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Chapter 3

Intrathecal Immunoglobulin Synthesis in MS—A Complete Reappraisal

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Abstract

Multiple sclerosis (MS) is characterized by the intrathecal synthesis (ITS) of immunoglobulins (Igs), which, although nonspecific, is the strongest biological marker. Since no specific target has been elucidated, this synthesis is considered to be disease-irrelevant. We demonstrate that this synthesis provides pertinent information about the pathophysiological processes involved. Quantification of ITS is based on an approximation intrinsically underestimating its level and it remains constant in MS, albeit sometimes at a low level. B-cell maturation seems to be initiated within the cervical lymph nodes and B-cells traffic on both sides of the blood-brain barrier by rounds of bidirectional traffic. During this process, they undergo somatic hypermutation, which is the hallmark of antigen-driven antibody maturation, suggesting that most of the ITS is probably directed against as yet unknown targets. Alternatively, examining"non-disease-relevant" ITS in the light of meningeal tertiary lymphoid organs provides new insights into the pathophysiology of MS. Although no specific target has yet been identified in MS, recent developments in the search for targeted antigens point to non-conventional antigens (posttranslationally modified proteins or oxidized products) of which a few are promising for future research.

Keywords: multiple sclerosis, meningeal inflammation, intrathecal synthesis, immunoglobulins, autoantibody, epitopes, antigens

1. Introduction

The normal central nervous system (CNS) is completely devoid of resident plasma cells, so finding infiltrating B-cells in the CNS is rather exceptional. Infiltrating activated CD20+CD23+ B-cells in brain parenchyma number to less than 0.1–1.5 cell/cm^2 [1–3]. Therefore, no intrathe-

cal immunoglobulin (Ig) synthesis (ITS) occurs in the basal state, and locally produced Igs testify to former or active CNS infection or inflammatory processes. Therefore, while it is easy to demonstrate high levels of ITS, quantifying low levels poses technical problems.

Although ITS is one of the most sensitive biological clues for MS, it was long considered to be nonspecific or a bystander of CNS inflammation since the latter mainly involves T-cells and no specific antibody target has yet emerged. The recent success of anti-CD20 therapy in relapsing and progressive MS sheds light on a more central role of non-CNS B-cells in the pathophysiology of MS. However, progressive MS pathophysiology is still poorly understood and CNS resident B-cells may play an even greater role. Since locally synthesized Igs are the main B-cell by-products, ITS offers a good opportunity for studying resident B-cells. The role of cerebrospinal fluid (CSF) B-cells has not yet been elucidated and is probably diverse as follows: (1) cytotoxic effect of antibodies; (2) local antigen presentation to T-cells; and (3) secretion of cytokines playing a complex regulating role, including a possible Ig-unrelated CNS cytotoxicity. Therefore, CNS-trapped B-cells not only synthesize oligoclonal bands (OCBs) but might also play a pivotal role in the slow-burning CNS inflammation.

2. Quantitative measures of CSF IgG

2.1. Pitfalls in assessing ITS level

Synthesis of Igs does not occur in the normal CNS and the tiny Ig concentration measured in normal CSF reflects a low-rate passive diffusion through the blood-brain barrier (BBB) to CSF. It was long thought that although virtually all the molecules may diffuse from serum to CSF, BBB permeability positively correlated with the molecular weight [4]. For example, the ratio decreases from 1:205 with albumin (65 kDa) to 1:440 with IgG (150 kDa) and 1:900 with IgM (970 kDa) [5]. Moreover, the permeability of the BBB commonly increases during CNS pathologies, leading to an increase in CSF concentrations of blood-borne proteins and Igs. As

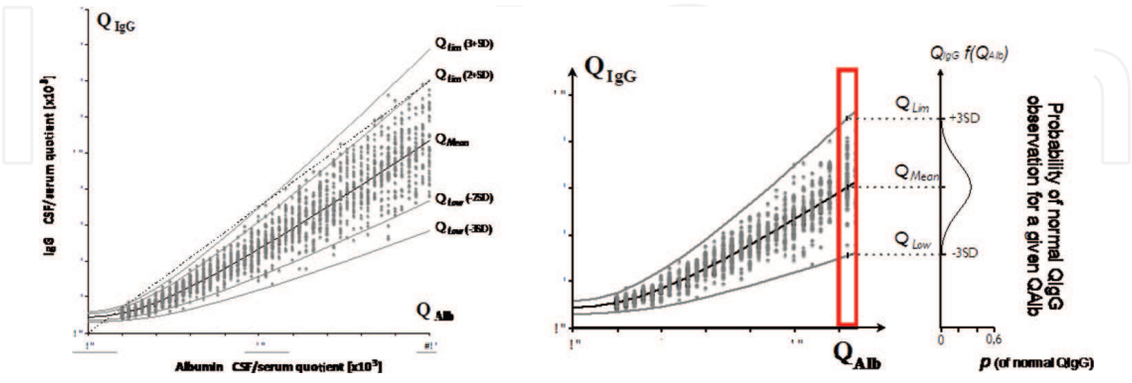

Figure 1. CSF IgG passively diffused from blood to CSF in normal population. Left panel (A). Plot of CSF/serum quotients with hyperbolic function of quotient ratios ("'Reibergram"). Reference range defined by Q_{Lim} : Q_{mean}±2SD or ±3SD involving, respectively, 96 and 99% of the normal population. Dotted line is the upper normal limit (>0.7) of IgG index, intersecting the Q_{Lim} curve at two points. **Right panel (B). Probability curve of basal Q_{IgG} for a given Q_{Alb}.** The maximum probability is obtained for Q_{mean}. Points are obtained from a simulated healthy population.

a consequence, the level of intrathecally synthesized Igs related to CNS inflammation is obscured by the passively diffused CSF Igs concentration and requires a mathematical approach that takes into account the permeability and blood concentration of the targeted molecules. The albumin quotient (or ratio), Q_{Alb} = $[Alb_{CSF}]/[Alb_{serum}]$, is indicative of BBB dysfunction and increases with its permeability. It is also influenced by the patient's age and underlying CNS pathologies. In the basal state, which is devoid of intrathecal IgG synthesis, the ratio $[IgG_{CSF}]/[IgG_{serum}]$ is proportional to Q_{Alb}.

The daily IgG synthesis rate can be assessed by Tourtellotte's formula (median value 29 mg/day), but inconsistent values are obtained at the individual level owing to assumptions [6]. The linear approach where the IgG index = Q_{IgG}/Q_{Alb} (normal values <0.7) does not take into account either normal Q_{IgG} variance nor nonlinear correlations with Q_{Alb}, leading to an approximation around the limit (**Figure 1**, left panel). Moreover, since Q_{Alb} increases with age, the IgG index is thought to decrease mechanically without any change in ITS [7]. Data are best fitted by an empiric hyperbolic function (the "Reibergram") [8] whose constant parameters were fitted with a large dataset of 4154 control patients [9]. From a theoretical point of view, the hyperbolic function is the application of Fick's laws of diffusion applied to albumin and Igs [9]. For a given Q_{Alb} in a population of normal patients, the distribution of Q_{IgG} follows a normal law around the mean curve as $Q_{mean}=f(Q_{Alb})$ (**Figure 1**, right panel) [9]. With intrathecal synthesis (ITS), the IgG concentration in the CSF is the sum of IgG passively diffused from the blood and synthesized intrathecally

