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Rituximab reduces B cells and T cells in cerebrospinal fluid of multiple sclerosis patients

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Abstract

Effects of B cell depletion by rituximab, a monoclonal antibody to CD20, were studied in patients with relapsing MS that had not responded optimally to standard immunomodulatory therapies. Flow cytometry demonstrated reduced cerebrospinal fluid (CSF) B cells and T cells in most patients at 6 months post-treatment. ELISAs demonstrated modest reductions in serum antibodies to myelin oligodendrocyte glycoprotein and myelin basic protein in some subjects. Beta-interferon neutralizing antibodies were reduced in three subjects, but developed anew after treatment in three others, suggesting caution in considering rituximab as a means to eliminate NABs. In summary, rituximab depleted B cells from CSF at 24 weeks after initial treatment, and this B cell depletion was associated with a reduction in CSF T cells as well.

Keywords

Multiple sclerosis; Treatment; B lymphocytes; Rituximab; Autoantibodies; Myelin oligodendrocyte glycoprotein antibodies; Myelin basic protein antibodies

1. Introduction

Evidence implicates B cells and antibodies (Abs) in the pathogenesis of MS (Cross et al., 2001). The most consistent laboratory abnormality found in MS patients is increased intrathecal production of oligoclonal immunoglobulin (Ig), present in > 90% of persons with definite MS (Walsh et al., 1985; Trotter and Rust, 1989). These Igs include IgG, IgA, IgM and IgD (Walsh and Tourtelotte, 1986). Several studies have correlated high levels of CSF Ig, including both IgG and IgM, with worse prognosis (Olsson and Link, 1973; Villar et al., 2002; Izquierdo et al., 2002). MS patients lacking CSF oligoclonal bands (OCBs) have a more benign course (Zeman et al., 1996), whereas higher numbers of OCBs are associated with a poor prognosis (Avasarala et al., 2001). These studies provide correlative data, but may reflect an altered humoral immune system rather than abnormalities fundamental to pathogenesis.

To better define the role of B cells in MS, we undertook an open-label Phase II clinical trial of B cell depletion in relapsing-remitting MS (RRMS) patients with suboptimal response to standard therapies. Serial measurements of serum and cerebrospinal fluid (CSF) Abs to the myelin proteins, myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) and serial measures of B and T cells in CSF were performed.

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2. Materials and methods

2.1. Study design

This Phase II trial was designed to study the use of rituximab as an add-on therapy in RRMS patients continuing to have MS activity, both clinically and by MRI, despite therapy with an FDA-approved medication. The study was approved by the Washington University Human Studies Committee (IRB). All subjects provided full informed consent prior to enrollment. The primary endpoint, still blinded, is to determine if the number of gadolinium enhancing lesions on brain MRI is reduced after administration of study drug. Additional aims of the study are to determine the effect of depletion of circulating B cells on the presence of Abs to human MOG and MBP and on CSF B cell numbers, T cell numbers, IgG concentration, IgG index, IgG synthesis rate and oligoclonal band numbers.

All patients enrolled have relapsing MS with EDSS ≤ 6.5. Because there is no placebo arm, clinical examinations were unblinded and performed primarily for safety. Rituximab, administered at the standard dose used in patients with B cell lymphoma (375 mg/m^2 weekly×4) was added to each subject's immunomodulatory therapy. Enrollment criteria were an MS exacerbation within the 18 months prior to enrollment despite receiving Avonex®, Betaseron®, Copaxone®, or Rebif®, and at least one gadolinium-enhancing lesion on any of three pre-treatment brain MRIs. Past treatment with an immunosuppressive agent at any time was exclusionary.

Patients underwent CSF and blood sampling 1 week prior to and 24 weeks following the initial dose of rituximab. CSF was assessed for IgG concentration, presence and number of oligoclonal bands (OCBs), IgG synthesis rate (Tourtellotte et al., 1980), and IgG Index (IgG CSF×Albumin serum/IgG serum×Albumin CSF; normal < 0.68). These tests were performed by the Barnes-Jewish Hospital (BJH) laboratory. The BJH laboratory performed CSF electrophoreses for OCB determinations pre- and post-treatment on the first eight subjects. These were counted in blinded fashion by AHC. For the remaining subjects, OCB determinations were performed and bands counted by Mayo Clinic laboratories.

