Repeated Treatment With Rituximab Based on the Assessment of Peripheral Circulating Memory B Cells in Patients With Relapsing Neuromyelitis Optica Over 2 Years

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Objective: To evaluate the efficacy and safety of repeated rituximab treatment based on the assessment of peripheral circulating memory B cells over 24 months in patients with relapsing neuromyelitis optica (NMO).

Design: Prospective open-label study.

Setting: Institutional referral center for multiple sclerosis.

Patients: Thirty patients with relapsing NMO or NMO spectrum disorder.

Intervention: Treatment protocol of rituximab consisted of an induction therapy (375 mg/m² once weekly for 4 weeks or 1000 mg infused twice, with a 2-week interval between the infusions) followed by maintenance therapy. The maintenance therapy was repeated treatment with rituximab (375 mg/m², once) whenever the frequency of reemerging CD27⁺ memory B cells was more than 0.05% in peripheral blood mononuclear cells by flow cytometric analysis.

Main Outcome Measures: Annualized relapse rate, disability (Expanded Disability Status Scale score), anti–aquaporin 4 antibody level, and safety of rituximab treatment.

Results: Of 30 patients, 28 showed a marked reduction in relapse rate while taking rituximab over 24 months. The relapse rate was reduced significantly, by 88%, and 70% of patients became relapse-free over 24 months. Disability either improved or stabilized in 97% of patients. Anti–aquaporin 4 antibody levels declined significantly following treatment with rituximab, consistent with the clinical response and the effect on CD27⁺ memory B cells. Repeated treatment with rituximab was generally well tolerated, and no clinically relevant adverse event leading to discontinuation of treatment was observed.

Conclusion: Repeated treatment with rituximab appeared to produce consistent and sustained efficacy over 24 months with good tolerability in patients with NMO.
rituximab retreatment subsequently experienced an exacerbation, and 48% of patients had relapses after initial rituximab treatment. Thus, it seems likely that repeated treatment with rituximab may be necessary to prevent a relapse. Consequently, questions remain how and when patients with NMO should receive further treatment. Subsequent cycles of rituximab treatment have been recommended in patients with rheumatoid arthritis (RA) based on clinical disease activity, including a return of RA symptoms, and the investigator’s discretion. However, the symptoms of an NMO relapse do not only gradually present like RA, but 1 relapse can cause severe neurological impairment. Thus, ideally, the patient with NMO would be retreated before clinical relapse. Furthermore, repeated treatment with rituximab at 6- to 9-month intervals was not sufficient to prevent recurrence of NMO in every patient. Accordingly, reliable biomarkers must be identified for better stratification of repeated rituximab treatment to fit the needs of patients with NMO.

Memory B cells are generated in germinal centers in response to T cell–dependent antigens. These cells have undergone somatic hypermutation of their rearranged immunoglobulin V region genes and rapidly differentiate into high-affinity plasma cells following a reencounter with the immunizing antigen. Depletion of memory B cells can indirectly affect the production of anti–AQP4-Ab by short-lived plasma cells. Accordingly, we hypothesized that it may be possible to determine when additional rituximab should be administered by assessing the frequency of memory B cells in peripheral blood mononuclear cells (PBMCs).

This study was performed not only to assess the clinical efficacy and safety of rituximab following repeated treatment in patients with NMO, but also to evaluate the effect of memory B cell depletion on clinical response and anti–AQP4-Ab levels.

METHODS

PATIENTS

Patients were eligible for the study if they had relapsing NMO according to the 2006 diagnostic criteria or NMO spectrum disorders. An additional eligibility criterion was that at least 1 relapse had occurred during the 12 months before the start of rituximab therapy. Of the 30 patients enrolled, 24 (80%) were previously treated with maintenance immunotherapies but had experienced ongoing relapses. Of the 24 patients, 23 patients stopped their previous immunotherapies immediately before starting rituximab, and 1 patient had stopped the previous treatment for 3 months before rituximab treatment. Patients with cardiac dysfunction, hepatic or renal disease, a history of cancer and chronic infection, or abnormal complete blood cell count, pregnant women, and those of reproductive age who were not willing to use contraception were excluded. Pretreatment testing for serum JC virus was required. Rituximab was provided as an off-label, unsponsored medication, and all patients were informed of potential adverse effects and risks. Treatment protocols were approved by the institutional review board of the National Cancer Center and written informed consent was obtained from all patients.

