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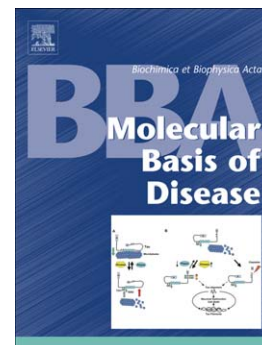
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MS and the B cell controversy

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Key words: Multiple sclerosis; B lymphocytes; antibodies; rituximab; therapy

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Abstract

The contribution of B cells and their products to the pathogenesis of MS has long been debated. The presence of B cells, plasma cells and excess immunoglobulins in MS lesions and in the cerebrospinal fluid of MS patients implicate the humoral immune system in the disease. Correlations of higher levels of CSF B cells and immunoglobulins found in some studies with a more aggressive clinical course of MS has bolstered the notion that the humoral immune system is involved in MS pathogenesis. However, until the advent of rituximab, a monoclonal antibody therapy that specifically lyses B cells, confirmation of the key role of B cells and their products in MS had been lacking. Development of this therapeutic monoclonal antibody to CD20, a cell surface molecule confined to B cells, allowed determination of the effects of B cell depletion. Perhaps somewhat unexpectedly, depletion of circulating B cells led to rapid and profound reduction in gadolinium enhancing lesions on brain MRI in three separate studies in relapsing MS subjects. When examined, depletion of B cells in the blood was accompanied by depletion of B cells in CSF. Notably, reduction of enhancing brain lesions was not accompanied by reduction in CSF immunoglobulins. Whether the critical role of B cells occurs in the periphery, in the CNS, or in both locations has not yet been determined.

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1. Introduction.

More than 90% of multiple sclerosis (MS) patients have increased intrathecal production of immunoglobulins, a fact that has been known since the 1940's [1]. Because of this, for many years research on MS focused on the humoral immune system. Nonetheless, in recent decades (1980's and 1990's) greater emphasis was placed on the potential pathogenic role of T lymphocytes in the pathologic process in MS. This emphasis on T cells was based on the presence of activated T lymphocytes in MS plaques, the strong genetic risk for MS conveyed by certain MHC-II (HLA-DR2, critical for presentation of antigen to T cells), and the use of an animal model for MS, experimental allergic encephalomyelitis (EAE), which can be initiated solely by myelin-reactive T cells [2].

Support for a critical role in MS for B lymphocytes, plasma cells, and the antibodies (Abs) that these cells produce is found in the pathology of MS, which reveals the presence of plasma cells, B cells, antibody and complement. Recently, several studies have focused on the formation of ectopic B cell containing lymphoid tissue in MS CNS, a finding first reported by Prineas in 1979[3]. Increased concentrations of Abs in CSF of MS patients have been shown in several different studies to correlate with more aggressive types of MS, and with episodes of MS worsening [4]. However, in addition to generation of plasma cells and Abs, B cells have many other potential roles in the pathogenesis of MS. These include antigen uptake, processing and presentation to T cells, T cell co-stimulation, and production of cytokines, chemokines and neurotrophic factors. They may also play a regulatory role, through production of regulatory cytokines (IL-10, TGFbeta)[5].

The first part of this paper describes the evidence, both old and new, implicating B cells, plasma cells, and Abs in the pathogenesis of MS. We review data that suggest that autoantibodies (autoAbs) directed against CNS myelin may contribute to MS pathogenesis. We also point out that autoAbs may be secondary to the liberation of antigens with CNS damage, or non-specific due to nonspecific B cell activation. A little more than a decade ago, rituximab, a monoclonal Ab drug specifically targeting B cells, became available to directly test the hypothesis that B cells are critical to the pathogenesis of MS. Part two of this paper focuses on several studies using this monoclonal Ab in MS patients that have concluded recently. These studies are of great interest because they have paved the way not only for new MS treatments, but have also confirmed that B cells play a key role in the pathogenesis of the inflammatory, relapsing subtype of MS. However, the means by which B cells affect MS remain unclear.

2. Part 1: Evidence implicating B cells, plasma cells and antibody in MS

2.1. The Neuropathology of MS.

The presence of plasma cells, myelin-specific Abs and, to a lesser extent, B cells in both chronic MS plaques and acute MS lesions [6; 7] strongly implicates their involvement in its pathogenesis. Immunoglobulin (Ig)G and complement deposition in MS tissues are typically found in areas of active myelin breakdown, suggesting a role in causation. Studies using electron microscopy and immunohistochemistry have provided the greatest details and will be summarized here.

Three decades ago, Esiri used immunohistochemistry to study 100 MS plaques (expanded in a later paper to 247 plaques) and to compare these lesions with 100 adjacent areas of normal-appearing white matter (NAWM) in post-mortem CNS tissues [8]. Cells

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in which Abs could be detected were far more numerous in MS plaques than in NAWM, and also were more numerous in active than in non active plaques. Most of the cells in which Abs were detected had the morphologic appearance of plasma cells. Free light chains (unassociated with heavy chains) were commonly detected, with a higher kappa to lambda ratio found in active plaques than in NAWM. The majority of heavy chains were of the IgG isotype, but IgA and IgM were also detected.