2.2. Study drug

Rituximab is a genetically engineered chimeric murine/human IgG_1 kappa monoclonal antibody that targets the CD20 antigen, a transmembrane phosphoprotein expressed only by pre-B and mature B cells (Reff et al., 1994). Rituximab binds complement and thereby mediates B-cell lysis (Di Gaetano et al., 2003).

2.3. Flow cytometry of cerebrospinal fluid cells

The presence and identity of CSF cells was determined by flow cytometry. In all cases, staining and flow acquisition were completed within 5 h of the lumbar puncture (LP). 30–35 ml of CSF were obtained from each patient at time of LP. The first 10 ml was sent to the BJH laboratory. The remainder was kept cool on ice and was centrifuged (250×g, 10 min) within 30 min of the LP. Cells were re-suspended in medium (RPMI-1640 supplemented with 1% penicillin–streptomycin and 20% human AB serum) for staining. Equal aliquots of cells were placed into three separate microcentrifuge tubes and incubated with fluorescent Abs identifying T cells and B cells or with isotype control Abs. Following a 30 min incubation on ice with Abs, cells were washed 2× with 1 ml cold FACS wash (PBS, 1% FBS, 0.1% sodium azide) and re-suspended in FACS wash. T cells were identified with PerCP-labeled anti-human CD3 (clone SK7; mouse IgG_1). B cells were identified with APC-labeled anti human CD19 (clone SJ25C1; mouse IgG_1). Isotype-matched control Abs that were conjugated to the same fluorochromes as those used for cell staining were used in each experiment. Data was collected at a flow rate of 60 μl/60 s using a Becton Dickinson Biosciences FACSCalibur cytometer and analyzed with

CellQuest software (Becton-Dickinson). Total time of acquisition was used to determine the portion of the re-suspended volume that was counted by the cytometer. That number was extrapolated to obtain the total number of stained cells in the sample, and then divided by the total CSF volume to get cells/ml.

2.4. Flow cytometry of peripheral blood

Blood was collected into heparin laced tubes. White blood cells were isolated by Ficoll-Paque (Amersham Biosciences) density gradient (3500 rpm for 30 min at 22 °C). Cells were washed with 20 ml of medium (RPMI-1640 supplemented with 1% penicillin–streptomycin and 20% human AB serum), and resuspended in 1 ml of medium. 50 μl aliquots were placed into separate microcentrifuge tubes and incubated with fluorescent Abs identifying T cells and B cells or isotype control Ab. Following a 30 min incubation on ice with Ab, cells were washed 2× with 1 ml cold FACS wash (PBS, 1% FBS, 0.1% sodium azide) and re-suspended in FACS wash to a final concentration of at least 5×10^5 cells/ml. Tcells were identified with PerCP-labeled anti-human CD3 (clone SK7; mouse IgG_1). B cells were identified with APC-labeled antihuman CD19 (clone SJ25C1; mouse IgG_1). Isotype-matched control Abs that were conjugated to the same fluorochromes as those used for cell staining were used in each experiment. Data was collected and analyzed as for CSF cells.