TREATMENT PROTOCOL

The rituximab therapy consisted of induction and maintenance phases during the 24 months (Figure 1). Two regimens were used as an induction treatment: (1) 375 mg/m² infused once per week for 4 weeks (n=16) and (2) 1000 mg infused twice, with a 2-week interval (n=14). These regimens were based on the use of rituximab by patients with lymphoma and RA and the previously reported studies of patients with NMO. Because rituximab was provided as an off-label medication, we used both regimens according to patient compliance and economic situation. Peripheral blood samples were obtained at baseline, every 6 weeks through the first year, and every 8 weeks thereafter to evaluate lymphocyte subsets including CD27+ memory B cell and anti–AQP4-Ab levels. The therapeutic target of CD27+ memory B cell depletion was defined as being less than 0.05% in PBMCs. Whenever the frequency of memory B cells was 0.05% or more in PBMCs, patients were given an additional infusion of rituximab (375 mg/m²).

CLINICAL ASSESSMENT

The primary end point was annualized relapse rate per patient. The secondary end points were an assessment of neurological status using the Expanded Disability Status Scale (EDSS) score and the safety of rituximab. Attacks were defined as objective worsening of new neurological symptoms lasting at least 24 hours that increased the EDSS score by at least half a step (0.5) or increased 1 point on 2 different functional systems of the EDSS or 2 points on 1 of the functional systems (excluding bowel/bladder or cerebral functional systems). Clinical adverse events were recorded throughout the study. Complete blood cell count and measurement of serum glutamic pyruvic transaminase and serum glutamic oxaloacetic transaminase levels were performed prior to and after every infusion. Serum IgG level and JC virus status were evaluated at baseline and every 6 or 12 months, respectively.

FLOW CYTOMETRIC ANALYSIS

We used simple whole-blood staining to characterize the leucocyte and B cell subsets directly from the circulation. Triple-color immunofluorescent staining of whole-blood samples was performed within 60 minutes of blood drawing using antibodies directed against CD4/CD3/CD19 and CD27/CD19 with isotype controls, followed by red blood cell lysis and immediate acquisition and analysis by flow cytometry.

Figure 1. Treatment protocol. IV indicates intravenous; NMO, neuromyelitis optica.
SERUM LEVELS OF AQP4-Ab

Anti–aquaporin 4 antibody levels were measured by enzyme-linked immunosorbent assay, as described previously.22 Briefly, we cloned human AQP4, expressed the protein in a baculovirus system, and used purified recombinant human AQP4 as an antigen. Enzyme-linked immunosorbent assay plate wells (Nunc-Immuno plate, Maxisorp; Nunc, Roskilde, Denmark) were coated with 50 µL of human AQP4 (1 µg/mL) in 0.7% β-octyl-glucoside, 20mM TRIS-hydrochloride (pH 7.5), and 300mM sodium chloride and incubated overnight at 4°C. The plates were washed (polysorbate 20, 0.1%, fetal bovine serum, 5% [Thermo, Ontario, Canada]) in phosphate-buffered saline (pH 7.4) and blocked with fetal bovine serum, 10%, in phosphate-buffered saline overnight at 4°C. The plates were incubated for 1 hour at room temperature with 50 µL of serum diluted (1:1000) with fetal bovine serum, 5%, in phosphate-buffered saline. The plates were washed and incubated for 1 hour at room temperature with peroxidase-conjugated goat antihuman IgG (Jackson ImmunoResearch, West Grove, Pennsylvania) diluted (1:10 000) with fetal bovine serum, 5%, in phosphate-buffered saline. The plates were washed again and treated with 50 µL of TMB substrate solution (BD Pharmingen, San Diego, California) for 15 minutes at room temperature. Finally, 30 µL of ammonium sulphate was added to terminate the reaction. Optical density was measured at 450 nm. Each sample was corrected for its non-specific background binding by subtracting the optical density values obtained from the blocked wells without serum or antigen. Serum was considered to be anti–AQP4-Ab positive if the optical density was more than 3 SDs above the mean value for the 43 healthy control samples. Every plate included positive and negative controls, which were confirmed by both tissue-based and cell-based indirect immunofluorescence assay. The sensitivity and specificity of the assay were 72% and 98% for NMO, respectively. Intraplate and interplate reproducibility for optical density values expressed by intraclass correlation coefficient were 0.990 and 0.979, respectively.