Prineas and colleagues have studied the neuropathology of MS for several decades during which time they have frequently addressed the presence and frequency of plasma cells, B cells, immunoglobulin and complement within MS CNS. An electron microscopic study of autopsied CNS from 6 MS and one control subject (with amyotrophic lateral sclerosis) revealed large numbers of plasma cells in the MS tissues, including within chronic, inactive plaques [7]. Plasma cells were also observed in NAWM and in the leptomeninges. It was suggested that the numerous plasma cells were the source of excess IgG and oligoclonal bands in CSF of MS patients. The accumulation of plasma cells at sites of chronic inflammation is often cited as evidence of a local antigen-driven response[9]. However, accumulation of plasma cells may also occur due to presence of nonspecific activators. For example, stimulation of Toll-like receptors 2, 6, 7, 9, and 10 on human memory B cells by their corresponding agonists can lead to differentiation into plasma cells in an antigen non-specific manner[10]. Other investigators have demonstrated that a particular cytokine milieu can lead to plasma cell differentiation, in an antigen non-specific manner. [11]

2.2. Immunoglobulins in MS CNS tissues.

Lucchinetti et al. performed a detailed immunohistochemical study of active MS lesions from 83 biopsies and autopsies of MS patients, describing four different pathologic subtypes of active MS lesions. One subtype, Type II, involved the prominent presence of Abs and complement, in addition to T cells and macrophages. The Type II pattern of pathology was by far the most common (>70% of active lesions) pattern observed [12]. Lucchinetti and colleagues have suggested that each individual MS patient has only one of the four subtypes of active lesion pathology, but controversy exists regarding this, and even regarding the validity of these four subtypes [13; 14] [15].

A recent detailed immunohistochemical study compared the presence of immunoglobulins and activated complement components in autopsied CNS tissues from 25 MS patients and 24 patients with other neurological disease (OND) [13]. Although IgG was observed within myelin in MS lesions, and in macrophages containing myelin debris, it was also observed within astrocytes, axons (normal and abnormal), occasional neurons, and oligodendrocytes. Moreover, there was no difference in frequency or pattern of IgG deposition noted between MS and OND tissues. These investigators interpreted their findings as not supporting Ab-mediated demyelination in MS, but rather to reflect a finding associated with white matter damage in general.

2.2. Lymphoid-like tissues in MS CNS

Prineas first reported the presence of ectopic lymphoid –like tissues in MS CNS, using electron microscopy [3]. More recently, B-cell follicle-like structures have been described in the meninges of some patients with secondary progressive MS [4; 16] . The presence of such follicles indicates that a microenvironment promoting B cell expansion, maturation and Ab production can exist within the CNS. The presence of ectopic follicles

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in the meninges is associated with cortical pathology and an aggressive clinical course [4]. However, formation of ectopic follicles in chronically inflamed non-lymphoid organs is not specific for MS, as these have been described in other autoimmune diseases with a strong immune activation, such as myasthenia gravis, autoimmune thyroiditis, and rheumatoid arthritis [17; 18].

2.3. B cells and Plasma Cells in MS CSF

B cells are rarely observed in the CSF of healthy people, but commonly are found in low percentage in the CSF of patients with MS[19]. Most of the B cells in MS CSF are of the CD19+ CD27+, CD138- phenotype, but some cells are CD19+ CD138+, indicating that they are B cells undergoing transformation into plasma cells (plasmablasts) [19; 20]. CSF plasma blasts are especially increased in relapsing MS patients with clinically active disease or with gadolinium-enhancing lesions on MRI[19; 20]. Occasionally, mature plasma cells (CD19-, CD38+, CD138+) are observed in MS CSF, but this is not common [20]. A higher proportion of B cells relative to monocytes in CSF correlates with more rapid disease progression [21].

2.4. Immunoglobulins in CSF

A relatively sensitive finding often used in the diagnosis of MS is the presence in CSF of elevated concentrations of IgG, although elevated levels of IgM, IgD, and IgA are all found in MS CSF. [22; 23; 24; 25] [26] Due to the blood-CNS-barrier breakdown observed in active MS lesions and the potential contamination of CSF by serum proteins, the IgG index was developed to take into account the level of blood-brain barrier (BBB) integrity. The IgG index is calculated using the concentrations of serum and CSF IgG relative to serum and CSF albumin, because albumin, the most abundant protein in the

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blood, is not made within the CNS so its detection in CSF reflects seepage across the BBB[27]. One small study performed on autopsied MS brains with concurrent CSF showed a correlation between brain IgG and CSF IgG, with the brain IgG always higher, suggesting that the CSF IgG derived from the brain tissue itself [28]. Other more recent studies have suggested the CSF Abs may derive from plasmablasts within the CSF.