2.5. ELISAs to measure antibodies to human MBP and MOG

Whole Ig and IgM levels to human myelin basic protein (MBP) and recombinant human extracellular myelin oligodendrocyte glycoprotein (MOG) in serum were performed by ELISA. Serum samples obtained at baseline (week 0) and at week 24 were assayed for total Ig (IgG, IgM and IgA) and IgM alone recognizing rMOG or MBP. Pre- and post-treatment samples from each subject were always assayed in the same ELISA to eliminate interassay variability. Known positive and known negative samples were included with each assay to confirm interassay consistency. MOG and MBP were applied at 10 μg/ml (in excess) to separate plates in bicarbonate buffer overnight. Standards of human gamma globulin (Jackson ImmunoResearch West Grove, PA) at varying concentrations (from 570 ng/ml to 9 ng/ml) in duplicate wells were included in each assay. The next morning, plates were washed mechanically with PBS four times (Wellwash4 MK2, Thermo Electron Corp) and PBS–3% BSAwas added for 2 h at 22 °C. Plates were again washed, after which diluted serum samples (1:250 and 1:500 for MOG, 1:500 and 1:1000 for MBP) were added in duplicate for 1 h at RT to the wells containing the excess protein. After washing, 1:3500 goat–antihuman poly-valent Ig-HRP (Sigma, St. Louis) or 1:20,000 goat–antihuman IgM-HRP (ICN Biomedicals Aurora, OH) was applied for 1 h at 22 °C. Plates were then washed four times, and 100 μl freshly prepared tetramethylbenzidine substrate (BD Biosciences, San Diego) was added per well for 30 min at 22 °C, protected from light. The reaction was stopped by addition of 100 μl of 2.5 N sulfuric acid LabChem Inc. Pittsburgh). Plates were read within 30 min at Abs_{450} nm BioTek ELX800 ELISA plate reader and data analyzed with KC junior software (BioTek). To consider a serum sample to be positive, two required criteria had to be met: (1) a dilution effect (i.e., level at 1:1000 must be approximately 1/2 of level at 1:500) and (2) absorbance at 450 nm must be ≥ 0.1.

2.6. Antigens

Recombinant MOG was prepared as previously published (Lyons et al., 1999). Human MBP was extracted from human brain tissue obtained at autopsy of a patient without known neurologic disorder, according to published methods (Deibler et al., 1972). Purity of MBP was confirmed by SDS-PAGE.

2.7. Neutralizing antibodies to beta-interferon (NABs)

Serum NABs were assayed by Athena Diagnostics (Worcester, MA) using the NAb Feron Antibody Test, a neutralization assay based on a published method (Grossberg and Kawade, 1997). As a check on reliability of these determinations, a positive serum sample was sent twice on different dates, yielding consistent results (1:43 and 1:41).

2.8. Clinical assessments

Per study protocol, Expanded Disability Status Scores (EDSS) were determined by the principal investigator (AHC) twice prior to receiving medication, on the day of the fourth infusion, and at monthly intervals thereafter (Kurtzke, 1983). These assessments were performed primarily for safety.

3. Results

3.1. Patients receiving rituximab

As of January 2006, 16 patients (10F, 6M) had been fully treated with rituximab, after screening of 36 (Table 1). Of these, 4 were on intra-muscular beta-interferon 1a, 2 were on subcutaneous beta-interferon 1a, 7 were taking subcutaneous beta-interferon 1b, and 3 were taking glatiramer acetate. One of the subjects (021) refused LP after an initial attempt failed, but remained in the study and was treated with study drug.

3.2. Blood

Circulating B cells were depleted to zero in all subjects that received full treatment. The first signs of B cells recovery were noted in 2 subjects at 6 months post-treatment, but most patients still had no B cells at 7 months post-treatment. Of four subjects tested at 12 months post-treatment, three still had reduced CD19 counts compared with baseline (50%, 70% and 80% reduced) and one had returned to baseline levels. Levels of serum IgA and IgG remained stable in all subjects at week 24 following treatment. Serum IgM concentration declined by 30% or more from pre-treatment level in 4 subjects (021, 025, 028 and 035), but remained within the normal range.

3.3. Serum antibodies to MBP and MOG

Serum ELISAs were performed at weeks 0 and 24. Prior to treatment, six subjects (001, 005, 017, 025, 031 and 035) had unequivocally elevated serum levels of total Ig to rMOG. There were an additional three subjects with borderline positive levels (026, 032, 036). After treatment, all except one borderline positive case (032) continued to have elevated levels, and one negative case had become borderline positive (028). Of the six unequivocally positive subjects at pre-treatment, three (025, 031, 035) had decreased significantly following treatment (Fig. 1A). Two subjects (001, 005) also had positive IgM responses to rMOG that remained positive following treatment (Fig. 1B).

