Assessment of the Effects of Rituximab Monotherapy on Different Subsets of Circulating T-Regulatory Cells and Clinical Disease Severity in Severe Pemphigus Vulgaris

Rajsmita Bhattacharjee a Dipankar De a Sanjeev Handa a Ranjana W. Minz b Biman Saikia b Neha Joshi b
Departments of a Dermatology, Venereology and Leprology and b Immunopathology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

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Abstract
Background: Robust evidence for the efficacy of rituximab monotherapy in pemphigus is lacking. The effects of rituximab on T-regulatory cells (Tregs) in pemphigus have not been studied. Objective: The primary objective was to assess the efficacy of rituximab monotherapy in severe pemphigus vulgaris. The secondary objectives were to assess whether counts of different subsets of Tregs in the peripheral blood correlate with baseline clinical severity and whether clinical response in severe pemphigus is associated with an alteration in the Treg count. Methods: Eighteen eligible subjects with severe pemphigus vulgaris were recruited and were treated with 1 g of intravenous rituximab on days 0 and 15. Efficacy was assessed in terms of disease control, time to disease control, complete remission off therapy, and relapse. Flow cytometric analysis of CD4+CD25+FoxP3, IL-10-secreting Tr1, and TGF-β secreting Th3 regulatory cells was performed. Clinical evaluation and flow cytometric analysis of Tregs was performed periodically until follow-up at 26 weeks. Results: Rituximab monotherapy was able to induce complete remission in all but 5 (68.75%) patients and was well tolerated. No direct relationship between clinical severity and CD4+CD25+FoxP3 cell counts was found. There were inverse correlations between serially measured values of the cutaneous and mucosal Autoimmune Bullous Skin Disorder Intensity Score (ABSIS) and Th3 cell count. Conclusion: Rituximab is a safe and effective monotherapy option for severe pemphigus. As the immunological findings were somewhat different from those observed in other autoimmune conditions treated with rituximab, further studies are required to substantiate the findings of our study in pemphigus patients.

Introduction

Pemphigus is an autoantibody-mediated epidermal immunobullous disease of the skin and mucous membranes. Though these antibodies are produced by B cells, they are under regulatory control of several lineages of T cells. Modulating these T cells may prove to be a useful approach in the management of pemphigus.
During the development of ‘central tolerance’ of T cells against self-antigens in the thymus during fetal development, some may escape clonal deletion. Activation and expansion of such autoreactive T cells is kept in abeyance in the periphery (peripheral tolerance) by CD4+/CD25+ regulatory T cells known as T-regulatory cells (Tregs). These cells express FoxP3 (forkhead box P3), a transcription factor that plays a critical role in their development and function [1, 2]. There are other types of Tregs, the adoptive Treg (iTreg), which include FoxP3iTreg, IL-10-secreting T-inducible regulatory type-1 (Tr1), and TGF-β-secreting Th3 cells [3].

T cells play an important role in the regulation of B-cell activation. Both B and T cells are in turn under the regulatory control of Tregs. Treg counts in peripheral blood have been assessed in myriad autoimmune diseases like systemic lupus erythematosus, rheumatoid arthritis, and idiopathic thrombocytopenic purpura [4–6]. However, no evidence exists in the literature on whether counts of Tregs in the peripheral blood correlate with the severity of pemphigus vulgaris (PV) and whether response to rituximab is associated with an increase in Treg count in the peripheral blood. We planned to assess whether Treg counts of different subsets in the peripheral blood correlate with baseline severity of PV and whether clinical response in severe PV patients treated with rituximab is associated with an alteration in the Treg count in the peripheral blood.

Rituximab has shown efficacy as a corticosteroid-sparing agent in the treatment of resistant cases of pemphigus. Preliminary reports suggest that rituximab as monotherapy may be effective in pemphigus [7–9]. Assessment of the efficacy of rituximab monotherapy on the clinical severity was another objective of our study.