IgG and IgM in MS CSF typically display an oligoclonal band (OCB) pattern when these proteins are separated by electrophoresis[26]. Strong circumstantial evidence exists associating higher levels of Ig in MS CSF with more aggressive disease. In most published studies, elevated OCB numbers, IgG and IgM levels, and kappa light chain concentrations in CSF all correlate with worse prognosis[29; 30; 31; 32; 33] [34] [35]. However, there are reports of OCB-positive MS having a better prognosis than OCB-negative MS, albeit confounded by a higher proportion of males in the OCB-negative group [36].

2.5. Autoantibodies targeting myelin proteins in cerebrospinal fluid of MS patients.

Despite a multitude of studies, the OCBs in MS have not consistently been shown to target myelin, other CNS components, or any particular infectious agent. This was elegantly demonstrated by Owens and colleagues by recombinantly generating 53 Abs from CSF B cells, none of which reacted with the major myelin proteins [37]. Only rarely have Abs to myelin been identified within OCBs[38]. AutoAbs to myelin that do not correspond to the OCBs detected by electrophoresis have been detected in MS CSF. Appropriate controls are critical in these types of studies, as immunoglobulins can non-specifically bind to myelin [39].

AutoAbs to several CNS antigens that do not represent major myelin proteins have been detected in CSF. AutoAbs to myelin-oligodendrocyte glycoprotein (MOG), a highly immunogenic but minor component of myelin [40] and to transaldolase, an enzyme which, in the CNS, is specific to oligodendroglia [41] have been detected with increased prevalence in MS. Evidence exists for cross-reactivity between epitopes of transaldolase, MBP and MOG with epitopes of infectious agents, allowing for the possibility of molecular mimicry in the development of these autoAbs [42; 43].

2.6. Antibodies extracted from MS CNS tissue are often autoreactive with myelin.

One possibility of why Abs found in CSF of MS patients are not often identified as autoAbs to myelin or CNS antigens is that the relevant autoAbs are largely bound to their tissue targets, rather than freely soluble within CSF. Several lines of evidence support this idea. In a series of studies, Warren and Catz isolated affinity purified IgG directly from MS plaques, and identified MBP as a target in most cases. The MBP (83-97) peptide appeared immunodominant since it strongly inhibited autoAb binding to MBP in almost 100% of cases [44]. In a subsequent study, CNS tissues from eleven of twelve MS cases yielded IgG directed toward MBP. In ten of the twelve cases, 10-20% of the total IgG extracted from the CNS was anti-MBP (83-97) peptide and in one case it represented almost 50% of the total extracted IgG [43]. Thus, in these MS autopsies, MBP (83-97) was the dominant autoAb target of tissue-bound IgG. This same region of MBP also contains the dominant T cell epitope. Microbial peptides were identified that were also bound by purified IgG extracted from MS CNS tissue, with strong cross-binding to peptides of human papillomavirus types 7 and 13. Purified IgG extracted from

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CNS of MS autopsies also bound peptides from several other viral and bacterial pathogens, but less well. [43].

Recently, O'Connor and colleagues have expanded this concept by eluting IgG from plaque regions in 14 MS cases, and from CNS tissues from 6 normal controls and 4 other inflammatory CNS disease controls [45]. They demonstrated specific binding with conformation-dependent epitopes of native human MOG by IgG that had been extracted from seven of 14 (50%) MS samples, one of the four inflammatory control samples (a case of SSPE) and none of the normal samples[45].

Some of the strongest, but still circumstantial, evidence of autoAbs being involved in myelin damage in MS derives from studies using gold-labeled peptides of MBP and MOG[46]. These peptides bound specifically to areas of myelin damage in sections of active MS lesions. Control gold-labeled bovine serum albumin peptides did not bind. On serial sections of the same CNS lesions, Abs to human IgG localized to the same regions, providing suggestive evidence that the binding of the peptides was due to autoreactive IgG at the sites of myelin destruction.

It should be noted that Abs eluted from CNS tissue may represent specific autoAb binding to antigen, but might also represent binding to tissue components nonspecifically [39] or via Fc receptors on glial or mononuclear cells. Moreover, the above reports are merely associations and do not prove a role for immunoglobulins or B cells in the pathogenesis of MS.