$$Q_{IgG} = \left(\left[IgG_{CSF\_passive}\right]+\left[IgG_{CSF\_Loc}\right]\right) / \left[IgG_{serum}\right] = Q_{IgG\_basal}+Q_{IgG\_Loc,}$$

where Q_{IgG_basal} is the Q_{IgG} of the same patient before the onset of ITS. In clinical practice, only Q_{IgG} is directly available but not Q_{IgG_basal}, so the exact IgG_{CSF_Loc} concentration can only be approximated by using Reiber's discrimination curve. The upper limit of the reference range, Q_{Lim}, is usually set arbitrarily as Q_{mean}+3SD and involves >99% of the normal population. By applying this definition, intrathecal IgG synthesis is considered to be present when $Q_{IgG} > Q_{Lim}$. The major drawback of this reference range is the loss of sensitivity for cases displaying a low level of ITS ($Q_{Lim} > Q_{IgG} > Q_{IgG\_basal}$). In common practice, demonstrating ITS in such cases requires a CSF-restricted OCBs positivity. As expected, restricting the reference range to Q_{Lim}+2SD instead of +3SD increases the percentage of abnormal Q_{IgG} in MS cohorts by 6–10% for IgG and up to 20% for IgM, which increases the risk of false positivity to 4% [7].

The true amount of intrathecally (or locally) synthesized IgG should be calculated as follows:

$$IgG_{Loc}\left(mg / L\right) = \left(Q_{IgG} - Q_{IgG\_basal}\right) x \left[IgG_{serum}\right].$$

However, since Q_{IgG_basal} is unavailable, it can be replaced by either Q_{Lim} or Q_{mean}. As previously demonstrated, replacing Q_{IgG_basal} by Q_{Lim} confers maximal specificity in single patient studies but with the drawback of an unavoidable underestimation of ITS. The range of IgG_{Loc}

underestimation may vary widely from 1 to 50 mg/L according to Q_{Alb} (unpublished results). In cohort studies, Q_{IgG_basal} is advantageously replaced by Q_{mean}, which provides a closer estimation of the exact IgG_{Loc} [7].

Lastly, inter-assay variability may directly impact Q_{IgG} or the IgG index. For example, a 10% decrease in serum IgG directly means a 10% increase in IgG index, and 10% variations of the IgG index from day to day are commonly reported [7, 10]. As a consequence, minor fluctuations in IgG levels may be translated into normal or abnormal Q_{IgG} results, although the intrathecal IgG synthesis rate is not really impacted. A final pitfall of ITS assessment relates to the properties of CNS-targeting antibodies themselves, which are capable of brain adsorption that can potentially abolish low levels of specific antibodies that are synthesized locally or spill over to the CSF [11].

2.2. Predicted changes in ITS measures in response to treatments

Using formulas and normal values obtained from the literature, we simulated results of a cohort with tunable ITS level (unpublished results). This model provides the advantage of being able to compare the calculated (approximated) IgG_{Loc} with the fixed IgG_{Loc}.

IgG_{Loc} estimation based on Q_{mean} fitted well with the exact IgG_{Loc}, even in small cohorts and for small ITS (<1 mg/L). On the other hand, individual or cohort estimations of IgG_{Loc} based on Q_{Lim} were strongly biased in a range dependent on Q_{Alb}.

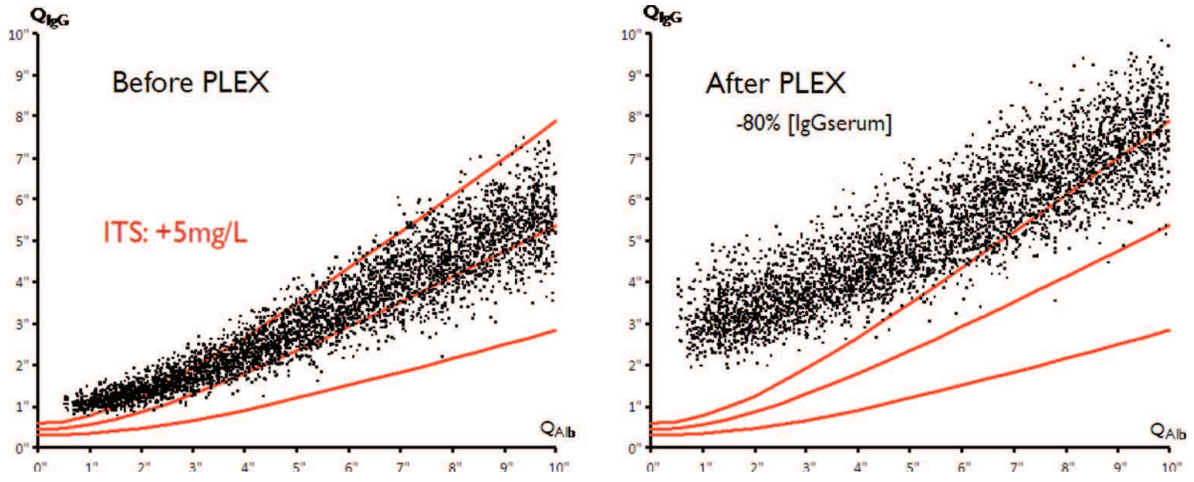

Figure 2. Effect of plasma exchange on Q_{IgG}. Simulated population with ITS +5 mg/L. Q_{IgG} values increase to abnormal range whereas ITS level remains unchanged.

Plasma exchange depletes both serum IgG and IgG passively transferred through the BBB. Therefore, assuming that ITS remains constant during the procedure, the contrast in locally synthesized and passively diffused IgG in CSF is dramatically tuned by plasma exchange (**Figure 2**). For example, after a 90% decrease in $[IgG_{serum}]$, IgG in CSF originates almost entirely

from local synthesis. The IgG index increases whereas the precision of the IgG_{Loc} calculation (based on Q_{mean}) remains unchanged.

For a given level of ITS, a decrease in BBB permeability decreases Q_{IgG_basal} in a nonlinear response whereas Q_{Loc} remains constant. Therefore, Q_{IgG} and the IgG index may shift to abnormal values and $IgG_{Loc}(Q_{Lim})$ increases incorrectly, whereas $IgG_{Loc}(Q_{mean})$ remains constant.

As a consequence, monitoring of the ITS level should be based on the unbiased $IgG_{Loc}(Q_{mean})$.

3. Qualitative measures of CSF IgG—method and limitations of oligoclonal band analysis

3.1. Oligoclonal bands

CSF OCB analysis is based on an isoelectric focalization (IEF) technique fractionating Ig into multiple bands according to their respective pI. Structurally diverse antibodies sharing the same pI may cofocus in a single band [12]. Studying IgM OCBs is harder owing to the dissociation of pentamers before the F fragment and the arbitrary reassociation of the separated monomers [13].