**Materials and Methods**

For further details, see the supplementary materials (for all online suppl. material, see www.karger.com/doi/10.1159/000448031) [10–12] (fig. 1).
Results

Clinical Assessment

The mean cutaneous and oral Autoimmune Bullous Skin Disorder Intensity Scores (ABSIS) at baseline were 19.42 ± 10.32 (range: 9.5–40) and 5.4 ± 2.4 (range: 3–10), respectively. Sixteen patients completed the clinical follow-up of 26 weeks. Eleven patients (68.75%) were in complete clinical remission (both oral mucosal and cutaneous) at 26 weeks.

Cutaneous ABSIS after Rituximab (online suppl. fig. 1a–d)

There was a downward trend for the mean cutaneous ABSIS throughout the follow-up period from baseline to 26 weeks (online suppl. fig. 2a). By 14 weeks of follow-up (12 weeks after the second dose of rituximab), 10 patients (58.8%) had achieved cutaneous ABSIS of 0. Fourteen patients (87.5%) had achieved cutaneous ABSIS of 0 at the end of 22 weeks. Two patients (12.5%) had persistent cutaneous lesions at the end of 26 weeks and had failed to achieve clinical remission.

Oral Mucosal ABSIS after Rituximab

There was a downward trend for the mean oral ABSIS as well throughout the study period of 26 weeks (online suppl. fig. 2b). Of the 15 patients who had mucosal lesions to start with and completed the total intended study period of 26 weeks, 5 (33.33%) had persistent oral lesions and had failed to attain an oral mucosal ABSIS of 0. One of these patients also had persistent cutaneous lesions. The mean mucosal ABSIS at the end of the 26 weeks was 0.5 ± 0.7.

Flare after Rituximab

Out of a total of 17 patients who were given the second dose of rituximab, 8 (47.1%) experienced flare after the first dose of rituximab; none of the patients experienced any flare at any time point after the second dose.

Correlation of Flare after Rituximab with Baseline ABSIS

The cutaneous ABSIS at baseline of those patients who experienced flare of disease in the form of new lesions after receiving the first dose of rituximab was 26.19 ± 12.22 vs. 12.75 ± 1.98 for patients who did not have a flare. Using the Mann-Whitney U test, this difference was statistically significant (U = 12.5, p = 0.04), i.e. patients with a higher baseline pre-treatment ABSIS were more likely to experience flare after the first dose of rituximab. There was no statistically significant difference between the mean mucosal ABSIS of those patients who had cutaneous flare (6.13 ± 2.47) and those who did not (4.38 ± 2; p = 0.142).

Steroids Needed for Disease Flare

Eight patients experienced flare of the disease at some point of time. The mean cumulative dose of oral prednisolone (for control of flare) needed by these 8 patients was 682.5 ± 97.21 mg. No other adjuvant drugs (topical or oral) were given.

Efficacy Analysis

Time to disease control and complete remission off therapy

Time to disease control (TDC) was 9 ± 3 weeks in our study. Since 5 patients had persistent oral lesions even at the end of the study period, we calculated the time to cutaneous remission off therapy and complete (cutaneous and mucosal) remission off therapy separately. The mean time to cutaneous remission off therapy in our study was 18 ± 3.38 weeks. The mean time to complete remission off therapy was 23.2 ± 2.7 weeks.

Correlation between Baseline ABSIS and TDC

Spearman’s correlation co-efficient (r) analysing the relation between the mean cutaneous ABSIS at 0 weeks (baseline) and TDC was 0.532 (p = 0.034), which is statistically significant (online suppl. fig. 3a). This means that with an increase in the baseline cutaneous ABSIS, the TDC also increased.

Similarly, Spearman’s correlation co-efficient (r) analysing the relation between the mean cutaneous ABSIS at baseline and time to cutaneous remission off therapy (defined earlier) was also statistically significant (online suppl. fig. 3b).

Adverse Effects Assessment

One patient experienced mild infusion reaction (not requiring treatment abandonment) at the time of the first dose of rituximab. No adverse effects were observed at the time of the second dose. No post-rituximab adverse effects were observed in any patient up to the completion of the 26-week study period.

Relapse

No relapse occurred in any patient who achieved cutaneous remission in the relatively short study period of 26 weeks.
Immunological Assessment

Immunological data was available for the entire 26-week study duration for only 14 patients. The statistical analysis for immunological assessments was done for these 14 patients.

