ORIGINAL CONTRIBUTION

Effect of Rituximab on the Peripheral Blood and Cerebrospinal Fluid B Cells in Patients With Primary Progressive Multiple Sclerosis

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Background: Rituximab, an anti-CD20 monoclonal antibody that depletes $CD20^+$ B cells, has demonstrated efficacy in peripheral neurological diseases. Whether this efficacy can be translated to neurological diseases of the central nervous system with possible autoimmune B-cell involvement remains unknown.

Objective: To determine the effect of rituximab on cerebrospinal fluid B cells in patients with multiple sclerosis.

Design: Four patients with primary progressive multiple sclerosis were treated with rituximab. Cerebrospinal fluid and peripheral blood B-cell subsets were identified by flow cytometry from each patient before and after rituximab treatment.

Results: The B cells in cerebrospinal fluid were not as effectively depleted as their peripheral blood counterparts. Rituximab treatment temporarily suppressed the activation state of B cells in cerebrospinal fluid. The residual B cells underwent expansion after rituximab treatment.

Conclusion: The effect(s) of rituximab on the cerebrospinal fluid B-cell compartment is limited in comparison with the effect(s) on the B cells in the periphery, but this finding will need to be confirmed in a larger group of MS patients.

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MULTIPLE SCLEROSIS (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS) that likely involves an autoimmune response directed against myelin-associated antigens. Evidence exists that possibly implicates B cells in the development and perpetuation of MS.[1-11] Thus, efforts to address whether B-cell–depleting therapies may have a place in therapeutic regimens for patients with MS (hereafter referred to as MS patients) have begun.

Rituximab (Genentech, Inc, San Francisco, Calif; IDEC Pharmaceuticals Corp, San Diego, Calif) is a chimeric murine/human IgG1 κ monoclonal antibody. It targets CD20, a transmembrane phosphoprotein expressed on most B cells, except for plasma B cells.[12-14] Rituximab depletes B cells by binding to the CD20 molecule and thereby initiating complement-dependent cytolysis or antibody-dependent cell-mediated cytotoxicity. Rituximab has clear clinical benefit for patients with neoplastic B-cell–mediated diseases[15] and treatment of anti-GM1 polyneuropathy.[16] Whether this efficacy of rituximab in peripheral neurological diseases with autoimmune B-cell involvement can be translated to neurological diseases of the CNS with possible autoimmune B-cell involvement remains unknown.

The objective of this study was to determine the effect of rituximab on B cells in the peripheral blood (PB) and cerebrospinal fluid (CSF) of MS patients.

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METHODS

STUDY POPULATION

Five patients were recruited for rituximab therapy at the University of Texas Southwestern Medical Center at Dallas on the basis of previous response to plasmapheresis and continued disease progression. All patients signed a consent form approved by the institutional review board. Their clinical presentation is summarized in **Table 1**.

THERAPY

All 5 patients were treated with a weekly dose of intravenous rituximab (375 mg/m^2) for 4 consecutive weeks.

Table 1. Patient Summary

Patient No./ Sex/Age, y	Type of MS	Time Since Initial Symptoms, y	Time Since Diagnosis, y	EDSS Before Rituximab	MRI Findings	OCB	IgG Synthesis, mg/d*
M116/F/61†	PP	23	20	7	Periventricular T2 lesions	Negative	8.36
M154/F/59	PP	26	22	6	Periventricular T2 lesions	Positive	0.85
M188/F/54	PP	5	3	8	Periventricular T2 lesions	Negative	0.00
M191/F/52	OND	2	1	NP	Normal	Negative	NP
M602/M/44	PP	24	17	7	Cervical atrophy	NP	NP

Abbreviations: EDSS, Expanded Disability Status Scale; MRI, magnetic resonance imaging; MS, multiple sclerosis; NP, not performed; OCB, oligoclonal banding; OND, other neurological disease; PP, primary progressive.
*The reference range for IgG synthesis is 0-12.
†This patient also had elevated anti-GM1 and antisulfatide antibody levels and a coexistent peripheral neuropathy.