Moreover, owing to differential posttranslational modifications (PTMs) (glycosylation or amino acid modifications), each particular IgG clone may display several pKs and may run in multiple bands in IEF conditions [14, 15]. Monoclonal antibodies occurring naturally (monoclonal gammopathy) or commercially available ones are resolved in multiple bands on IEF gel runs (ladder pattern) [15–17]. In a series of 20 myelomas, IEF identified 5–10 OCB in serum [18]. Commercial monoclonal antibodies produced by hybridomas (monoclonal mouse antibody) or monoclonal IgG produced in vivo by plasma cell tumors (multiple myeloma) are highly heterogeneous and present a clone-specific profile of glycosylation [17, 19]. Lastly, each OCB is a visual optical interpretation of a local contrast of color density differentiating OCB from the surrounding polyclonal background. Apart from the relative subjectivity of the technique, faint and ambiguous OCB may be obscured by a dense polyclonal background. Capillary IEF seems to increase sensitivity and demonstrated OCB in negative patients with classical IEF [20]. Therefore, immunoblotting against known antigens, for example, in association with viral encephalitis, unmasks specific OCB in cases failing to demonstrate any OCB with conventional IEF [21]. In MS patients explored for specific antibody anti-viral synthesis, OCB against multiple viruses may occur in the same migrating zone on IEF [22] and CSF OCB against myelin basic protein (MBP) or measles obtained with immunoblot detection does not comigrate with OCB obtained by IEF [23].

There may be several consequences of this as follows: (1) each OCB may be composed of multiple distinct IgG, which may share by chance the same isoelectric point [15]; (2) multiple OCB in MS may derive from one or a small number of cell clones [17]; (3) OCB count cannot predict the number or variety of B-cell clones; and (4) clonally expanded B-cell clones may precede OCB detection, thus reflecting the difficulty to detect very low levels of ITS.

ITS in MS is characterized by numerous unambiguous OCBs (≥10 OCB) that are highly specific of MS [24]. Monoclonal bands are exceptional and should be considered carefully. Repeated LP in these atypical cases demonstrates a broadening of ITS or a different diagnosis [25]. Owing to their lack of specificity [25], ≥2 OCB are required in most studies to define an oligoclonal pattern with optimal specificity.

Absence of OCB (type 1 pattern) is observed in less than 10% in recent studies, whereas most MS patients display the type 2 pattern and only some have type 3 (**Figure 3**). Among OCB+ patients, patterns 2 and 3 are observed in about 90 and 10% in relapsing-remitting (RR) and secondary progressive (SP) patients, respectively, whereas the distribution is 40 and 60% in primary progressive (PP) patients [27], suggesting that pattern responses may be linked to pathophysiology.

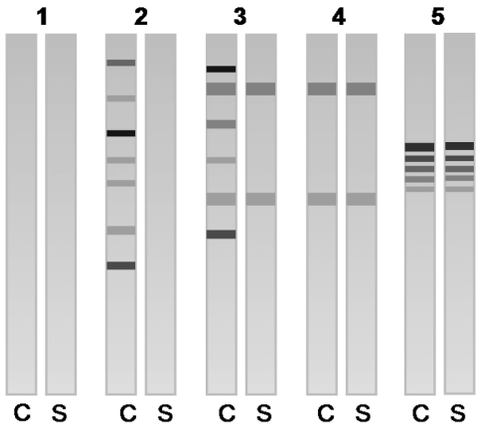

Figure 3. Types of isoelectric focusing patterns on agarose gels [26]: Type 1, no OCB in CSF or serum (normal pattern). Type 2, OCB restricted to CSF, absent in serum. Indicates low-level ITS. Typical pattern in MS. Type 3, Identical OCB in both serum and CSF with extra bands in CSF. Pattern seen during systemic synthesis associated with intrathecal synthesis. Seen in MS. Type 4, OCBs in CSF mirror those in serum. Indicates a systemic IgG synthesis and passive transfer of OCB from blood to CSF, without any local synthesis. From a purely theoretical point of view, a low local synthesis with similar B-cell clones cannot be completely deciphered from this situation. Type 5, Ladder-type identical OCB in both serum and CSF typically associated with monoclonal IgG proteins. Peripheral IgG synthesis without local synthesis.

3.2. Absence of OCB in MS patients is a technical limitation

OCB are almost always present when $Q_{IgG} > Q_{Lim}$, but are also commonly present when Q_{IgG} Q_{Lim} [26, 28, 29]. Nonetheless, about 5–10% of MS patients fail to demonstrate any OCB or an elevated IgG index. Nevertheless, the question remains whether ITS is really absent from such patients' CSF. Our short review argues for a probable faint ITS in the rare 'CSF-negative' patients, making ITS the most valuable marker of MS to date.

A high CSF IgA synthesis has been demonstrated in a few patients lacking the classical IgG synthesis [30, 31]. Free light chain (FLC) sensitivity seems to be near 100% but the (expected) specificity lower than OCB makes FLC less useful for routine clinical purposes [32, 33]. Oligoclonal κ-FLCs are detected in about 50% of MS patients without OCB [34, 35]. Isoelectric focusing with affinity blotting against known antigens overcomes the limitation induced by

the background and unmasks specific ITS. OCBs against the neurotropic MRZ viruses, measles, rubella, and varicella zoster virus (VZV), which are commonly observed in MS for reasons that remain unclear, were present in the CSF of 72% of MS patients who otherwise failed to demonstrate OCB with IEF [36].

The specific antibody index (AI) is calculated by using the ratio of specific antibodies Q_{Spec}=[$Spec_{CSF}$]/[$Spec_{serum}$] in the formula, AI = Q_{Spec}/Q_{IgG}, where AI values >1.3–1.5 indicate the presence of ITS. An MRZ pattern, which is defined as an elevated AI against ≥2 neurotropic viruses, is observed in up to 90% of MS patients [37, 38]. However, the AI is not usually assessed in patients with Q_{IgG} Q_{Lim} and negative OCB, because they are thought to be MRZ-negative since AI correlates with Q_{IgG} [29]. When multiple AIs (VZV, herpes simplex virus (HSV), cytomegalovirus (CMV), measles, rubella, and Borrelia) were systematically assessed in MS patients without apparent ITS (Q_{IgG} Q_{Lim} and negative OCB), all of them showed ≥1 MRZ reactivity and up to 47% of patients had OCB against ≥1 MRZ [29, 36, 39–41]. Interestingly, AI results are not completely congruent with those obtained by IEF with affinity blotting, thus increasing the prevalence of ITS detected by ≥1 technique to 64% in the "CSF-negative" subgroup of MS patients [36]. Since the MRZ reaction is common but not exclusive and because reactions against many other viruses have been confirmed in MS (see below), a larger antigenic test panel might improve the frequency of ITS detection [29], for example, by the systematic use of antigen array [42] and the systematic assessment of IgM.

Studies combining all currently available techniques should be undertaken in MS patients, especially in those with "negative CSF", in order to establish the true prevalence of ITS in MS, especially in patients of non-Caucasian descent who are thought to harbor a lower CSF positivity [43, 44]. Future studies could combine quantitative techniques (IgG_{Loc}, FLC, AI against MRZ) and highly sensitive qualitative techniques (OCB) until the discovery of putative antigenic targets such as anti-CCP (cyclic citrullinated peptide) in rheumatoid arthritis. Criteria for standardizing intrathecal Ig synthesis should be based on the simultaneous normalization of all the tests. In view of the fluctuations of ITS in individual patients (up to 30% of IgG_{Loc} [45]), the action of a drug upon ITS should be statistically demonstrated in groups. To be able to demonstrate a successful intrathecal reset, a null Ig synthesis should be confirmed by using several techniques together.

4. Intrathecal Ig synthesis is a robust and predictive marker in MS

4.1. Factors influencing ITS level

About 95% of patients display OCB but a quantifiable IgG synthesis only occurs in about 70% of them. Several studies have shown that the level of ITS is highly variable from one patient to another, which suggests a genetic determinism. Females are more prone to OCB positivity (92% vs 84%) and to a higher IgG index (1.32 ± 0.92 vs 1.03 ± 0.54) [46, 47]. An ethnic influence on IgG index level and OCB status has also been confirmed by several studies. Black-ascending patients are more prone to a higher IgG index, a higher rate of IgG synthesis, and OCB positivity [48, 49]. The IgG index level is still higher in Afro-Americans after

adjustment for OCB status [49]. The association of female sex to higher IgG index was also confirmed in this group [49].