An absorbance of ≥ 0.30 was set for positive total Ig to MBP due to lack of a consistent dilutional effect below that absorbance. Prior to rituximab, elevated total Ig to MBP were noted in three subjects (017, 028 and 032). Levels were reduced but not to normal after therapy in patients 028 and 032 (Fig. 1C). Three additional subjects (005, 016, 035) were borderline positive pre-treatment, all of which dropped into the negative range post-treatment. Two subjects displayed elevated IgM Abs to MBP (005, 025), one of whom (005) dropped to below the absorbance cut-off of 0.10 after treatment (Fig. 1D).

3.4. Neutralizing antibodies (NABs) to beta-interferons

Thirteen of the subjects were taking beta-interferon therapies before and during the study. Of these, subjects 019, 021 and 032, all three on Betaseron®, had positive NABs with titers of 72, 168 and 43 prior to rituximab treatment, respectively (Table 2). These titers declined: to < 1:20 in the first two and to 1:34 in patient 021. However, subjects 012 (Betaseron®), 020 (Rebif®), and 026 (Rebif®) with negative titers prior to treatment developed positive titers following treatment.

3.5. Spinal fluid

The study protocol included lumbar punctures performed at weeks 0 and 24. In two cases the second LP was delayed due to scheduling problems. The second LP was done at week 36 in subject 012 and at week 28 in subject 036. There were no significant differences in IgG concentration, IgG index, IgG synthesis rate or oligoclonal band number when comparing pre-treatment to post-treatment values (Fig. 2A–D). The CSF IgG concentration declined by 10% or more in 5, increased by 10% or more in 5, and was stable in 5 subjects (Fig. 2A). The IgG synthesis rate decreased by 30% or more in 6 of 15 subjects with pre- and post-treatment CSF values, but it increased by 30% or more in 3 subjects (Fig. 2B). The post-treatment reduction in IgG synthesis rate of 21% was not statistically significant.

Flow cytometry was used to determine the number and percentage of lymphocytes that expressed CD3 (T cells) and CD19 (B cells) in the CSF. Prior to treatment, B cells constituted a small number of CSF lymphocytes. In 5 subjects, $CD19^+$ B cells were too low to be accurately counted in both pre- and post-treatment CSF. These were assigned a value of 0. In 9 subjects, $CD19^+$ B cells were lower after treatment, whereas in only one case (003) were CSF $CD19^+$ B cells higher post-treatment compared to week 0. Overall, compared to pre-treatment, CSF $CD19^+$ B cells were reduced by 90% in the post-treatment spinal tap (mean 16.7 versus 1.7 $CD19^+$ cells/ml; p=0.03 paired t-test) (Fig. 3A).

Following rituximab therapy, the number of CSF T cells decreased in 12/15 subjects (p=0.003, paired t-test; Fig. 3B). Pre-treatment, $CD3^+$ T cells averaged 633 $CD3^+$ cells/ml (median 646) whereas at 24 weeks post-treatment the average was 283 $CD3^+$ cells/ml (median 171). In more than half of the subjects (001, 005, 012, 019, 020, 025, 032 and 035) the decline was greater than 50%. In 3 of the 15 subjects (017, 026, 031), $CD3^+$ T cells had increased in the post-therapy LP.

3.6. Clinical results

Clinical results should be considered with caution, as subjects and examiners were unblinded. The EDSS of most subjects remained stable, as expected in a short trial such as this. Two subjects showed sustained improvement on EDSS by > 1 point (021, 028) and one (026) showed sustained deterioration during the period of B cell depletion (Fig. 4).

4. Discussion

The role of B cells and Abs in MS pathogenesis is unknown. Several published studies have suggested that increased immunoglobulins in CSF portend a worse course. The presence and number of OCBs, the level of CSF free kappa light chains, and an elevated IgG index all have been correlated with a worse prognosis (Rudick et al., 1995). Published data indicate that patients with serum IgM Abs to MBP and MOG may have a more aggressive type of MS (Berger et al., 2003). However, these data do not prove that B cells or antibody has a *causative* role in the pathogenesis of MS lesions.