STATISTICS

The annualized relapse rate, EDSS score, and serum anti–AQP4-Ab levels were compared before and after 24 months of rituximab treatment using the Wilcoxon signed-rank test and the 2-sided sign test. All statistical analyses were performed using GraphPad PRISM (GraphPad, La Jolla, California), and \( P < .05 \) was considered statistically significant.

RESULTS

PATIENT CHARACTERISTICS

Thirty patients were enrolled and treated (27 women, 3 men). Twenty-one patients were positive for anti–AQP4-Ab, and 9 seronegative patients satisfied the NMO diagnostic criteria.18 The average age for starting rituximab

Table 1. Baseline Clinical Characteristics of the Patients Treated With Rituximab

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean (SD) (N=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>38.4 (10.5)</td>
</tr>
<tr>
<td>Onset age, y</td>
<td>33.9 (10.7)</td>
</tr>
<tr>
<td>Female, No. (%)</td>
<td>27 (90)</td>
</tr>
<tr>
<td>Disease duration, y</td>
<td>4.5 (3.8)</td>
</tr>
<tr>
<td>No. of total attacks prior to rituximab treatment</td>
<td>6.8 (5.4)</td>
</tr>
<tr>
<td>No. of attacks in 1 y prior to study entry</td>
<td>2.9 (1.3)</td>
</tr>
<tr>
<td>EDSS score</td>
<td>4.4 (2.1)</td>
</tr>
<tr>
<td>Seropositivity for anti–aquaporin 4 antibody, No. (%)</td>
<td>21 (70)</td>
</tr>
</tbody>
</table>

Abbreviation: EDSS, Expanded Disability Status Scale.

Figure 2. Relapses in patients with neuromyelitis optica before and after treatment with rituximab. On the x-axis, 0 indicates the start date of treatment. Each interrupted line on the y-axis represents a patient.
therapy was 38.4 years (range, 23-58 years) and the median interval from the onset of NMO to treatment with rituximab was 4.5 years (range, 0.5-12.9 years). The median interval between the last relapse and the start of rituximab treatment was 2.8 months (range, 1-10 months). Clinical and demographic profiles of the patients are outlined in Table 1.

TREATMENT EFFICACY

Of the 30 patients, 28 showed a marked reduction in relapse following rituximab treatment over 24 months. The mean pretreatment annualized relapse rate was 2.4 (range, 0.4-8.0 relapses), and the mean posttreatment annualized relapse rate was 0.3 (range, 0-4 relapses; P < .001). The number of relapses during the preceding 2 years before rituximab treatment was 121 and it became 14 during the 2 years after starting rituximab therapy. Twenty-one patients (70%) became relapse-free during rituximab treatment (Figure 2). The EDSS scores improved in 24 patients and stabilized in 5. The mean EDSS score before starting treatment was 4.4 (range, 1.0-8.5), whereas it was 3.0 (range, 1.0-7.5; P < .001) at the end of treatment. However, 1 patient (patient 26) had 4 relapses despite rituximab treatment and switched treatment from rituximab to mitoxantrone hydrochloride 9 months after starting rituximab and 1 patient (patient 16) had 3 relapses during treatment in which 2 relapses were associated with delayed retreatment because the patient did not keep to the follow-up schedule.