Prevalence of abnormal OCB status and IgG_{Loc} is lower in Asian patients than in Caucasian patients [50–52]. In Asian patients having the strongest genetic risk factors (DRB1*1501), the prevalence of OCB positivity remains lower than in Caucasians (<72% vs 97%) [53, 54]. Moreover, irrespective of genetic background, extreme latitudes may exert a positive influence on OCB status whereas tropical latitudes exert a negative influence [53, 55].

Several studies have confirmed a strong association between OCB status and human leukocyte antigen (HLA). The DRB1*15 allele confers a higher risk of OCB positivity whereas the genotypes DRB1*04:04/*04:05 or DRB1*03:01/*04:01 are associated with OCB negativity [56, 57]. Interestingly, DRB1*1501 also increases the prevalence of abnormal IgG and IgA indexes but not of the IgM index [54]. In Asian patients, even though these determinants are conserved, OCB status is also driven by a strong interaction with latitude [53].

Genetic markers of γ chains (GMs) display 18 serologic specificities and all of the variants but two are expressed in the Fc region of γ chains. Some of these variations have been shown to strongly influence antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [58]. In an animal model of rheumatoid arthritis, the recognition of collagen-II epitopes by VH chains strongly depended on GM haplotypes [59]. Interestingly, GM haplotypes are in almost total linkage disequilibrium among world populations and are therefore typical of geographic origin unless there is a genetic admixture [60]. Gm21* haplotypes were associated with high ITS in a Caucasian population although Gm5*;3 haplotypes are associated with a low level of ITS[96, 97]. Studies are required concerning non-Caucasian (African and Asian descent) haplotypes. Gm haplotypes are neither correlated with serum IgG level nor with the risk of MS [61, 62]. The causes of interaction between GM allotypes and ITS level remain elusive and the relationship between allotypes and CNS FcRn function (which enhances IgG clearance from CSF via Fc interaction) is unknown. Other non-HLA unknown genes also influence OCB status [56, 63].

Healthy siblings of MS patients display a hyperimmune condition termed "MS immunopathic trait" that is characterized by (1) one or more CSF OCB, (2) an exaggerated response to a variety of viral antigens, and (3) an increased BBB permeability [64, 65]. CSF OCB were found in up to 19% of MS siblings in contrast to 4% of an unrelated control population [66, 67], some of them also displaying an elevated IgG index [66] and up to half of these siblings having both OCB- and measles-specific IgG local synthesis [64, 67]. It is not clear whether ITS observed in these cases reflects a presymptomatic MS in high-risk patients, elite non-progressors, or an unrelated predisposition to ITS.

4.2. ITS is robust over time

ITS occurs as a very early disease event. In pediatric cohorts with very early onset MS (before 6 years), only 8% showed IgG OCB, a figure increasing to 90% with 69% of intrathecal production in two early onset cohorts (<15 y) and even more after a further relapse [68, 69]. In adults, the proportion of OCB positivity tends to appear at re-examination [70] or increases as

the disease progresses (RR = 325/360, SP: 25/25, and PP: 39/39) [27]. In a longitudinal study of CSF from 19 patients taken at multiple time points up to 12 years (mean 8 years), OCB never abated with time and demonstrated a robust pattern [71]. Most of the changes concerned band intensity and the acquisition of a new band [25, 72], although band loss was sometimes described in earlier reports [25, 71]. The same stability of OCB over time was observed with IgG, IgM, and IgA [13, 71, 73].

When focusing on the clonal repertoire of CSF Ig, the highest number of clones was found in patients with the longest disease duration, suggesting a continuous clonal expansion over time [74]. However, the low number of patients included cannot totally rule out disease heterogeneity [74]. In a longitudinal study of CSF B-cells in two MS patients, clonal rearrangements on VH (VH3 and VH4), D segment, JH, and HCDR3 sequences were conserved at two time points 1 and 4 years apart [75]. The antibody index of phagotopes from the entire panel of clones extracted from a single patient was stable at 2 years [76]. The peptidic targets of the OCB IgG are constant over time [72] and the activity index (AI) against infectious agents also remained stable over several years [77, 78]. These findings are in line with a slightly delayed oligoclonal immune response immediately after the onset of MS. However, once initiated, CSF IgG secretion persists over time indefinitely and is little altered qualitatively at the clonal level [71]. Other CSF parameters such as albumin index, IgG, and IgM index remained essentially the same in the clinically isolated syndrome (CIS), RR-MS, and progressive MS groups [27, 79].

A few exceptions should be mentioned as follows: (1) IgM OCBs behave differently, if absent at onset, no further IgM OCBs appear [73]; when present, they tend to disappear after a few years (RR: 109/360, SP: 15/25, PP: 0/39) [27]. When oligoclonal IgMs are present, the disease lasts for less than 5 years in 90% of patients, but when IgMs are absent, it lasts for more than 5 years in 60% of patients. (2) Anti-MBPs in CSF are high during relapses in about 90% of patients but vanish during the 6 weeks following onset, relapse treatment appearing to precipitate this event [80, 81].

Each patient has a private and unique CSF OCB pattern, the so-called OCB fingerprint [82, 83]. The pattern persists even though the total CSF IgG is significantly reduced, for example, by steroids [83]. The CNS IgG synthesis rate remains roughly stable over several months [6]. When steroids are used to reduce the IgG index, pretreatment values are recovered in a few weeks or months [83]. Intrathecal steroids rarely obliterate OCB for more than 1 month [71], but this modification can be corrected when equal amounts of CSF Ig are applied in IEF.

Unfortunately, OCBs provide basic information and the future development of microarrays may provide more precise insight into the intimate evolution of clonality. For example, the involution of the antibody response against heat shock protein (HSP) in serum is a central modification in the immune response during the transition from RR to the progressive form [84], but the outcome of this immune signature in CSF is unknown.

The persistence of OCB suggests a local IgG production by long-lived plasma cells residing inside the CNS, suggesting that the B-cell fostering properties of the CNS deserve consideration [85]. On the other hand, minor changes in OCB pattern are in accordance with competition between plasma cells for a limited number of survival niches, as observed in the

bone marrow. It seems unlikely that such a discrepancy between brain volume and the small number of CNS plasma cells can be explained only by the B-cell fostering properties of the CNS. Moreover, the intrathecal IgG synthesis rate is roughly stable over time, even though persistent local inflammation would be expected to induce a gradual increase in synthesis rate. These data suggest that the number of CNS niches is limited and that they are quickly saturated. Therefore, the putative role of CNS tertiary lymphoid organs (TLOs) deserves further consideration.

4.3. None of the available MS drugs deplete intrathecal Ig synthesis

In a complete review of the literature, we demonstrated that none of the available (approved or experimental) treatment proved to abate intrathecal Ig synthesis (review in reference [86]). A null or minimal (<20%) impact on intrathecal IgG synthesis was observed after the administration of azathioprine, β-interferons, cytarabine (intravenous or intrathecal), lomustine, 5-fluorouracil, CNS irradiation, mitoxantrone, methotrexate, cyclophosphamide, cyclosporin A, cladribine, alemtuzumab, daclizumab, fingolimod, or stem cell transplantation following myeloablation.