Indirect support for a causative role comes from treatment results. A role for humoral factors is suggested by the successful treatment of severe cases of acute CNS demyelination with plasma exchange (Weinshenker et al., 1999). On the other hand, some Abs enhance remyelination in mouse models of demyelination (Rodriguez and Lennon, 1990). These Abs were polyreactive towards a variety of antigens, including an as yet unidentified surface antigen on oligodendrocytes. An anti-idiotypic network of Abs might regulate the immune reaction in MS as it appears to do in the animal model for MS, experimental autoimmune encephalomyelitis (Zhou and Whitaker, 1993). Thus, some Abs produced by MS patients may be beneficial, making it important to clarify the role of Abs and B cells.

The existence of a relatively safe drug that transiently depletes circulating B cells provided the opportunity to begin to examine whether B cells, and their attendant products, might be pathogenic in MS. In this ongoing study that was initiated in 2002, circulating B cells were depleted in all MS subjects that received the full course of treatment. Depletion of circulating B cells did not lead to a consistent reduction of circulating Abs to MOG and MBP at 24 weeks following therapy, although in about half the cases levels were reduced. All six subjects with unequivocally elevated total Ig to rMOG prior to treatment remained positive 6 months after treatment, although levels had decreased substantially in three. Two subjects had positive total Ig and IgM to rMOG that remained present at similar level after treatment. Five subjects had Abs to MBP prior to treatment. After treatment, one became negative and two others were reduced. One of two subjects with positive IgM to human MBP dropped into the negative range following treatment.

Elimination of circulating B cells did not eliminate NABs to beta-interferon. This finding was not unexpected, because it was known that serum Abs to tetanus do not change following rituximab (Pestronk et al., 2003). However, it was unexpected and disappointing that three subjects developed circulating NABs to beta-interferon following therapy. An explanation for this occurrence is not readily apparent, but deserves further study.

Similarly, CSF IgG levels, IgG index and oligoclonal band numbers were not consistently altered at 24 weeks in the 15 subjects that underwent post-treatment LPs. Following rituximab, CSF IgG synthesis rate diminished by 30% or more in 6/15 subjects, but it increased by 30% or more in 3 subjects. This was not surprising because the majority of Ig is made by plasma cells which do not express CD20.

On the other hand, long-term humoral immunity is now believed to be due to the periodic non-specific activation of memory B cells, which do express CD20 (Bernasconi et al., 2002). Memory B cells, but not naïve B cells, express toll-like receptor 9 via which they can periodically be nonspecifically reactivated. Long-term elimination of memory B cells therefore may result in reduced Ab levels after shorter-lived plasma cells have died off (Bernasconi et al., 2003). In the present study, a single round of therapy eliminated B cells for 6 to more than 9 months. However, B cells had returned partially or fully in all subjects followed to 12 months post-treatment. Perhaps this is too short a time frame to discern an effect on Ab levels.

An unexpected and intriguing finding was that CSF Tcells were reduced post-treatment in most subjects, with an overall decrease of 55%. CSF Tcells were reduced by more than 50% in 8/15 (53%) subjects, decreased by lesser degrees in four, and increased in only three subjects, yielding an overall group decrease of more than 55% after treatment. The reason for this reduction is not known. One possibility is that B cells of CNS myelin specificity “home” to the CNS and in so doing alter the blood–brain barrier or secrete chemokines that recruit T cells as well. For example, B cell receptor triggering induces human B cell production of two T cell chemokines, macrophage inflammatory protein-1 beta (MIP-1 beta) and MIP-1 alpha (Krzysiek et al., 1999). Another possibility is that B cells entering the CNS secrete complement-

binding Abs which lead to local activation of complement proteins including C5a and C3a. C5a is an especially potent chemo-attractant for monocytes, macrophages and neutrophils, and also upregulates the production of several chemokines by endothelial cells (Sellebjerg et al., 1998; Guo and Ward, 2005).