2.7. Autoantibodies in the blood of MS patients and controls.

Serum Abs targeting myelin proteins have been reported by some as elevated in MS patients. One report of 103 subjects with clinically isolated demyelinating syndrome

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(CIS) at high risk to develop MS suggested that the presence of serum IgM Abs detected by Western blot to recombinant non-native MOG (prepared in E.coli) and to MBP was predictive of more rapid development of the second attack, marking the diagnosis of MS [47]. Subsequent studies of hundred of MS and CIS subjects from around the world have not been able to reproduce an association between more rapid onset of subsequent attacks and the presence of anti-MOG or anti-MBP IgM or IgG antibodies.[48; 49; 50]. The results from subsequent studies, which have typically utilized ELISAs rather than Western blots, collectively do not support a pathogenic role for these antibodies, but instead suggest that their presence may be secondary to the liberation of CNS antigens upon damage to CNS tissues.

An elegant and sensitive assay for Abs to native MOG was developed based on binding to Jurkat cells stably transfected with a MOG-green fluorescent protein construct [51]. Serum samples from 131 pediatric MS cases, 254 adult-onset MS cases, 30 clinically isolated syndrome (CIS) patients, and numerous control subject groups including pediatric neurologic and non-neurological control subjects, pediatric diabetes patients (n=28), neuromyelitis optica cases (n=13), viral encephalitis cases (n=29) and healthy adult controls (n=86) were tested [51]. The samples derived from patients at many different MS centers in several countries around the globe. Very few of the adult-onset patients had serum anti-MOG Abs (11/254, 4.3%). Only two of 30 CIS serum samples contained anti-MOG Abs which was of low binding affinity. Interestingly, Abs to MOG were detected in 28/131 pediatric-onset MS samples, with most positive samples deriving from the patients with the youngest ages of onset. In this study, in the small number of cases with positive serum anti-MOG Abs in which CSF was also tested, CSF

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was either negative or of lower binding than serum. Abs to MOG were never detected in the small numbers of samples from NMO subjects, or in the viral encephalitis cases. Thus, only a minority of MS patients harbored Abs targeting native human MOG. This finding suggested that autoAbs directed against native MOG are not involved in the primary pathogenic mechanisms of MS, or that they denote only a minor subset of primarily pediatric MS patients. It was notable that anti-native MOG Abs were *not* detected in several other destructive CNS disorders, such as viral encephalitis.

2.8. Antibodies to non-protein CNS antigens in MS.

AutoAbs against myelin lipids, especially glycolipids such as galactocerebroside, GM4 gangliosides, and GM1 gangliosides, can be detected in the serums and CSFs from some MS patients [52; 53]. CSF antibodies to myelin lipids have been associated with a more aggressive MS course. [33] MS patients in the progressive stage are reported to have a higher incidence of anti-lipid Abs than patients with a relapsing-remitting course. However, these anti-lipid Abs can also be found in other diseases, such as systemic lupus erythematosus and stroke, making their significance unclear [54].

Recently, IgM antibodies to alpha-glucose-based glycans have been found in approximately 30% of relapsing (RRMS or SPMS) patients, including early patients with a first demyelinating episode[55]. The IgM Abs are found in a much smaller proportion of PPMS subjects, but can also be found in healthy controls. Their incidence in other immune disorders is not reported[56]. The presence of these serum IgM Abs may indicate a more rapid time to next relapse, but their significance is as yet unknown.

In summary, B cells and plasma cells can be found in autopsied and biopsied CNS tissues from MS patients. Often they are found at the sites of active lesions. Likewise,

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autoAbs to myelin proteins, particularly MOG and MBP, can be extracted from MS tissues and also are present in the CSF and blood of some MS patients. However, autoAbs to myelin in blood and CSF are not specific to MS, and often can be detected in OND subjects and other control populations. The latter suggests that such autoAbs may develop in response to tissue destruction and are not the primary cause of MS. Even so, autoAbs targeting CNS components may contribute to the ongoing tissue destruction in MS, as they have been shown to do in relevant animal models [57].

3. Part 2: Therapeutic Depletion of B Cells in MS patients.

3.1. B cell Depleting Agents

A little more than a decade ago, a therapy that specifically depletes B cells in humans became available, providing the opportunity to test the hypothesis that B cells are critical to the pathogenesis of MS. This monoclonal Ab drug is rituximab (MabThera[®]/Rituxan[®], Genentech Inc., San Francisco, CA; IDEC Pharmaceuticals Corp, San Diego, CA; Roche, Basle, Switzerland) (US license No. 1048). It is a genetically engineered glycosylated immunoglobulin G1 κ chimeric mouse/human antibody that binds to the CD20 antigen present on the majority of circulating B cells [58]. It contains murine light- and heavy-chain variable regions and human gamma 1 heavy-chain and kappa light-chain constant regions. Rituximab has an approximate molecular weight of 145 kD. Expression of CD20 is restricted to the B cell lineage from the pre-B cell stage until prior to the terminal differentiation into plasma cells (Table 1).