Steroid infusion decreases blood Ig levels in a few days [87]. However, high-dose steroid infusions have little effect on IgG index and OCB number [88–90], irrespective of the dosage or the mode of administration (intramuscular, intravenous, and intrathecal) [91]. Even if steroids can transiently lower the IgG index in most but not all patients, the decrease in the range of CSF IgG synthesis is low [90, 92]. The mean antibody reactivity and the mean number of targeted antigens in CSF are decreased in the 2 months following steroids [93].

Rituximab depletes CSF B-cells but fails to significantly decrease intrathecal IgG synthesis (<-20%) [45, 94], possibly because ITS may partly depend on plasma cells (CD20-), which are constitutively resistant. This failure to lower intrathecal IgG secretion was predictable from the absence of the effect of blood-infused rituximab upon serum IgG and IgA levels, contrary to a minor effect upon IgM levels [45, 95]. No data are available on intrathecal IgM synthesis after rituximab treatment.

Given the low diffusion of rituximab to CSF (<0.2%) and the growing body of evidence demonstrating the safe use of intrathecally infused rituximab, a rationale to infuse intrathecal rituximab in progressive MS recently emerged (review in reference [96]). Data obtained from a single patient receiving intrathecal rituximab (10 mg per month for 2 months) showed a major effect upon CSF cytokine levels although intrathecal IgG synthesis was unchanged [97, 98] (unpublished).

An unexpected partial repression of intrathecal IgG synthesis was obtained with natalizumab treatment in some series [99–101] but results were null or lower in two others [102, 103]. These findings need to be replicated in another large cohort. Three non-mutually exclusive explanations may be put forward. First, $\alpha 4\beta 1$-integrin is expressed by B-cells (CD19+ and CD138+) at higher levels than CD3+ T-cells, and natalizumab impedes B-cell trafficking to the brain and renewal of the CNS plasmablast pool. Second, apart from cytokines, T-cells also play a supportive survival role in the survival niches of plasma cells [104]. Third, natalizumab inhibits

the CNS migration of dendritic cells, which in turn may affect the maintenance of CNS lymphoid tissue [105], and dendritic cell counts in MS brains were lower than expected [106]. Of note, a significant decrease in IgM (≈-45%) (and less significantly in IgG1≈-18 to 36%) plasma levels also occurs during natalizumab treatment but is not correlated with treatment duration, suggesting that it effectively perturbs the IgG synthesis pathway [103, 107]. Future experiments should examine the kinetics of intrathecal IgG secretion after withdrawal of natalizumab. Data obtained from a single patient devoid of OCB under natalizumab and discontinuing treatment for progressive multifocal leukoencephalopathy (PML) showed that OCB returned shortly with a slightly modified pattern [101], whereas a sustained negativation was observed at 6 months in two other patients [99]. However, in a series of 23 patients, no increase of ITS or OCB was observed at 14 months after natalizumab discontinuation [108], suggesting that the impact of natalizumab on ITS is probably low.

A single patient treated by EBV-specific adoptive immunotherapy (autologous CD8+ T-cells activated against EBV) regained a normal level of IgG index at month 4 [109]. This single case needs to be replicated.

4.4. Prognostic value of ITS

Although the prognostic value of OCB positivity is not consensual, ITS is consistently associated with a worse outcome. In CIS patients, none of the IgG, IgA, or IgM indexes are predictive of clinical conversion to MS [110]. On the other hand, OCB positivity in CIS increases the risk of having a second attack irrespective of magnetic resonance imaging (MRI) results [111] and the number of OCB is an independent risk factor for relapses [112], whereas their absence is associated with a lower clinical conversion at 5 years and with a better clinical outcome [113]. Interestingly, double OCB positivity (IgG and IgM) is associated with an odds ratio (OR) of second relapses higher than fulfilling of Barkhof's criteria (OR 55.7 vs 3.5) [114]. The MRZ pattern is also an independent risk factor of clinical conversion [115] and all CIS patients displaying OCB targeting lipid antigens relapse in the first year [113].

In clinically defined MS patients, the IgG synthesis rate correlates with MRI burden and the IgG1 index correlates with expanded disability status scale (EDSS). Free light chains in CSF are predictive of impairment [116]. CSF IgM and IgM OCB are associated with a poorer clinical outcome [73, 117] and a lower brain volume [118, 119]. IgM OCBs reacting against myelin lipids are associated with a higher relapse rate, lower efficiency of IFNβ, and a faster EDSS increase [120, 121]. Time and probability to reach EDSS 3 and 4 are strongly correlated with the presence of IgM in CSF [122, 123]. The IgM index in CSF at onset is strongly correlated with a higher last follow-up EDSS [117, 124]. The time to conversion to the secondary progressive phase is shorter in the presence of IgM BOC or an elevated IgM index [73, 123, 124]. These attractive findings concerning IgM were established in a single group of patients. Stauch et al. [13] did not find any influence of IgM OCB on the evolution of MS in a pediatric group and raised some technical concerns about the IgM tests used previously, which may invalidate the results. Another group claimed negative results but they also obtained a shorter, albeit nonsignificant, time to relapse after CIS in OCB-IgM+ patients [125]. In a large retrospective study, disease severity appeared to be identical between OCB-positive and -negative patients [126], whereas

in a study including 1800 MS patients, the number of OCB was considered to correlate with the course of the disease [127]. The mean number of OCB in the group of benign patients was 2.9 ± 3.6 compared to 5.7 ± 4.9 in the severe group ($p < 0.06$) [127]. Therefore, the evidence points to a poorer clinical prognosis in MS patients in association with ITS. On the other hand, OCB negativity correlates with a better outcome in many studies [70, 127–131], even after demographic adjustment [132].

5. Intrathecal Ig synthesis in animal models—technical limitations

IgG synthesis level in CSF is highly correlated with CD19+CD138+ plasmablast levels [133], but although ITS is commonly thought to be associated with CSF floating cells, this minute cell number may account for less than 0.1% of the whole ITS, meaning that the bulk of IgG synthesis is provided by resident IgG-secreting cells, residing either in the meninges or in the perivascular areas [134]. In experimental allergic encephalomyelitis (EAE) models, the parenchymal level of B-cell infiltration increases from null to 134 B-cell/cm^2 [135] and the whole number of infiltrating B-cells is in the range of 10^3–10^5 cells per mouse or rat brain [136, 137]. In MS, B-cells remain a minor proportion of the infiltrating lymphocytes (<5%), are virtually absent from the parenchyma, and are mostly observed around the small veins and meninges (4.6–6 cells/mm^2)[1, 138]. Plasma cells are occasionally found in demyelinated areas. No data are available regarding a possible role of meningeal B-cell aggregates in ITS.

IT Ig synthesis in animal models has received little attention since the very small volume of CSF does not facilitate sampling. Although older experiments based on low-sensitivity techniques failed to demonstrate any ITS during EAE, optimal techniques (IEF followed by anti-IgG staining) have subsequently confirmed the local synthesis of OCB and/or an elevated IgG index [139–142]. In viral models of demyelination (i.e., measles or JHM strain of coronavirus infection), a mirror pattern of OCB is common but local OCBs are always revealed by immunoblot against viral antigens [21, 143]. We are not aware of any EAE experiments aimed at demonstrating the nonspecific pattern of ITS observed in MS, especially the animal counterpart of the human MRZ pattern using common animal viruses or vaccines. From a theoretical point of view, animals receiving intrathecal vaccination with foreign antigens such as albumin are able to mount a strong intrathecal response [144–146]. In a unique case challenged intrathecally with ovalbumin (OVA), an increase in AI-herpes was also obtained apart from the expected increase in AI-ovalbumin, suggesting that animal models are promising for future studies of nonspecific ITS [145].