Table 2. Marker Expression on B-Cell Subsets

B Cells	CD19/20	FSC/SSC	CD38	IgM	IgD
Immature	Bright	Low	+	+	+
Naive	Bright	Low	–	+	+
Recently activated	Bright	Low	–	+	–
Advanced memory*	Dim	High	–	–	–
Plasma B cells	Dim/–	High	+	–	–

Abbreviations: FSC/SSC, forward scatter vs side scatter; minus sign, no expression; plus sign, expression.
*Includes postswitch memory and preplasma B cells.

Table 3. $CD19^+$ Frequencies in the PBL Samples of Patients With MS Receiving Rituximab

Patient Code, Time of Sampling by Rituximab Treatment	$CD19^+$ in PBL, %
M116	
Before	18.43
1 mo after	0.14†
18 mo after*	4.42†
20 mo after	7.91†
M154	
Before	3.80
2 mo after	0.02†
14 mo after	7.42†
M191	
Before	6.3
14 mo after	1.58†
M602	
Before	6.5
2 mo after	0.32†

Abbreviations: MS, multiple sclerosis; PBL, peripheral blood leukocytes.
*Patient M116 was retreated with rituximab immediately after this sampling.
†Statistically different from the pretreatment $CD19^+$ frequency in the PBL, according to χ^2 analysis.

B-CELL FREQUENCY ASSESSMENT

Four of the 5 patients consented to donate a PB sample before and after completion of rituximab therapy. Approximately 10 mL of heparinized PB was collected at each sampling. Mononuclear cells from the PB were isolated by Ficoll-Hypaque sedimentation as described.[17] Two of these patients also consented to donate CSF at the same time points as the blood samplings. The CSF cells were isolated by centrifugation of the CSF. The PB and CSF cells were stained with a phycoerythrin-labeled anti-CD19 monoclonal antibody and other markers that identify B-cell subsets (**Table 2**). The cells were collected using a flow cytometer (FACStar Plus; Becton Dickinson, San Jose, Calif) and the $CD19^+$ B-cell frequencies and light-scatter characteristics analyzed using CellQuest software (Becton Dickinson). We used the χ^2 test to compare the frequencies of $CD19^+$ B cells (before and after rituximab treatment) in the PB and CSF.

RESULTS

EFFECT OF RITUXIMAB ON PERIPHERAL B-CELL FREQUENCY

The PB and CSF samples were collected from MS patients M116 and M154 before and after rituximab treatment. Only PB was sampled from MS patient M602 and a single patient with progressive myelopathy (patient M191). The samples were stained for CD19, a B-cell marker whose expression parallels that of CD20.[18]

The $CD19^+$ B cells were effectively depleted in the periphery after rituximab therapy in all 4 patients (**Table 3**), as expected. Patient M116 had 18.43% $CD19^+$ PB B cells before rituximab therapy, which decreased to 0.14% 1 month after rituximab therapy (Table 3). The other 3 patients demonstrated similar significant decreases in $CD19^+$ PB B-cell frequencies after rituximab therapy. Patient M116 had a second course of rituximab, and 2 months after this second course, the $CD19^+$ B-cell population was not depleted (4.42% vs 7.91%). This resistance to depletion on retreatment with rituximab has been reported previously.[19]

The time frame in which $CD19^+$ PB B-cell frequencies recovered varied between the patients (Table 3). The $CD19^+$ B-cell frequency in patient M116 had increased to 4.42% 18 months after rituximab treatment, but did not fully recover to the pretreatment B-cell frequency of 18.43%. Patient M191 also did not recover to the pretreatment B-cell frequency at 14 months after rituximab treatment. In contrast, patient M154 had a larger posttreatment frequency than that observed before rituximab treatment. Thus, there will likely be individual variability in the time frame and extent of $CD19^+$ PB B-cell frequency recovery after rituximab therapy.

Table 4. CD19⁺ Frequencies in the CSF of Patients With MS Receiving Rituximab

Patient Code, Time of Sampling by Rituximab Treatment	CD19⁺ in CSF, %
M116	
Before	1.03
1 mo after	0.63†
18 mo after*	5.63†
20 mo after	9.24†
M154	
Before	1.74
2 mo after	3.44‡
14 mo after	3.31‡

Abbreviations: CSF, cerebrospinal fluid; MS, multiple sclerosis.
*Patient M116 was re-treated with rituximab immediately after this sampling.
†Statistically different from the pretreatment CD19⁺ frequency in the CSF, according to χ^2 analysis.
‡Not statistically different from the pretreatment CD19⁺ frequency in the CSF, according to χ^2 analysis.