The Fab domain of rituximab binds to CD20 on B cells and the Fc domain recruits immune effector functions to mediate B-cell lysis *in vitro*. Possible mechanisms of cell lysis *in vitro* include complement-dependent cytotoxicity and antibody-dependent cell mediated cytotoxicity

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and possibly apoptosis. The relative extent to which these individual mechanisms account for the observed depletion of B cells *in vivo* is unknown but likely depends on the tissue where depletion is occurring. Depletion in the peripheral blood is rapid and dramatic. A recent case report in a patient with lymphoma suggests that B cell depletion therapy is associated with the absence of B cells in the perivascular spaces [59]. It is unclear if this effect was a direct or indirect effect of rituximab therapy.

Rituximab was approved in the United States in November 1997 for the treatment of patients with relapsed or refractory low-grade or follicular CD20⁺ B-cell non-Hodgkin's lymphoma (NHL) at a dose of 375 mg/m² weekly for four doses. In April 2001, the FDA approved additional claims for the treatment of low-grade NHL: retreatment (weekly for four doses) and an additional dosing regimen (weekly for eight doses). In February 2006, rituximab was FDA-approved for the treatment of rheumatoid arthritis patients with inadequate response to anti-TNF α therapy. Useful insights into administration, safety and tolerance profiles have been gained through the more than 1 million patient exposures to rituximab, as either monotherapy or in combination with immunosuppressant drugs for up to 2 years[60; 61].

3.2. Rituximab in MS: Open-label add-on study in breakthrough RRMS.

In an open-label investigator-initiated trial of rituximab as add-on therapy to IFNB or GA in subjects with RRMS and suboptimal response despite standard therapy, 30 subjects on first-line disease modifying therapies completed the regimen of four weekly infusions of 375 mg/m² each [62] [63]. The primary endpoint of this trial was change in number of gadolinium-enhancing lesions on three post-treatment versus three pre-treatment brain MRIs scans. MRI results showed 88% reduction in mean number of gadolinium-enhancing (Gd⁺) lesions (Naismith et al submitted; p<0.0001). Although B cell numbers in CSF declined by 95% on

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average between baseline and 20 weeks following the 4th dose, there was no significant change observed in OCB number, IgG index or CSF IgG concentration. The number of T cells in the CSF decreased by 50% on average [63]. A similar effect of rituximab on CSF B cells has been reported in subjects with PPMS and RRMS [64; 65]. It is unclear whether decreased B cell numbers in CSF relates to depletion of a trafficking pool of B cells that enter the CNS from the periphery or to entry of rituximab into the CNS. Although the number of B cells in the CSF was greatly reduced after rituximab therapy, the proportion of B cells that also expressed costimulatory molecules CD80 and CD86 was increased. In the peripheral blood, unlike what was seen in the CSF, and in contrast with the dramatic B cell depletion, T cells were only 12% lower after therapy[63]. Modest reductions in serum antibodies to MOG and MBP were seen in some subjects after therapy (week 24)[62]. After rituximab, CSF CXCL13 and CCL19 were selectively decreased. CXCL13 decrease correlated modestly with CSF T cell reduction, while CCL19 did not [63]. These two chemokines are involved in the organization of lymphoid follicles. CXCL13 is also a chemoattractant for both B and activated T cells. Dendritic cells are one of the sources of CXCL13 within human lymphoid tissues. In the CNS, dendritic cells or monocytes/macrophages are believed to produce it. CCL19 is involved in the migration of lymphocytes and mature dendritic cells. Finally there was a negative correlation between the changes in T cell numbers and the changes in CSF MCP-1. The underlying biological mechanisms leading to these chemokine changes after B cell depletion are unclear.

3.3. Phase I trial of Rituximab in relapsing MS

A total of 26 subjects with RRMS were enrolled in this open-label trial [66]. Subjects received two courses of rituximab (one course = 2,000 mg delivered as 1,000mg each on two days, two weeks apart) over one year (infusions on weeks 0 and 2, and week 24 and 26).

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Enrolled subjects had a mean age of 40.4 ± 8.7 years. At baseline, mean EDSS score was 2.3.

Subjects had a mean of 1.27 relapses during the previous year. Over the 72 weeks, 21 (80.8%) subjects remained relapse-free; 4 subjects (15.4%) reported 1 relapse and 1 subject (3.8%) reported 2 relapses. The unadjusted annualized relapse rate was 0.25 and 0.18, from baseline to Week 24, and from baseline to Week 72, respectively.

The mean number of Gd+ lesions decreased from 1.31 (baseline) to 0.73 (Week 4) after the first course of rituximab. After the second course, the mean number of Gd+ lesions decreased to 0.05 (Week 48) and 0 (Week 72). The mean number of new T2-bright lesions decreased from 0.92 at Week 4 to 0 at Week 72.

3.4. Phase II placebo-controlled trial of Rituximab in RRMS

A multicenter trial of rituximab in RRMS (funded by Genentech, Inc. and Biogen Idec, Inc.) enrolled 104 subjects, 69 were randomized to rituximab and 35 to placebo[67]. Of 104 subjects, 96 (92.3%) completed 24 weeks, and 79 subjects (76%) completed 48 weeks, including 84.1% in the rituximab group and 60% in the placebo group. Twelve of the 25 subjects who discontinued the study treatment period early completed the safety follow-up, including 10.1% of subjects in the rituximab group and 14.3% of subjects in the placebo group[67].