Clinical results in this study were unblinded and performed primarily for safety purposes. No dramatic clinical improvement or deterioration was observed while patients were B-cell depleted. Occasional patients appeared to improve in walking speed or EDSS score after treatment during the time when circulating B cell levels were low. One subject had sustained deterioration (026) and one had non-sustained deterioration (031) based on EDSS during the period of peripheral B cell depletion. Interestingly, these were two of the three subjects that had increased CSF T cells post-rituximab. Small trials of rituximab therapy have been reported in patients with neuromyelitis optica (NMO) and primary progressive MS (Cree et al., 2005; Monson et al., 2005), and in a case report of one subject with relapsing-remitting MS (Stuve et al., 2005). Although these reports suggested that this therapy was beneficial, it should be noted that assessments of clinical efficacy in these studies and in the present one were unblinded. Therefore, the use of rituximab in demyelinating CNS diseases should be considered experimental. Larger, randomized, blinded studies of B cell depletion in patients with inflammatory demyelinating CNS disorders are needed to fully assess its usefulness. It will be particularly interesting to determine whether subgroups of patients with evidence of humoral components (e.g. high numbers of OCBs, IgG index 1.0, etc.) might be responsive to B cell depletion.

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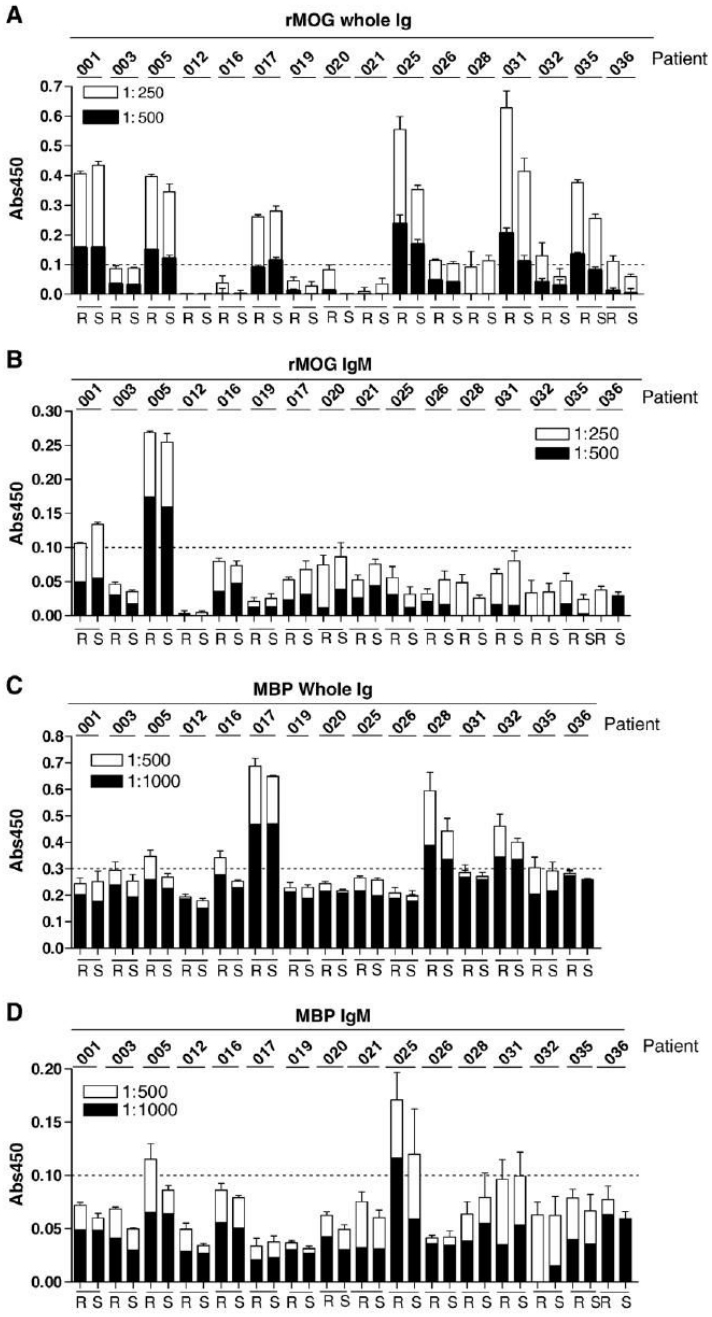

Fig. 1.