EFFECT OF RITUXIMAB ON B-CELL FREQUENCY IN THE CSF

Only 1 of the patients demonstrated a significant decrease in CD19⁺ CSF B-cell frequency after rituximab therapy (1.03% vs 0.63% in patient M116; $P < .001$) (**Table 4**). In contrast, the CD19⁺ CSF B-cell frequency in the CSF of patient M154 after rituximab therapy increased (1.74% vs 3.44%; $P = .14$). Thus, depletion of CSF B-cells after rituximab therapy cannot be achieved as consistently as in the periphery.

The CD19⁺ B-cell frequency in patient M116 at 18 and 20 months after rituximab therapy was significantly greater than that in the pretreatment sampling, whereas the CD19⁺ B-cell frequency of patient M154 at 14 months after rituximab therapy remained the same as the posttreatment sampling results 2 months after rituximab therapy (Table 4). Thus, recovery of the CD19⁺ CSF B-cell frequencies after rituximab therapy is likely to vary among individuals. To provide an accurate time frame in which rituximab depletes B cells most effectively in the CSF, a thorough kinetic analysis of B-cell frequencies in a larger patient population would be required.

ANALYSIS OF THE RESIDUAL B-CELL POPULATION IN PATIENT M116

There was no decrease in the CD19⁺ B-cell frequency 2 months after patient M116 had received a second course of rituximab (Tables 3 and 4). To discern what B-cell subtypes survived the second rituximab course, we stained the PB and CSF with CD19, CD38, IgM, and IgD. Recently activated B cells express CD19/CD20 very brightly and are $CD38^-IgM^+IgD^-$, whereas advanced memory (postswitch and preplasma B cells) dimly express CD19/CD20 and are $CD38^+IgM^-IgD^-$ (Table 2).

The CD19⁺ PB B-cell population of patient M116 could be subdivided into 2 separate populations: $CD19^{bright}$ and $CD19^{dim}$. Most of the CD19⁺ B cells that remained after rituximab therapy were $CD19^{dim}$ (87%) (**Figure 1**A) and IgM^-IgD^- (Figure 1B) and did not express CD38 (see rectangular gate in Figure 1C). Thus, the residual B cells were postswitch B cells that had not yet differentiated to a plasma B-cell phenotype. There was also a population of cells that were $CD19^{bright}$ $CD38^+IgM^+IgD^+$ (see oval gate in Figure 1C), which are likely immature B cells (Table 1) that have recently emerged from the bone marrow to begin repopulating the peripheral B-cell pool.

Approximately 80% of the $CD19^{dim}$ CSF B cells were $IgM^-IgD^-CD38^+$ (Figure 1E and rectangular gate in Figure 1F), which stratified them as plasma B cells. The other approximately 20% of the $CD19^{dim}$ B cells were $IgM^-IgD^-CD38^-$, and had thus not differentiated to a plasma B-cell phenotype. The $CD19^-CD38^+$ population would include plasma B cells as well, but this population also contains T cells, so an accurate analysis of the plasma B cells in this population could not be ascertained.

ACTIVATION STATUS OF PERIPHERAL B CELLS IN RESPONSE TO RITUXIMAB

Activated B cells (such as plasma B cells) are generally larger and more granular than their naive counterparts.[20-23] This phenomenon can be observed by flow cytometric light-scatter characteristics, looking at the placement of B cells in the forward scatter vs side scatter (FSC/SSC) plot, which separates cells by size and granularity, respectively. To identify B-cell subpopulations after rituximab therapy, we reanalyzed the B-cell compartments in the CSF and PB from the time point before and 2 time points after rituximab therapy in patients M116 and M154.

