B-cell malignancies, may be used to overcome rituximab resistance in autoimmune diseases.

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Executive summary

Variability in biological response to rituximab: what does it mean & why does it matter?

- Interindividual variability in clinical response to rituximab occurs both in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).

Interindividual variability in depletion & clinical response

- Highly sensitive flow cytometry is a valuable tool to assess peripheral B-cell depletion.
- The degree of depletion and the pattern of B-cell reconstitution may serve as surrogate markers of rituximab resistance and/or refractory disease.
- Serum rituximab levels are remarkably variable between individuals with RA and SLE.
- Factors such as Fc gamma receptor polymorphisms that influence the host's immune system play an important role in determining biological and/or clinical response to rituximab in both RA and SLE.
- Several potential biomarkers of rituximab response are currently being evaluated for clinical utility.
- A better understanding of the variability in response to rituximab therapy is likely to provide new insights to optimize the use of rituximab and move us a step closer towards delivering personalized therapy.

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