Primary Endpoint (Intent-to-treat analysis)

Rituximab-treated subjects demonstrated a dramatic reduction in total Gd+ lesion counts at weeks 12, 16, 20, and 24 compared with placebo-treated subjects ($p < 0.001$). Those receiving rituximab had a mean of 0.5 Gd+ lesions, compared with 5.5 in those receiving placebo, a relative reduction of 91%. Beginning at week 12, compared with placebo, rituximab significantly reduced Gd+ lesions at each subsequent study visit ($p = 0.003$ to < 0.001).

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Key Secondary Endpoints (Intent-to-treat analysis)

The proportion of subjects with relapses was reduced in the rituximab group compared with placebo at week 24 (14.5% versus 34.3%, $p=0.02$) and week 48 (20.3% versus 40.0%, $p=0.04$). At week 24, placebo recipients were at double (2.31) the risk of relapse compared with rituximab-treated subjects; at week 48, the relative risk of relapse was 1.90.

Beginning at week 12, rituximab also significantly reduced the number of new Gd+ lesions at each study week ($p=0.002$ to <0.001).

Immunologic studies

B-cell depletion, both *ex vivo* and *in vivo* resulted in significantly diminished pro-inflammatory Th1 and Th17 responses of both CD4 and CD8 T cells. Soluble products from activated B cells of untreated MS subjects reconstituted the diminished T-cell responses observed following *in vivo* B-cell depletion in the same subjects, and this effect appeared to be largely mediated by B-cell lymphotoxin and tumor necrosis factor-alpha[68]. These findings of decreased peripheral T cell activation and proliferation in the absence of B cells would be expected to result in lesser T cell trafficking into the CSF compartment.

3.5. Phase II/III trial in primary progressive MS

A phase II/III trial in PPMS (OLYMPUS, funded by Genentech, Inc. and Biogen-Idec, Inc.) tested the effect of rituximab versus placebo in 450 subjects for a total of 2 years of therapy[69]. Using 2:1 randomization, 439 PPMS subjects received either two 1,000 mg rituximab infusions or two placebo infusions (separated by 2 weeks) intravenously every 24 weeks, (4 treatment courses) through 96 weeks. Mean baseline EDSS was 4.8. Half of the subjects were males, and median age at baseline was 51 years.

Primary endpoint: time to confirmed disease progression (CDP), defined by a pre-specified increase in EDSS sustained for 12 weeks (increased by at least 1.0 point from baseline if EDSS between 2.0 and 5.5 inclusive; increased by at least 0.5 point if EDSS > 5.5). The difference in time to CDP between treatment arms was not statistically significant ($p=0.14$, stratified log-rank test). Kaplan-Meier estimates for the proportion of subjects with CDP at 96 weeks were 38.5% for placebo and 30.2% for rituximab (e.g. a 24% reduction in the risk of progression in rituximab recipients).

Secondary imaging endpoints: Rituximab recipients had less T2 lesion volume increase than placebo recipients ($p<0.001$) while brain volume change was similar in both groups ($p=0.62$). Median increase in the T2 lesion volume was 302.95 mm³ for rituximab treated subjects and 809.50 mm³ for placebo treated subjects.

Exploratory endpoints: compared with placebo, rituximab treated subjects had less worsening in the timed 25-foot walk at weeks 48, 96 and 122. Other MSFC items showed no difference between groups.

Pre-planned sub-group analysis showed that subjects under 51 years of age, who had Gd+ lesions at baseline or who had progression the year before study participation were more likely to benefit from treatment.

3.6. Clinical Safety and Tolerability

Side effects of rituximab:

The most common side effects of rituximab are infusion reactions including fever, chills, rigors, hypotension and general flu-like symptoms. These generally occur during the first infusion and the majority is classified as mild or moderate. Rarely, severe and fatal infusion-related reactions

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have occurred in lymphoma patients. Risk factors for serious infusion reactions in lymphoma patients include high tumor burden, high circulating lymphocyte counts, and concurrent cardiovascular or pulmonary disease. Rituximab does not appear to increase significantly increase the rate of serious infections in most studies to date [60; 61; 70]. However, several cases of progressive multifocal leukoencephalopathy (PML) have been reported in patients receiving rituximab and concurrent or prior therapy with other immunosuppressive therapies for lymphoma, rheumatoid arthritis or systemic lupus erythematosus. Thus far, no PML cases have been reported in patients with MS, or in patients with other conditions receiving rituximab as a monotherapy who had not been exposed to prior immunosuppression or did not have another potential reason to develop PML.

Rituximab side effects in MS.