There were 2 populations of CD19⁺ PB B cells (bright and dim) in patient M116 (Figure 1A), so we analyzed them separately for their light-scatter profiles. Most of the B cells in the PB of patient M116 before rituximab therapy were $CD19^{bright}$ (78.8%) (Figure 1A), and were mostly contained in the typical lymphocyte gate (ie, low FSC/SSC) (red dots) (**Figure 2**A). The $CD19^{dim}$ B cells (green dots) (Figure 2A) were also mostly contained in the typical lymphocyte gate (ie, low FSC/SSC). This indicated that the $CD19^{bright}$ and $CD19^{dim}$ B cells were largely in a resting state.

One month after rituximab therapy, the $CD19^{bright}$ B cells (red dots) were largely depleted (Figure 2B), as expected. However, the $CD19^{dim}$ B cells (green dots) remained and maintained their resting state (low FSC/SSC). Eighteeen months later, the $CD19^{bright}$ B cells (red dots) had repopulated the B-cell pool (Figure 2C) and were in a resting state, as had been observed before rituximab therapy. Interestingly, 18 months after rituximab therapy, the $CD19^{dim}$ B-cell population (green dots) had not only expanded (3.9% of CD19⁺ B cells before vs 96.4% of CD19⁺ B cells after rituximab treatment), but had increased their FSC (ie, were larger in size) to a position above the lymphocyte region (Figure 2C). This light-scatter profile is consistent with a more activated state of the $CD19^{dim}$ B cells than that observed before rituximab therapy.

The peripheral B cells from patient M154 behaved in a similar manner (**Figure 3**), with the $CD19^{bright}$ B cells (red dots) located in the typical lymphocyte region before rituximab treatment (Figure 3B), followed by depletion of the $CD19^{bright}$ B cells 2 months after rituximab therapy (Figure 3C), and finally, repopulation of the typi-