The frequency and interval to retreatment varied between individual patients. After the induction treatment, patients received a mean of 3.7 retreatments (range, 2-7 retreatments), for a mean cumulative dose of 4153 mg over 24 months. The mean interval to retreatment was 23.6 weeks (range, 6-41 weeks). There was no difference in their clinical efficacy and the retreatment interval depending on the previous treatment (eg, interferon vs azathioprine vs prednisolone).

The anti–AQP4-Ab levels declined or maintained a low level after rituximab treatment in the majority of 21 seropositive patients (Table 2 and Figure 3 and Figure 4). However, 1 patient (patient 26) who showed a poor clinical response to rituximab had an increase in anti–AQP4-Ab level despite frequent reinfusion of rituximab (Figure 3H). Additionally, 1 patient (patient 16)
Figure 3. Association of clinical relapse with frequency of CD27⁺ memory B cells and anti–aquaporin 4 (AQP4) antibody level in 9 patients with neuromyelitis optica. The cutoff optical density (OD) for a positive anti-AQP4 antibody level was 0.170.
did not show a significant change in anti–AQP4-Ab level after 2 years, compared with the baseline level. In the early phase of treatment, anti–AQP4-Ab level decreased, but delayed retreatment caused an increase of the anti–AQP4-Ab level accompanied by clinical relapses (Figure 3E). Among the 9 patients who were initially seronegative, 6 maintained seronegative status throughout the 24 months without a relapse (Figure 4D), and 2 patients (patients 9 and 25) were seropositive transiently at the time of relapse (Figure 3A and G). However, 1 patient (patient 13) had a relapse following a reemergence of B memory cells but was still seronegative, even at relapse (Figure 3D).

When the patient (patient 26) who stopped treatment 9 months after starting rituximab was excluded, there were no significant differences between patients treated with the 2 induction regimens in the number of relapses during treatment (P = .65), the percentage of relapse-free patients (P = .73), and mean anti–AQP4-Ab level (P = .58) 2 years after rituximab treatment. The second group of 14 patients needed a subsequent cycle earlier than the first group did (mean, 11.8 weeks vs 18.7 weeks; P = .03), so retreatment with rituximab was more frequent in the second group during 2 years (4.2 vs 3.3 times; P = .048), but mean cumulative dose did not show a significant difference (P = .31).

FLOW CYTOMETRIC ANALYSIS

At baseline, the frequency and total number of CD3+ T cells, CD14+ monocytes, CD19+ B cells, CD27+ B memory cells, and CD27− naive B cells in peripheral blood did not differ significantly among the patients who were being treated with interferon beta, azathioprine, and prednisolone. The frequency of CD27+ memory B cells in the peripheral blood decreased promptly from a median of 2.19% (range, 0.35%-8.02%) to a median of 0.025% (range, 0.00%-0.36%) at 6 weeks after rituximab induction treatment. After every rituximab retreatment at a dose of 375 mg/m² once, CD27+ memory B cell frequency decreased again to the level of our therapeutic target in 29 patients; 1 patient had continual relapses (patient 26). The reemergence of CD27+ memory B cells after rituximab retreatment had a characteristic pattern. After rituximab retreatment, CD27+ memory B cells maintained their therapeutic depletion status during a certain period. When the CD27+ memory B cell frequency reached near 0.05%, the percentage increased abruptly by the next scheduled monitoring (Figure 3 and Figure 4).