All three trials in RR and PPMS of rituximab monotherapy have confirmed a side effect profile similar to that previously reported in rheumatoid arthritis and other autoimmune disorders. The most common ($\geq 10\%$) drug-related adverse events (AEs) in the rituximab group were infusion-associated. Most infusion-related AEs occurring with the first infusion in MS subjects were mild or moderate ($>90\%$), while 7% were grade 3 and none were grade 4. Rates of infusion-related AEs were comparable to placebo upon successive infusions. In the phase II trial in RRMS, grade 4 events were reported in 3 rituximab subjects: ischemic coronary artery disorder (1), malignant thyroid neoplasm (1), and acute and progressive MS symptoms (1). Serious AEs (SAEs) were reported in 14.3% of subjects in the placebo group and 13% of subjects in the rituximab group. A total of 5.7% placebo subjects and 4.3% of rituximab subjects withdrew from the study due to AEs. One death (homicide) occurred in the rituximab group.

In the phase II RRMS trial, the incidence of infection was similar in the placebo (71.4%) and rituximab (69.6%) groups. The most common ($\geq 10\%$) infections in rituximab-treated subjects were nasopharyngitis, upper respiratory tract infections, urinary tract infections, and sinusitis. Urinary tract infections (14.5% rituximab vs. 8.6% placebo) and sinusitis (13.0% vs. 8.6%) were more common in rituximab-treated subjects. No opportunistic infections were reported. Infectious SAEs were reported by 5.7% of placebo subjects and 2.9% of rituximab subjects. Of the 2 serious infection-related AEs in the rituximab group (gastroenteritis, bronchitis), both resolved without sequelae; the last recorded values for immunoglobulin levels prior to these infections were above the lower limit of normal in both subjects.

In the PPMS phase II/III trial, infection-related SAEs were higher in the rituximab group than the placebo group (4.5% vs. $<1.0\%$). Three subjects died, one from each group with pneumonia, and one from the placebo group due to cardiopulmonary failure.[69] Although likely too small to be conclusive, there was no evidence for increased incidence of infection or other adverse events in subjects with Ig levels below the lower limit of normal (LLN).

3.7. Dosing and Pharmacokinetics.

Dosing

Rituximab was initially approved in lymphoma at a dose of 375 mg/m² weekly for four doses. Subsequently, the regimen was changed to a fixed dose of 2,000mg (two infusions of 1,000mg, 2 weeks apart) based on safety, pharmacokinetic, pharmacodynamic and efficacy data in lymphoma and rheumatoid arthritis (Edwards 2004).

Pharmacodynamics and Immunogenicity in MS trials

One uncontrolled phase I and one randomized phase II trial in RRMS, and one randomized phase III study in PPMS have provided detailed information on the pharmacodynamics and immunogenicity of rituximab in MS subjects [66; 67]. In these trials, rituximab induced near-complete depletion of CD20-positive peripheral B-cells (measured by CD19 expression) (>95% reduction from baseline) by Week 2. In the phase II trial (one course of rituximab at weeks 0 and 2 vs placebo), depletion was sustained through 24 weeks; by week 48, CD19-positive cells had returned to 30.7% of baseline values. CD3 T lymphocytes in the blood were not appreciably altered by rituximab. Similarly, in the phase I study (two courses of 1,000mg rituximab at weeks 0 and 2, and at weeks 24 and 26) depletion was complete up to Week 48, with reconstitution to mean 34.5% of baseline by Week 72. The majority of reconstituting cells were CD19-positive/CD27-negative naïve B cells (mean 51% of baseline) rather than CD19-positive/CD27-positive memory B cells (mean 14% of baseline) suggesting that naïve B cells appear to recover faster than memory B cells. Circulating B cells are replenished from bone marrow pro-B cells within 4 to 12 months after depletion, sometimes longer.

In the phase II monotherapy study, median IgM, IgG, and IgA levels remained above the lower limit of normal (LLN) in both treatment groups throughout the trial. At any time through week 48, IgG levels were below LLN in 7.9% of rituximab-treated subjects and 3.0% of placebo-treated subjects, and IgM levels were below the LLN in 22.4% of rituximab subjects and 8.6% of placebo subjects. No subject had IgA levels below the LLN during the study. In the phase I study, serum IgG titers for tetanus generally remained unchanged over 72 weeks and no subject who was seropositive for mumps or rubella at baseline became seronegative during the study. In the phase III OLYMPUS study, IgG and IgA levels were below LLN range in <5% of subjects in

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either treatment arm. IgM were below LLN at some time during the study in 31.7% of rituximab recipients and 5.9% of placebo recipients[69].