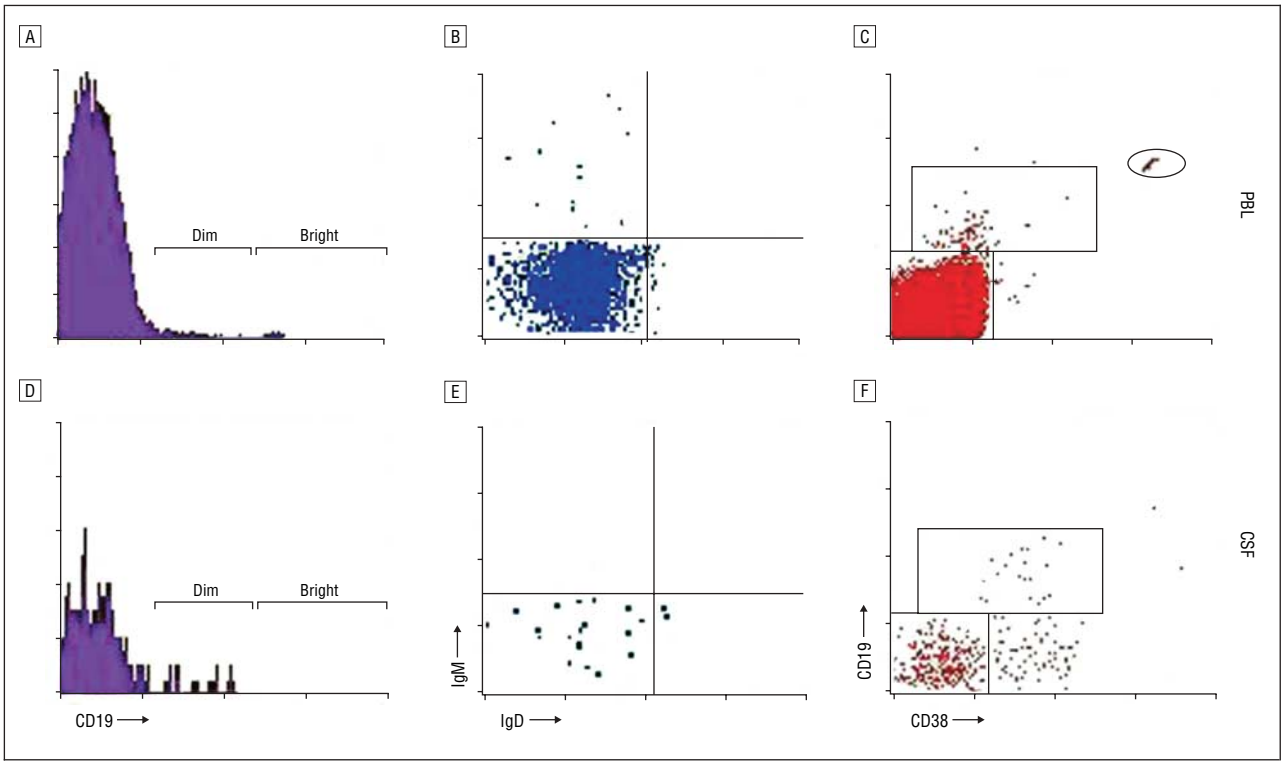

Figure 1. Representation of B cells in patient M116 after retreatment with rituximab. Peripheral blood leukocytes (PBL) (A-C) and cerebrospinal fluid (CSF) (D-F) B cells were stained for CD19, IgM, IgD, and CD38. Panels A and D are histograms, demonstrating the position of $CD19^{dim}$ and $CD19^{bright}$ populations within the lymphocyte population. The y-axis is cell count and x-axis is log fluorescent intensity. Panels B and E illustrate the IgM and IgD expression of the $CD19^{+}$ B cells. Panels C and F illustrate the coexpression of CD38 on the $CD19^{+}$ B cells. The rectangular gate indicates the $CD19^{dim}$ B-cell population discussed in the "Analysis of the Residual B-Cell Population in Patient M116" subsection of the "Results" section. The oval gate in panel C indicates the $CD19^{bright}$ $CD38^{bright}$ population. The square gate in panels C and F indicates cells negative for CD19 and CD38. In panels B, C, E, and F, the axis values for the indicated markers are given in fluorescence intensity.

cal lymphocyte region 14 months after rituximab therapy with $CD19^{bright}$ B cells (Figure 3D). The $CD19^{dim}$ B cells (green dots) were also present before rituximab therapy (Figure 3B), but remained largely in the typical lymphocyte region (low FSC/SSC), and remained so 2 months after rituximab treatment (Figure 3C). Fourteen months later, the $CD19^{dim}$ B-cell population had expanded (20.1% of $CD19^{+}$ B cells before vs 91.9% of $CD19^{+}$ B cells after rituximab therapy) and were in a more activated state.

ACTIVATION STATUS OF CSF B CELLS IN RESPONSE TO RITUXIMAB

We have observed that most of the CSF B cells are not located within the typical lymphocyte region, but instead have increased light-scatter profiles to reflect a more activated phenotype. The CSF B cells from patient M116 are a typical example of that, having few $CD19^{bright}$ B cells (red dots) relative to PB (compare Figure 2A and Figure 2D), and most $CD19^{dim}$ B cells (green dots) were located above the resting lymphocyte region (Figure 2D) before rituximab treatment. One month after rituximab therapy, most of the CSF B cells were $CD19^{dim}$ (green dots), and had relocated to the resting lymphocyte region (Figure 2E). Twenty months after rituximab therapy, some $CD19^{bright}$ CSF B cells (red dots) were observed within the typical lymphocyte region. However, the $CD19^{dim}$ B cells (green dots) had increased light-scatter profiles, indicating a heightened state of activation (Figure 2F) similar to their light-scatter profile before rituximab therapy. In addition, the $CD19^{dim}$ B cells (green dots) had expanded in comparison with the population before rituximab treatment (55% of $CD19^{+}$ B cells before vs 77% $CD19^{+}$ B cells after rituximab therapy).

Most of the CSF B cells from patient M154 before rituximab therapy were $CD19^{dim}$ (77.8%), and had a high light-scatter profile (Figure 3F). We had observed the same profile of CSF B cells from patient M116, and consider this pattern typical for CSF B cells, which are more activated than their peripheral counterparts. Two months after rituximab therapy, some $CD19^{bright}$ CSF B cells (red dots) were still detectable (Figure 3G), but the $CD19^{dim}$ B cells (green dots) remained and, interestingly, displayed a low light-scatter profile, indicative of a resting state (Figure 3G). However, 14 months after rituximab therapy, some $CD19^{bright}$ CSF B cells (red dots) had repopulated. The $CD19^{dim}$ B cells (green dots) had expanded significantly (20.2% of $CD19^{+}$ B cells before vs 94.4% of $CD19^{+}$ B cells after rituximab therapy) and had assumed their activated state (high light-scatter profile) (compare Figure 3H and Figure 3F).