Comparison of Different Tregs in Cases at Different Time Points and Controls (Once Only at Baseline)

In order to compare the mean Treg count in cases at different time points with controls, the Mann-Whitney U test was used. We found that except for TGF-β-secreting Th3 cells, the other two subsets of Tregs were less in pemphigus patients at baseline compared with controls (online suppl. table 1). Except at baseline, Tr1 cells in patients were significantly less compared with controls at all other time points. The CD4+CD25+FoxP3+ cells were not significantly different between cases and controls at any of the observed time points.

Changes in Tregs with Rituximab over Different Time Points of Assessment

No specific trend was observed in the CD4+ CD25+FoxP3 Treg counts after rituximab infusions, though a substantial increase in the mean count was observed at 14 weeks, the time by which the majority of the study subjects achieved clinical remission.

The mean count of Tr1 cells increased at the 2-week assessment and then gradually declined over the remaining study period. Similarly, though no definite trend was observed with the mean values of TGF-β-secreting Th3 cells, there was an increase in the mean value at the 14-week assessment.

Correlation between Pemphigus Disease Severity and Different Subsets of Tregs

In order to assess the correlation between pemphigus disease severity (in terms of cutaneous and mucosal AB-SIS) and the different subsets of Tregs at baseline and at different observed immunological time points, the trendlines between the parameters were analysed using the generalized estimating equation method of longitudinal panel data, with cutaneous and mucosal ABSIS as dependent variables and the 3 types of Tregs measured as time series variables (online suppl. fig. 4a–c).

We observed that the trendline for CD4+CD25+FoxP3 cell count increased from baseline to the 26-week follow-up, whereas line plot for actual ABSIS (cutaneous and mucosal) spiralled down over the follow-up time period. A sharp decline in the cutaneous ABSIS was observed from week 2 onwards, while the CD4+CD25+FoxP3 cell count showed an increase with a peak at 14 weeks. However, there was no direct relationship between serial values of cutaneous ABSIS and CD4+CD25+FoxP3 cell counts at any of the time points.

Similarly, the trendline for cutaneous ABSIS was directly influenced by the Tr1 trendline, and this direct relationship between serial values of cutaneous ABSIS and Tr1 cell counts was almost statistically significant (p = 0.097). There was no statistically significant relationship between serially measured mucosal ABSIS versus Tr1 cell counts at any of the time points.

On analysing the correlation between the trendlines for cutaneous and mucosal ABSIS with TGF-β-secreting Th3 cells, we found an inverse correlation between serially measured values of cutaneous ABSIS and Th3 cell count, and this was statistically significant (p = 0.026). There was an inverse relationship between serially measured TGF-β-secreting Th3 cells and mucosal ABSIS as well, which was also statistically significant (p = 0.037).

Discussion

Rituximab has been used in pemphigus mainly as an adjuvant to corticosteroids or immunosuppressives. Of late, considering the unfavourable side effect profile of long-term high-dose steroids and other immunosuppressives, rituximab monotherapy as the frontline treatment is emerging as a viable option, even for patients presenting with severe pemphigus. However, the evidence is not robust. Craythorne et al. [7] treated a PV patient with rituximab at a dosage of 375 mg/m^2 for 8 weekly infusions, followed by monthly infusions of 375 mg/m^2 for 4 months. He was completely clear of all PV lesions, was in remission after the sixth infusion, and remained disease free without the need for adjuvant corticosteroid or other immunosuppression until 16 months later.

We set out to study rituximab as monotherapy in patients with PV. Though there was provision for the use of oral prednisolone in the case of severe disease flare, 8 patients (of the 16 who completed the clinical follow-up) did not require oral steroids at all and achieved clinical remission. The mean total dose of prednisolone required for control of flare was only 682.5 mg. In the study by Sharma et al. [13], where prednisolone was started at 0.5 mg/kg/day (and tapered over a period of 3–4 months) along with
either azathioprine or cyclophosphamide, the total cumulative dose of prednisolone used was 3,535.64 mg.