In the phase II trial at week 48, of 58 subjects who completed the treatment period, 14 (24.1%) tested positive for human anti chimeric antibodies (HACA) to rituximab; no placebo subject tested positive at any time. There was no apparent association between HACA positivity and the type or severity of adverse events or efficacy response at weeks 24, 36, or 48 but this trial did not evaluate the efficacy of rituximab re-administration. In the Phase II add-on trial, 13% of subjects developed HACA by 24 weeks after therapy. (Naismith et al., in press). Surprisingly, in the PPMS trial[69], 7% of rituximab recipients and 6.3% of placebo recipients were positive for HACA. The reason for the presence of HACA in placebo recipients in this study is less clear.

3.8. Off-label B cell depletion in MS patients.

Rituximab is not approved for use in MS patients. Nonetheless, it is currently used off-label by some physicians for MS patients failing approved therapies for MS. The optimal frequency of rituximab administration and how long subjects should be retreated is unclear. Moreover, the risks of long-term B cell depletion in MS patients are unknown. It remains to be seen whether the rate of infections increases with long term B cell depletion. Furthermore, no data are available regarding the efficacy of vaccinations performed in patients undergoing chronic B cell depletion.

The next generation of anti-CD20 monoclonal Ab is under clinical evaluation. Ocrelizumab is a humanized Ab which has a lower rate of potentially neutralizing human antihumanized Abs development and infusion reactions than rituximab. Ocrelizumab is being

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tested in MS. Other humanized antiCD20 molecules are being developed and include both monoclonal Abs and small molecules. Other therapeutic strategies that aim to eliminate B cells later in B cell development (atacept), or modify B cell response (BAFF-receptor Ig) are being developed. These have not yet been tested in MS.

3.9. What have we learned from B cell depletion trials in humans?

The four trials of rituximab that have been performed in RRMS and PPMS have all shown a benefit in terms of brain MRI Gd⁺ lesion reduction. From the studies in RRMS, Gd⁺ lesion reduction appears to be very prompt in onset (i.e. beginning within a few weeks after treatment administration). The phase II placebo-controlled trial in RRMS also suggested a substantial clinical reduction in relapses during the 12 months following treatment, when subjects were depleted of circulating B cells. While the effect of rituximab on the inflammatory component of the disease is obvious, the effect on insidious disability progression such as occurs in PPMS is unclear.

B cell depletion in MS subjects did not significantly alter CSF IgG levels; IgG Index, IgG concentration, IgG synthesis rate and oligoclonal bands did not change significantly after treatment. [62] [63]. However, B cell depletion induced a delayed and marginal reduction of IgG and IgM blood levels. This effect likely requires several rounds of treatment over several months, as the Ab-secreting plasmocytes become CD20 negative upon differentiation from B cells. Thus, mature plasma cells are not directly impacted by rituximab. However, as rituximab depletes the pool of B cells differentiating into plasma cells secreting Ig, chronic B cell depletion appears to initially affect IgM and subsequently IgG levels. Whether these changes participate in disease modification is unknown. With respect to the notion of using rituximab based on its

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potential to impact autoAb production, it is important to keep in mind reports that some CNS autoAbs may actually have positive effects on remyelination[71; 72].

While B cells return several months after B cell depletion, there seems to be a differential impact on various B cell subtypes. Naïve B cells (CD27-) return much earlier than memory B cells (CD27+) [73]. This observation has raised the question whether rituximab might be “resetting” the B cell immunologic clock.

4. Conclusion and Future Perspectives

The dramatic reduction of Gd+ lesions on brain MRI in RRMS that has now been observed in three separate trials of B cell depletion using rituximab has confirmed a pathogenic role of B cells in the development or maintenance of inflammatory MS lesions. The mechanisms by which the effect occurs remain unknown. The rapid impact of rituximab in RRMS argues for a main effect related to antibody-independent B cell functions. One of the striking findings in the open label add-on trial was the dramatic reduction in B cells in the CSF, with a lesser reduction in CSF T cells. In the context of the marginal reduction in peripheral T cells [63] and the diminished pro-inflammatory response of both CD4 and CD8 T cells [68], the 50% reduction of CSF T cells suggests a decreased ability of peripheral T cells to enter the CSF compartment. Thus, B cells may be directly or indirectly critical to the ability of T cells to traffic into the CNS. The effect of B cell depletion in terms of antibody independent immune response is likely complex, and as suggested by Piccio et al. and Bar-Or et al., could be mediated via an effect on decreased T cell migration and proliferation, and decreased release of pro-inflammatory cytokine by B cells and possible indirect consequences on dendritic cells. Future studies to analyze the

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effects of B cell depletion on antigen uptake, processing and presentation to T cells in MS patients promise to provide further understanding of the role of B cells in MS.

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Table 1: Expression of cell surface antigens CD19 and CD20 at various stages of development of the B cell lineage.

	Stem cell	Pro-B cell	Pre-B cell	Immature B cell	Mature B cell	Activated B cell	Memory B cell	Plasma cell
CD19	0	+	+	+	+	+	+	0
CD20	0	0	+	+	+	+	+	0