RELAPSES DURING RITUXIMAB TREATMENT

Fourteen relapses occurred in 9 of 30 patients over 24 months (Figure 3). First, 5 relapses in 5 patients (pa-
patients 10, 16, 20, 25, and 27) occurred during the early treatment stages when depletion of memory B cells following rituximab therapy was not sufficient yet. Second, 3 relapses in 2 patients (patients 11 and 16) were associated with delayed retreatment. In particular, patient 16 was retreated after the frequency of CD27+ memory B cells was confirmed to be significantly high (0.4%), but a relapse occurred 2 weeks after the retreatment. Third, 2 relapses in 2 patients (patients 9 and 13) occurred despite following the treatment protocol. Finally, 1 patient (patient 26) had 4 relapses during 9 months of rituximab treatment that were associated with incomplete depletion of CD27+ memory B cells despite frequent rituximab infusions. The severity of neurological impairment following a relapse during rituximab treatment was much less than that at pretreatment except for 3 relapses in 2 patients (patients 9 and 26).

The median frequency of CD27+ memory B cells at the 14 relapses was 0.07% (range, 0.05%-0.58%). Thirteen of the 14 relapses occurred with sustained high titers of anti–AQP4-Ab, mostly at the early stage of treatment or followed by an increase in anti–AQP4-Ab titers compared with the previous value. However, anti–AQP4-Ab levels at relapses varied between and within individuals and rising antibody titers did not always lead to relapse.

**ADVERSE EVENTS**

The most common infusion-related reactions, noted in 12 of 30 patients (40%) during the first infusion, were transient hypotension and mild to moderate flulike symptoms, such as a febrile sense, headache, and skin rash. These adverse events were transient and managed well with methylprednisolone. Approximately 40% of patients experienced at least 1 infection during the study period. The most frequently reported infections included nasopharyngitis, upper and lower respiratory tract infection, and urinary tract infection. No case of serious infection or malignancy leading to discontinuation of treatment was observed. Serum IgG titers remained normal and no patient became seropositive for the JC virus over the 24 months.

**COMMENT**

Rituximab is one of the agents that has demonstrated efficacy in previous studies and case series of NMO.²⁻¹⁴,²₃ This study not only adds support to the efficacy of rituximab in NMO but also suggests that repeated rituximab treatment based on the assessment of CD27+ memory B cells in peripheral blood can maintain clinical efficacy over 24 months with good tolerability. The relapse rate was significantly reduced, by 88%, and disability either improved or stabilized in 97% of patients. Among the 14 relapses during the 2-year study period, 5 relapses occurred when depletion of memory B cells following rituximab therapy was not sufficient yet, 3 relapses were associated with delayed retreatment, and 4 relapses occurred in 1 patient in whom treatment failed. Only 2 relapses were observed after memory B cells were successfully depleted and the therapeutic depletion maintained by following the treatment protocol. A significant reduction in anti–AQP4-Ab levels was observed following rituximab treatment, consistent with the clinical response.

The temporal association of clinical relapses with increases in memory B cell and anti–AQP4-Ab levels when retreatment was delayed suggested the relevance of repeated rituximab treatment. In particular, relapses that occurred during the second year indicated that reemerging CD27+ memory B cells, even after repeated depletion with rituximab, may still be relevant in the immunopathology of NMO. Thus, sustaining the depletion of memory B cells through repeated treatment may be a key for the clinical effects of rituximab in patients with NMO. The relationship between memory B cell depletion and the clinical response to rituximab has been suggested in studies of patients with RA. CD27+ memory B cell depletion is connected to the duration of response and repeated CD27+ memory B cell depletion by rituximab is associated with a sustained reduction in the number of relapses.²⁴,²⁵

In the present study, a single retreatment with 375 mg/m² of rituximab was sufficient to induce depletion of CD27+ memory B cells again to the therapeutic target, with a sustained clinical response. In previous studies, patients with NMO were retreated with rituximab in two 1000-mg doses 2 weeks apart or 4 additional doses of 375 mg/m² when CD19+ B cells became detectable in the peripheral blood or at 6- to 12-month intervals.²⁴,²⁵ Although our patients were retreated more frequently, we used much less than a conventional retreatment dose. Therefore, the cumulative dose would be much lower than the expected dose of previous studies.²⁴,²⁵ More importantly, our treatment protocol showed a clinical benefit for up to 97% of patients from rituximab, compared with 75% to 80% of patients in previous studies.²⁴,²⁵ These findings suggest that frequent retreatment with rituximab can enable the disease activity to be controlled even with a lower dose and that more patients than previously thought may benefit from rituximab therapy if its use is refined.