6. Non-IgG ITS

6.1. General background

The IgG class accounts for the bulk of locally synthesized Igs in MS and most knowledge to date concerns this class. All Ig classes may give rise to ITS. IgG, IgM, and IgA are, respectively,

present in 100, 20–36, and 6–9% of MS patients [69, 78]. The relative absence of IgE is commonly interpreted as a clue to type I immunity [147].

6.2. IgM chains

Intrathecal IgM synthesis is always higher in MS than in controls [110]. IgM OCB may occur in about 20–75% of MS patients [78, 113, 118] and in 74% of CIS [125]. CD19+CD5+ lymphocytes are the predominant population of B-cells in CSF and are characterized by the T-cell independent secretion of IgM natural antibodies directed against phylogenetically conserved structures that target non-proteic epitopes. In a series of 53 CIS/relapsing-remitting multiple sclerosis (RRMS), 46 of them had oligoclonal IgM-recognizing lipid antigens, 30 targeted phosphatidylcholine (PC) alone, seven targeted PC and had additional reactivity against other myelin lipids (phosphatidylethanolamine, phosphatidylinositol, and sphingomyelin), and nine targeted myelin glycolipids, mostly sphingomyelin [113]. IgM OCB against lipids may be restricted to the RR and SP phases since they were not observed in PP patients [27]. Pentameric IgM strongly activates the complement cascade and is involved in the lesions of plaques (pattern II).

6.3. IgA

The IgA index is elevated in at least 9% of MS patients [54, 78, 148] and the mean intrathecal IgA index is modestly but significantly higher in patients than in controls [110]. Local synthesis of IgA may be the only clue to local Ig synthesis in some MS patients devoid of local synthesis of IgG [31]. About a quarter of Ig-secreting cells are IgA in choroid plexuses [149]. IgA cells are also common in plaques (3–9%) and demonstrate a high clonality (up to 80%) [149]. IgAs are mainly mucosal secretory antibodies but could be a major component of CNS immune responses in certain viral infections [148, 150]. OCB IgAs were reported to have a low frequency (2/33) in the earlier CSF studies [71, 150]. In a post-mortem analysis of plaques extracted from four MS brains, IgA+ plasma cells were recovered in 18/24 plaques and sometimes predominated on Ig+ plasma cells [148]. Clonality assessment in the plaques of two patients demonstrated a low diversity of clones and a clonal restriction confined to plaques [148]. The somatic mutations were highly concentrated in the complementary determining region (CDR) and FR3, which are considered critical for antigenic specificities, suggesting an antigen-driven maturation of affinity as observed for IgG. The locally synthesized IgA were demonstrated to target injured axons [148].

6.4. Free light chains

Light chains are normally synthesized more than heavy chains, with an FLCκ/λ ratio of 2:1 in normal blood. Serum circulating levels of FLC are about 1000-fold lower than Ig levels owing to rapid renal clearance (serum half-life of 2–6 h), whereas the rate of clearance from CSF is similar with Ig [151]. Therefore, the level of κFLC in MS is equal or higher in CSF than in serum. Although the absolute value of CSF FLC is variable among studies, the FLC level is always higher in MS than in controls, and the κFLC/protein ratio is even higher [152]. The κFLC index

is more sensitive (Se 95%, Sp 91%) than the IgG index and OCB [153]. Since elevated CSF FLC is predictive of impairment [116], it has even been proposed as a therapeutic target [47].

7. Clues suggesting an antigen-driven maturation of the intrathecal clonal population of B-cells

The IgG1 subclass is significantly elevated in most MS CSF although some authors recovered rare higher CSF IgG2 or IgG3 indexes [110, 154]. The Ig repertoire uses 51 functional germline genes and the normal repertoire of naïve peripheral blood CD19+ cells closely approximates the frequency of the VH family gene segments within the germline. On the other hand, over-representation of a single VH family is common in MS plaques and CSF but not in blood [155]. The VH4 family is observed in more than 35–95% of CD19+ or CD138+ cells. However, the VH1 family, which represents 20% of the functional germline, is nearly absent from CSF in MS [156, 157]. As observed for IgG, the VH4 germline predominates in CSF IgM [158]. This bias precedes the onset of OCB [155] and is typical of MS whereas different biases are observed in other CNS disorders [155]. The clonal expansion of a single ancestor gene was robustly demonstrated by the analysis of clonal diversification from an ancestor gene accumulating substitutions [156]. In CIS patients, the presence of a VH2-VH4 CSF bias either in CD19 or in CD138 cells, which is observed in 7/10 patients, was highly correlated with a clinical conversion to MS in the following 6 months. By contrast, none of the CIS patients without the repertoire bias had developed MS at 2 years of follow-up.

Somatic hypermutation (SHM) drives the maturation of the variable regions of the Ig genes under the control of activation-induced cytidine deaminase (AID), with a preference to the hotspot regions (RGYW/WRCY motifs) of the CDR. The antigen-driven affinity maturation process positively selects replacement mutations occurring in the CDR, which are in contact with the antigen, whereas silent mutations predominate in the framework regions. Therefore, the R/S ratio, which is lower than 2.9 before antigen-driven maturation, is expected to be higher in CDR and the same or lower in the framework regions. In MS, the R/S ratio in the CDR of Ig genes was unchanged in blood B-cells but elevated in CSF B-cells [149, 158]. This ratio is elevated for both IgG- and IgM-producing CSF B-cells and occurs to the same extent in CSF, whereas the blood IgM ratio is about half of the IgG ratio [158]. The extent of SHM is in the expected range in CSF naïve B-cells, whereas its level increases in switched memory B-cells and plasma cells [159].

It is widely admitted that AID expression is a stage-specific hallmark of germinal center B-cells undergoing SHM and gene conversion. However, an aberrant expression of AID was demonstrated in CSF B-cells producing IgG and IgM [158].

A signature score of hot/cold spots of higher/lower frequency replacement was established with six key positions, mostly situated in the CDR [160]. The signature score was 4.5 in the CSF of non-MS patients, 2.0 in the blood of MS patients, and 10.9 ± 2.0 (7.6–11.9) in the CSF of MS patients (cutoff predictive of clinically defined MS ≥6.8). This technique was applied to B-cells extracted from the autopsied brains of four MS patients. The scores ranged from 10 to

14.5, so B-cells from plaques and CSF share the bias [161]. Unfortunately, these bench results cannot be translated into common practice. Various clones displaying the same amino acid sequences but encoded by different nucleotides reinforce the hypothesis of antigen-driven affinity maturation [158].

In summary, B-cells in CSF demonstrate the cardinal features of an antigen-driven humoral immune response: clonal expansion and somatically hypermutated VH family sequences [155]. This suggests that these lineage cells were expanded by antigen and have undergone a germinal center reaction [162]. Moreover, there is evidence that the maturation process takes place at least partly in the CNS compartment [163].