COMMENT

It is evident that $CD19^{+}$ B cells were depleted in the periphery within 1 to 2 months after rituximab therapy in the PB of all 3 patients. Even patient M191 maintained a

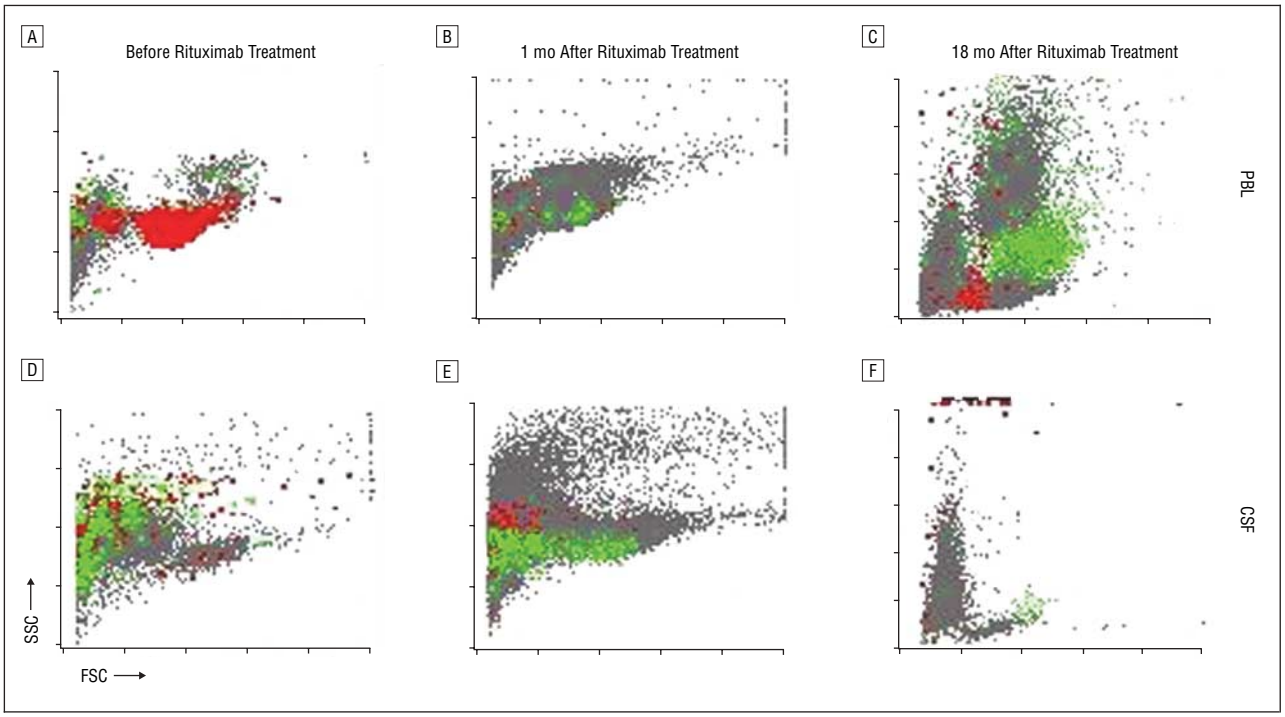

Figure 2. B-cell activation states in the peripheral blood leukocytes (PBL) and cerebrospinal fluid (CSF) of patient M116. Peripheral blood (A-C) and CSF (D-F) were stained for CD19. Red dots indicate the location of $CD19^{bright}$ B cells; green dots, the location of $CD19^{dim}$ B cells. Panels A and D indicate the forward scatter vs side scatter (FSC/SSC) designations for the PB and CSF cells before rituximab therapy. Panels B and E indicate the FSC/SSC designations for the PB and CSF cells 1 month after rituximab therapy. Parts C and F indicate the FSC/SSC designations for the PB and CSF cells 18 months after rituximab therapy. Axis values are given in fluorescence intensity. The y-axis scale for panels C and F is linear, rather than logarithmic.