The therapeutic efficacy of rituximab clearly supports the role of B cells in driving the NMO inflammatory process. Nevertheless, it is difficult to ascertain which of the B cell effects is primarily responsible for the benefit. Diminished production of anti–AQP4-Ab may be a contributing factor, but the extent of the decrease seems insufficient to support this assumption. Moreover, 9 patients who were seronegative at baseline also showed a significant clinical benefit following rituximab treatment, and 1 relapse occurred in a patient who was seronegative. Other plausible modes of action include an effect on costimulatory molecules required for clonal expansion of T cells, an inhibition of the antigen-presenting role of B cells, suppression of the cytokine network, or induction of immunoregulatory T cells by rituximab.²⁶⁻²⁸

Although most patients showed a good response to rituximab treatment, 1 patient did not benefit from rituximab treatment probably because of incomplete depletion of CD27+ memory B cells. Accordingly, insufficient depletion of memory B cells in peripheral blood despite repeated rituximab treatment may predict a poor response.
clinical response. Why rituximab appeared to show a variation in its ability to deplete peripheral B cells is not completely understood. Several factors, such as Fc receptor polymorphism and human anti-chimeric antibodies, have been suggested as potential contributors to inadequate depletion after treatment with rituximab.29,30

The safety profile of rituximab in this study was consistent with previous experience, including that of repeated courses in patients with RA and NMO, with no new or unexpected safety signals being observed.14-16,31,32 No concrete evidence indicates that repeated long-term use does not result in hypogammaglobulinemia. Popa et al33 recently described patients who received 5 to 7 courses of rituximab and in whom the IgG levels tended to decrease over time, but the infection rate did not increase. Moreover, the protective titers of antibodies to tetanus and antipneumococcal capsular polysaccharide were not affected by repeated rituximab treatment.34 Circulating antimicrobial antibodies and other immunoglobulins appear to arise from long-lived plasma cells, which do not depend on B memory cells to replenish their pool, but may also be responsible for the safety profile of the drug.26,35

Progressive multifocal leukoencephalopathy after rituximab treatment has been reported in a few patients with autoimmune diseases.36 However, these patients received other immunosuppressive agents prior to or in conjunction with rituximab. Further long-term consequences of repeated rituximab therapy in patients with NMO are unknown.

Because it was an uncontrolled and open-label study, it was difficult to provide a definitive evaluation of the clinical benefit of rituximab. However, given that controlled trials are difficult to organize because of the rarity of the disease and the high morbidity from relapse, these robust effects with good tolerability provide some rationale for the use of rituximab. A debate could arise based on the relevance of the 0.05% value, which was the percentage of memory B cells in PBMCs as a therapeutic target. This value was arbitrary based on our clinical experience prior to this study. Nevertheless, the results of the current study suggest relevance of the value as a quantitative threshold to determine retreatment. First, patients did not experience clinical relapse before the frequency of CD27+ memory B cells reached 0.05% in PBMCs. Furthermore, a characteristic reemerging pattern of CD27+ memory B cells was observed that abruptly increased after it recovered to the level of 0.05% in PBMCs. Finally, retreatment after significant repletion of CD27+ memory B cells could not prevent a clinical attack in patient 16. This result suggests that additional depletion of CD27+ memory B cells must occur prior to revitalization of disease activity. Recent studies in patients with RA have suggested a result that corresponds with our treatment strategy. Retreatment with rituximab is more effective when it is offered before disease symptoms flare, and an additional cycle of rituximab administered prior to total B cell repopulation enhanced B cell depletion and clinical responses.17,36

In conclusion, repeated treatment with rituximab appeared to produce consistent and sustained efficacy over 24 months with good tolerability in patients with NMO. Additionally, considering the various retreatment inter-
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