8. Maturating B-cells undergo bidirectional exchanges across the BBB

Authors are often reluctant to localize the SHM process inside the CNS and prefer models that posit peripheral antigenic stimulation followed by CNS migration, although evidence is accumulating in favor of a local maturation process. High-throughput sequencing techniques allow the analysis of clonal B-cell populations on both sides of the BBB. A common finding is the demonstration of IgG cell lineages either restricted to one compartment (CNS, blood, and lymph node) or overlapping multiple compartments. In a study pairing CNS and cervical lymph node (CLN) B-cells, about 6–15% of the IgG B-cell sequences in plaques were recovered

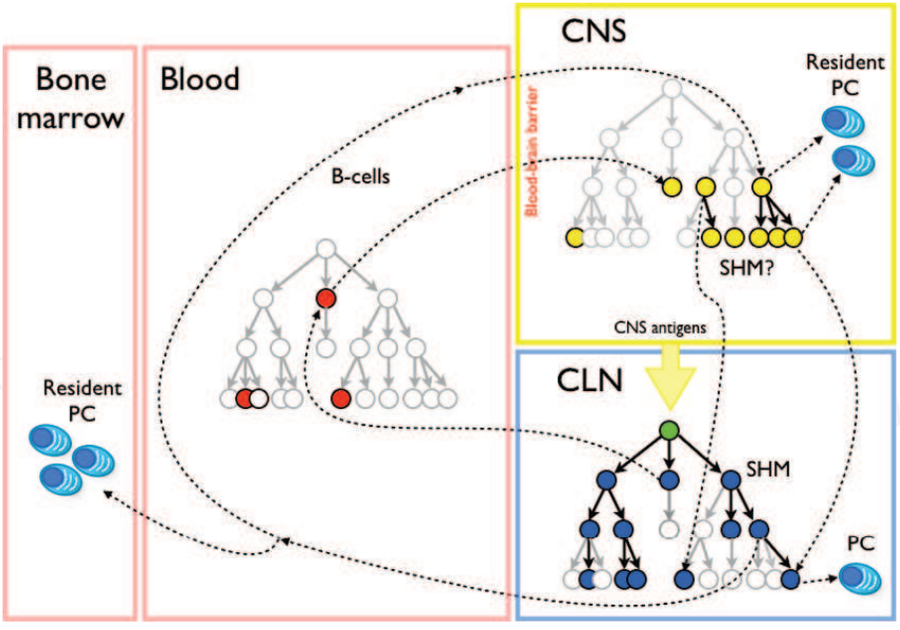

Figure 4. Schematic B-cell lineage tree and putative migration routes. Lineage founding B-cell is postulated in the CLN (green), which drains antigens and B-cells coming from the CNS. Immunoglobulins are subjected to somatic hypermutation (SHM) and proliferation in cervical lymph nodes (CLN). Migrating B-cell clones are sometimes encountered in blood on their way to the CNS. B-cell proliferation occurs both in CLN and in CNS. Evidence is growing that SHM may also occur inside the intrathecal compartment, possibly in meningeal tertiary lymphoid organs (TLOs). In such a model, rounds of B-cell maturation and proliferation may occur in a recursive way both in CNS and in CLN [14, 149]. Long-lived plasma cell (PC) differentiation occurs in the different compartments.

in the CLN [149]. Cell lineage analysis demonstrated that most of the founder cells originated in the CLN [149]. A tentative interpretation is that antigen-driven affinity maturation of B-cells takes place in the CLN, which drains CNS antigens [164], then B-cells migrate to colonies populating the CNS and continue to traffic between the CNS and the periphery, notwithstanding the possibility that a bidirectional traffic occurs in association with clonal expansion in both compartments (**Figure 4**) [149]. Clonal populations of CSF IgM- and IgG-secreting B-cells do not overlap and seem to have matured independently from each other [158]. This has been interpreted as a failure of IgM switching to IgG despite aberrant AID activity. However, only a partial overlapping of IgG and IgM populations has been demonstrated in various studies but it now needs to be replicated.

Naïve B-cells are probably not randomly recruited in the inflammatory CNS since the VH family is biased [159]. Although the fate of naïve B-cells emigrating to the CNS is still not clear, local maturation in the TLO structures and/or emigration to CLN are both probable (see below).

Most of the Ig peptides recovered from OCB by mass spectrometry match Ig-secreting CSF B-cells [14]. Moreover, clusters of related B-cells present in the CSF are also sometimes recovered from blood, the largest bi-compartmental proportion being observed in association with a recent relapse [14]. This observation may explain the existence of OCB "mirror" patterns 3 and 4 with overlapping B-cell lineage on both sides of the BBB.

8.1. Local synthesis also occurs in the eye

Inflammatory lesions are observed in the eye in association with MS and perivenous lymphocyte cuffings (i.e., periphlebitis) are commonly observed, reminiscent of lymphoid aggregates [165]. Therefore, besides the classical intrathecal Ig synthesis, local intraocular synthesis may also affect the eyes and glands. The MRZ reaction occurs with single specificity in up to 76% of cases and with ≥2 specificities in 82% [166, 167], but the ratio of F_s between Fuchs heterochromic uveitis syndrome (FHUS) and MS is about 40-fold, which is in the same range of ITS ratios as observed between MS and viral encephalitis. OCBs are always found in the CSF and aqueous humor of MS patients. Importantly, both OCB and MRZ patterns mismatch in most cases.

9. Meningeal tertiary lymphoid organs may play a role in B-cell traffic and maturation

Meningeal TLOs are ectopic follicles with germinal centers aggregating a reticulum of CD35+ and CXCL13+ stromal/dendritic follicular cells, proliferating CD20+/Ki67+ B-cells, Ig+ plasma/plasmablast cells, and CD138+ plasma cells [168]. The mantle zone is often lacking, whereas CD4+ and CD8+ T-cells infiltrate follicles [169]. CXCL13 immunoreactivity is confined to dendritiform cells inside intra-meningeal B-cell follicles [163]. The abundance of plasma cells is variable and proliferating cells in B-cell follicles, mostly CD20+ cells, are observed at rates varying from 0 to 43% [163]. CCL21 and adhesion molecule peripheral node addressin (PNAd),

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which selectively binds to naïve T and B lymphocytes and allows their homing to secondary lymphoid organs, are absent [163], suggesting that the homing is dependent on various markers specific to brain tissues (see reference [170]). TLO demonstration is associated with higher CSF cytokines (TNFα and IFNγ) [171] but further studies are needed. The functions of TLO have been well described in various models of peripheral inflammation. TLOs are able to mount a T-cell memory response in connection with the other lymphoid compartments [172]. In these models, the amount of Ig synthesis occurring in TLO is completely uncoupled from the blood response, meaning that local Ig-secreting cells are highly specific, although a nonspecific response is associated [173, 174]. In fact, TLOs commonly occur during EAE; AID is locally expressed in association with SHM and B-cell switching occurs [175–177]. The incidence of TLO in response to age insults is genetically determined in mice by two genes [178], but data are lacking in human and in EAE models.