(A–D) Serum ELISA results for: (A) total Ig (IgG, IgM and IgA) and (B) IgM to human myelin oligodendrocyte glycoprotein, and (C) total Ig (IgG, IgM, IgA) and (D) IgM to human myelin basic protein. Each subject is identified by study number and represented by 2 bars, the first collected at week 0 (pre-rituximab — R) and the second at week 24 (post-rituximab — S). The post-treatment (S) sample on subject 012 was not obtained until 36 weeks post-treatment. For (A) and (B), samples were diluted 1:250 and 1:500, and antibody specific for rMOG was detected via ELISA vs. recombinant protein. Samples with an $Abs_{450\,nm}$ greater than 0.1 (dotted line) were considered positive, based on results obtained with healthy donors. For (C) and (D),

serum dilutions of 1:500 and 1:1000 were used. An Abs_{450} of ≥ 0.30 was set for positive total Ig to MBP due to lack of a consistent dilution effect below that absorbance.

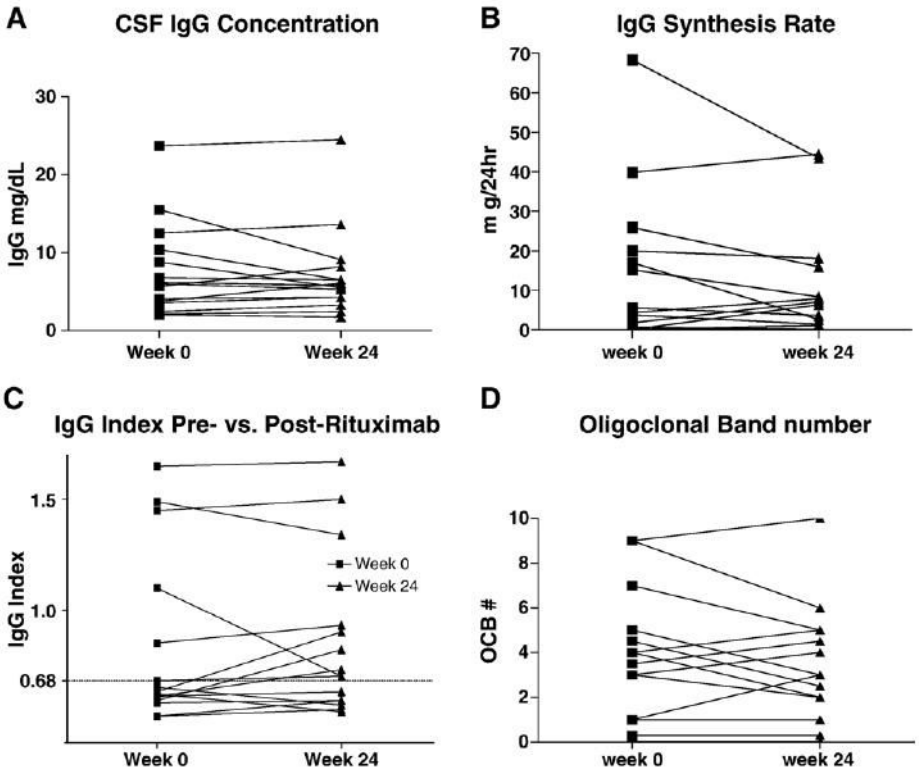

Fig. 2.

Results of CSF Immune analyses of CSF obtained prior to and 24 weeks after beginning rituximab therapy. (A) IgG concentration; (B) IgG synthesis rate; (C) IgG Index and (D) oligoclonal band number. The post-treatment sample on one patient (012) was not obtained until 9 months post-treatment.