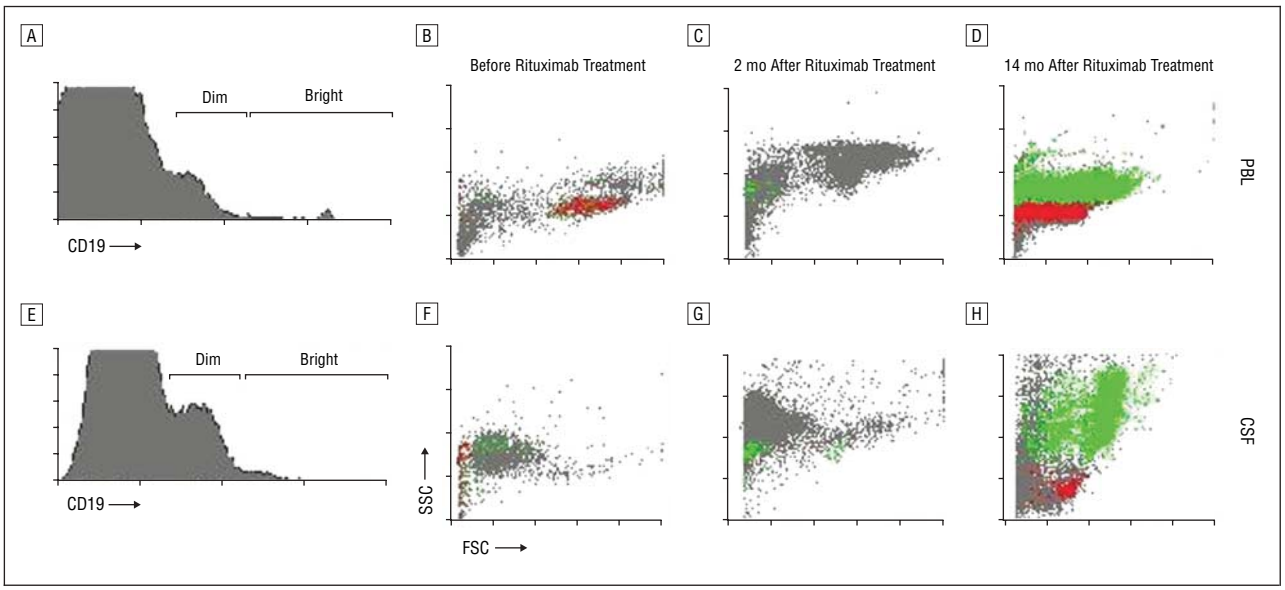

Figure 3. B-cell activation states in the peripheral blood leukocytes (PBL) and cerebrospinal fluid (CSF) of patient M154. Peripheral blood (A-D) and CSF (E-H) were stained for CD19. Red dots indicate the location of $CD19^{bright}$ B cells; green dots, the location of $CD19^{dim}$ B cells (B-D and F-H). Panels A and E are histograms indicating the $CD19^{dim}$ and $CD19^{bright}$ designations. Panels B and F indicate the forward scatter vs side scatter (FSC/SSC) designations for the PB and CSF cells before receiving rituximab. Panels C and G indicate the FSC/SSC designations for the PB and CSF cells 2 months after rituximab therapy. Parts D and H indicate the FSC/SSC designations for the PB and CSF cells 14 months after rituximab therapy. The y-axis scale for panels D and H is linear, rather than logarithmic.

depleted $CD19^+$ B-cell population in the periphery 14 months after rituximab therapy. However, the PB $CD19^+$ B-cell population was partially or fully reconstituted between 14 and 18 months after rituximab therapy, indicating that the inhibitory effect of rituximab on B cells no longer exists.