The mean time to control of disease activity in our study was 9 ± 3 weeks. The mean TDC in various earlier studies using rituximab with concomitant oral corticosteroids ranged from 6 to 8 weeks [14, 15].

Cutaneous remission off therapy in our study was achieved at a mean of 18 ± 3.38 weeks. The mean time to overall (cutaneous and mucosal) remission off therapy was 23.2 ± 2.7 weeks. Kim et al. [16] reported partial remission at 19 weeks and complete remission at 27 weeks in 27 patients who were unresponsive to conventional treatments and received weekly infusions of rituximab at a dose of 375 mg/m² body surface area. Kanwar et al. [17] observed that the TDC was approximately 7 weeks for both the low- and high-dose groups, while time to complete remission was paradoxically 25.2 weeks for the group receiving high-dose rituximab and 18 weeks for the low-dose group. In both of these studies, however, rituximab was not used as monotherapy. Thus, the time to achieve complete remission off therapy in our study was similar and comparable to that in previously published studies with rituximab.

Experience in rituximab monotherapy for pemphigus is limited. In a report of 5 cases [9], the mean time to achieve complete or nearly complete healing of the disease after the first infusion of rituximab was 15 weeks (range: 12–20 weeks). These patients also received daily applications of 5–20 g of clobetasol propionate and methylprednisolone mouthwashes [9]. In a study by Joly et al. [18], 5 of the 21 patients received rituximab without corticosteroids because of contraindications; 4 of them were in complete remission at 3 months, as were the 16 patients receiving corticosteroids.

The effect of rituximab on CD4+ CD25+ FoxP3+ cells has been studied in other autoimmune diseases like idiopathic thrombocytopenic purpura [6], lupus nephritis [19], myasthenia gravis [20], and rheumatoid arthritis [5]. Treatment with rituximab led to an increase in this subset of Tregs in all of these conditions. This increase in cell count was irrespective of clinical severity status.

It has been reported that PV patients have a decreased number of CD4+ CD25+ Tregs compared with healthy controls in the peripheral blood [21]. This is the first study to evaluate the different subpopulations of Tregs in pemphigus patients and the effects of rituximab on them at different time points.

In our study, we observed that both CD4+ CD25+ FoxP3+ and IL-10-secreting Tr1 Tregs were less in pemphigus patients at baseline compared with controls. We could not find any specific trend in the CD4+CD25+FoxP3 Treg counts after rituximab infusions. We also could not elucidate any direct relationship between serial values of cutaneous ABSIS and CD4+CD25+FoxP3 cell counts at any of the time points, though their trendlines did show divergent directions. Considering the fact that FoxP3 is thought to be the most reliable marker of Tregs, and CD4+CD25+FoxP3 Tregs play critical roles in maintaining immunological self-tolerance by suppressing autoreactive T cells, and the deficiency of Tregs causes autoimmune diseases, our results are probably influenced by our small sample size and limited follow-up. For comprehensive immunological evaluation, serial assessment of serum anti-desmoglein antibody levels and count of CD19 positive cells were desired but could not be performed due to constrained finances.

In conclusion, rituximab is a safe and effective monotherapy option for severe PV. There are two important observations made in this study. Firstly, there was a positive correlation between baseline cutaneous ABSIS and flare of the disease after the first infusion of rituximab, suggesting that those patients with a more severe disease at baseline may require systemic corticosteroids for control of their flare. Secondly, there was a positive correlation between baseline cutaneous ABSIS and TDC and time to cutaneous remission off therapy, suggesting that those having more severe disease at baseline would require a longer TDC. The effects of rituximab on Tregs in this study are somewhat different from those observed in autoimmune conditions other than pemphigus treated with rituximab. This could have been due to a number of factors: (1) the use of other concomitant immunosuppressants/immunomodulators in the other studies, (2) Tregs behave differently in pemphigus patients compared with other autoimmune conditions or (3) the sample size was relatively small in our study.

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**Statement of Ethics**

This study protocol was approved by Ethics Committee (Intramural) of the Postgraduate Institute of Medical Education and Research, Chandigarh, India.

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Disclosure Statement

None of the authors have any conflict of interest to declare.

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