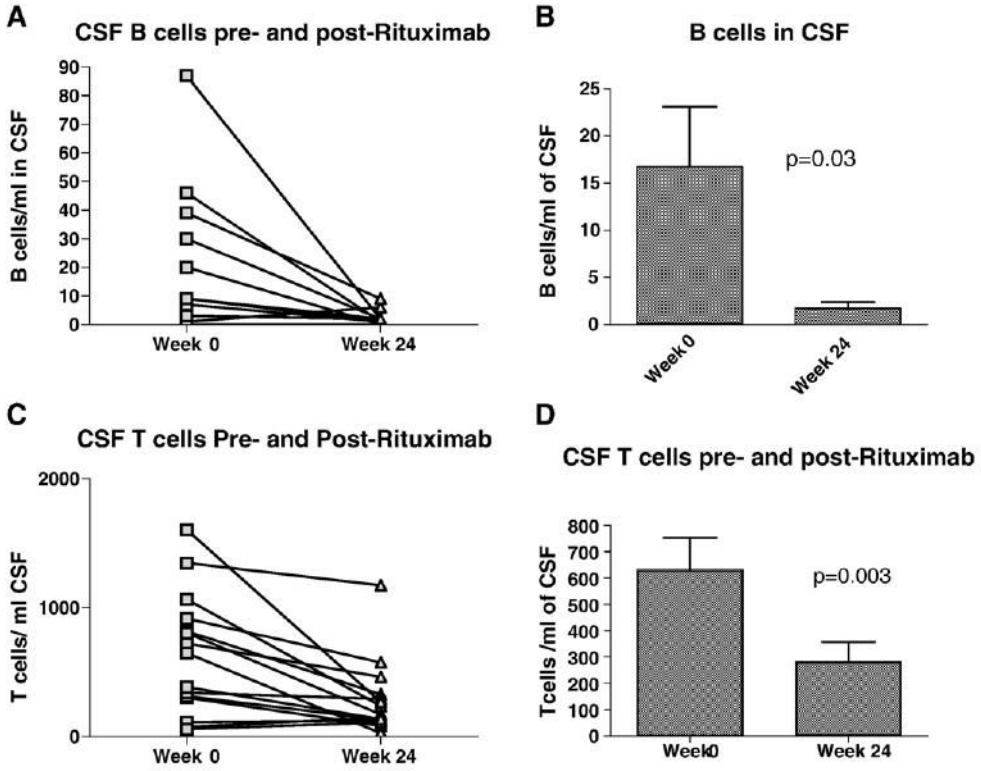

Fig. 3.

CSF concentration of lymphocytes pre- and post-rituximab therapy. Number of cells per ml of CSF for (A) B cells and (C) T cells determined on 20–25 ml CSF samples obtained at week 0 and week 24. (B and C) show the mean±S.E.M. for B and T cell numbers at weeks 0 and 24 after therapy. The post-treatment sample on one subject (012) was delayed until 9 months post-treatment. *P*-values derive from paired *t*-tests.

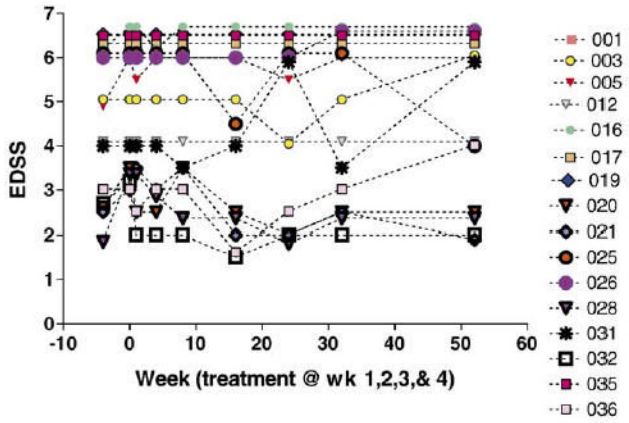

Fig. 4.

Serial unblinded clinical assessments. Expanded disability status scores (EDSS) for the first 16 patients in this open-label trial.

Table 1

Patients enrolled

	Females	Males
Number	10	6
Mean age±S.D.	42.7±6.7 years	41±6.5 years
Years with MS	11±6.7 (range 3–25)	11.3±6.4 (range 2–20)
No. of attacks in past 18 months	1.6±0.7 (range 1–3)	1.8±1.0 (range 1–3)
Beta-IFN 1b SC	5	3
Beta-IFN 1a IM	2	2
Beta-IFN 1a SC	1	1
Glatiramer acetate	2	0

Table 2

Neutralizing antibodies to beta-interferon

Patient	Pre-treatment NAB titer	Post-treatment NAB titer
012	<1:20	1:64
019	1:72	<1:20
020	<1:20	1:43
021	1:128	1:34
026	<1:20	1:106
032	1:43	<1:20