The effect of rituximab on the B-cell pool within the CNS tissue is not as well-defined, but it becomes an issue when considering rituximab as a therapeutic agent in B-cell–mediated diseases of the CNS. We have examined the effect of rituximab on $CD19^+$ CSF B cells in 2 MS patients, which likely represents a subset of B cells

that populate the CNS tissue. The $CD19^+$ CSF B cells showed a mild decrease 1 month after rituximab therapy, followed by a significant expansion 18 and 20 months after rituximab therapy that were 5- to 9-fold the $CD19^+$ B-cell frequency before rituximab therapy. However, the $CD19^+$ CSF B cells in patient M154 were not affected by rituximab. This was in direct contrast to what had been routinely observed in PB B cells in response to rituximab, and prompts the question of whether this lack of CSF B-cell depletion has any repercussions on the potential efficacy of rituximab treatment of MS.

There are at least 2 possible scenarios that could account for the lack of CSF B-cell depletion. First, the concentration of rituximab that reaches the CSF can be nearly 10-fold less than the concentration readily achieved in the PB.[24] Thus, if therapeutic levels of rituximab cannot reach the CSF, depletion of CSF B cells should not be expected. However, the observation that only 10% or less of the rituximab concentration that is reached in the PB is obtainable in the CSF is based on patients whose blood-brain barrier is not compromised, as it is in most MS patients. Thus, perhaps higher CSF rituximab concentrations can be achieved in MS patients whose blood-brain barrier has been compromised. This concept has not been rigorously tested. Second, rituximab is more effective at depleting B cells that have high expression of CD20, such as naïve B cells. However, most CSF B cells are advanced memory or plasma B cells, which express CD19/20 dimly,[18] rendering them resistant to rituximab.

Rituximab also appears to affect $CD19^{dim}$ B-cell activation states, because the initial activated phenotype of the $CD19^{dim}$ CSF B cells was suppressed after rituximab therapy. However, these $CD19^{dim}$ CSF B cells had reestablished their activation phenotype 14 and 20 months after rituximab therapy. It is unclear how the suppression of this activation state shortly after treatment occurs. It is possible that rituximab itself asserts some form of suppression on residual B cells, or that some other changes in the CSF environment in response to rituximab causes $CD19^{dim}$ B cells to enter a resting state. What effect this resting state of the residual $CD19^{dim}$ CSF B cells has on disease status remains unknown, although it is compelling to predict that this suppression of the residual B cells by rituximab (directly or indirectly) will translate to a clinical improvement for the patients. However, it is clear that at some point (presumably when rituximab levels drop below a certain concentration), the surviving CSF B cells resume their activated phenotype.

This activation suppression phenomenon we had observed with the CSF $CD19^{dim}$ B cells was not observed in the peripheral $CD19^{dim}$ B-cell population because these cells are typically resting. However, the $CD19^{dim}$ B cells that survived in the periphery had acquired an activated state after rituximab therapy. Whether this activation state of $CD19^{dim}$ peripheral B cells parallels that of the $CD19^{dim}$ CSF B cells will need to be investigated, as well as what effect this might have on the reconstituted B-cell pool.

We also observed an expansion of PB and CSF $CD19^{dim}$ B cells after rituximab therapy. Expansion of residual $CD19^{dim}$ B cells may be based on the theory of space threshold, which suggests that there is a finite amount of space where cells are allowed to populate, and once the $CD19^{bright}$ B cells were depleted, the $CD19^{dim}$ B cells took advantage of the extra space and expanded as observed in these 2 patients. Alternatively, the chemokine milieu may have shifted during rituximab therapy, resulting in an influx of $CD19^{dim}$ B cells from other compartments (such as the bone marrow), and subsequent expansion. Third, perhaps residual $CD19^{bright}$ B cells that survived rituximab treatment were able to rapidly become activated and differentiate. However, for this hypothesis to apply to the CSF B cells, the concept that B cells can differentiate and become activated outside the context of germinal centers must be substantiated.

CONCLUSIONS

We have observed typical depletion of PB B cells after rituximab therapy in patients with primary progressive MS. Rituximab does not deplete CSF B cells efficiently because most CSF B cells are highly activated advanced memory or plasma B cells that express little or no CD20. Recovery of the initial B-cell frequencies will likely vary among individuals. Alterations in activation and expansion status of PB and CSF B cells in response to rituximab requires further study to resolve the mechanism(s) underlying these phenomena.

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