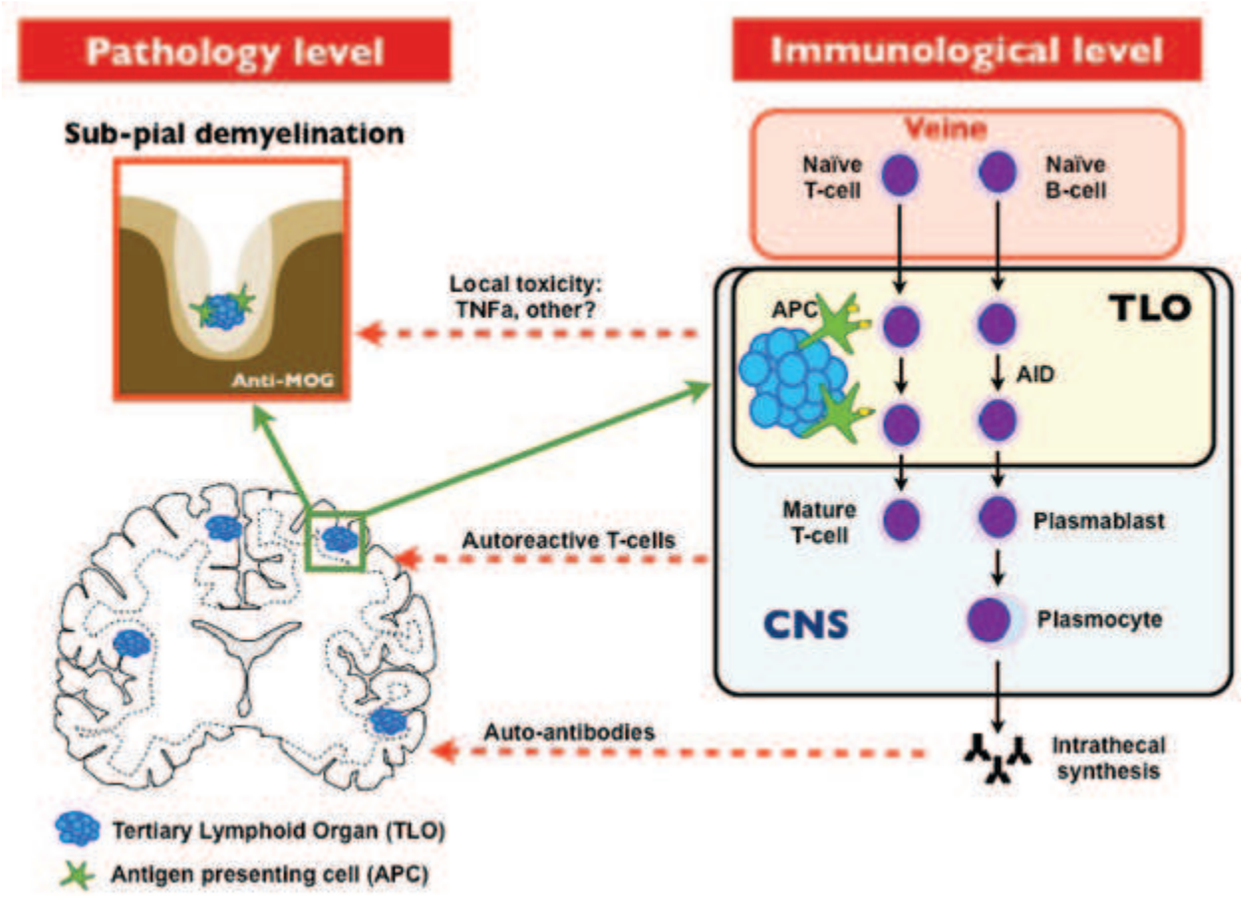

Figure 5. Schematic drawing of tertiary lymphoid organs (TLOs). A few TLOs are disseminated on the leptomeninges. At pathologic level, leptomeningeal TLOs are surrounded by subpial lesions (cell and myelin loss). The toxic mechanism is less certain and might involve TNFα secretion. At the immunological level, TLOs recruit naïve T- and B-cells that undergo antigen presentation, AID expression, affinity maturation, and proliferation of B-cells. Local Ig synthesis depends on plasmablasts/plasma cells and remains the only easily accessible bedside parameter indirectly informing about the presence of TLO (adapted from reference [179]).

In MS, the presence of TLO is associated with a more intense subpial demyelination (cortical lesions type III) and cell loss [169]. A strong topographical relationship is observed between TLO and subpial inflammation and an incidental observation showed that acute subpial lesions are associated with active local neurodegeneration (**Figure 5**) [180]. Owing to their small size and low number (about six follicles in positive blocks [168]), TLOs are probably largely underestimated by the sampling process of pathological examinations. Nevertheless, it was recently demonstrated that late post-contrast FLAIR sequences on 3T-MRI sequences might sample few leptomeningeal lesions (one lesion in 65% of patients, ≤6 in all of them) in a third of progressive patients [181], and this prevalence could be higher with 7T-MRI. Pathological examination of one of them confirmed the congruence of the MRI lesion with TLO. Given the massive underestimation of TLO lesions, the practical consequences of this technique are currently being examined for their predictive clinical value. The longitudinal evolution of these structures and the effect of immunosuppressive therapies on them are unknown. Up to now, TLO could only be examined in the removed CNS structures including brain and leptomeninges but excluding skull and dura mater. Therefore, the recently discovered dura mater lymphatic network, which drains CSF to CLN, has never been examined in the context of MS. Since lymphatics are closely associated with TLO in all other tissues, confirmation of these structures might open up unexpected avenues in studies of inflammatory cell studies trafficking and maturation [182].

10. Epitope spreading

Epitope spreading in EAE and MS is mainly documented for T-cells where it occurs in association with TLO formation [176]. The contribution of B-cells to epitope spreading, especially for their intrathecal counterpart, is less well known. The intimate mechanisms driving epitope spreading are speculative and it remains unclear to what extent SHM contributes to this process.

It was observed some years ago that the ITS of IgG becomes enriched during the early phase after MS onset [27, 42, 68–70, 74]. The frequency of the MRZ pattern increases, OCB-negative patients become positive, and more OCBs occur in the few patients initially displaying a low number of OCB. Assessment of IgG synthesis with multiplex antigen arrays now allows epitope spreading against CNS antigens to be monitored over time. Serum studies have shown that both intramolecular and intermolecular spreading occurs early after the very first demyelinating index event in children but only in patients demonstrating a further clinical MS conversion [42]. By contrast, pediatric patients remaining monophasic fail to diversify their antigenic response. A failure to regulate the aberrant autoimmune response is tentatively thought to explain this observation. Studies on early harvested CSF would give rewarding clues about CNS B-cell epitope spreading. A longitudinal description of the network of interactions in B- and T-cell epitopes (functional immunomics) at both serum and CSF level, and their interaction with HLA polymorphisms, might shed light on the pathophysiology of MS [183].

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11. The problem of Ig directed against nonspecific targets

11.1. The "MRZ pattern"

ITS may occur in supposedly healthy patients or in those suffering from nonimmune CNS disorders, but in such cases, synthesis is always directed against a former healed CNS infection such as Lyme disease, VZV, or HSV [22].

The long-known "MRZ pattern" is an ITS of Ig directed against measles, rubella, and varicella zoster virus, the herpes virus sometimes being included ("MRZH"). Although the IgG fraction that belongs to this specific response in the CSF is estimated to represent less than 2% of the total amount of CSF IgG [78, 184, 185] and only a minor fraction of total OCB [77, 186, 187], the reaction is considered to be highly specific of MS. None of the MS patients in whom the MRZ pattern has been reported suffered from a history of clinical encephalitis involving these viruses. The proportion of MS patients with an elevated antibody index against ≥1 of these viruses increases over time and MS evolution [78]. This MRZ reaction is sometimes present in OCB-negative MS patients and in CIS patients who will convert and it becomes more pronounced over time, as demonstrated by follow-up LP [39, 188, 189].

ITS is polyspecific in a quarter of MS patients (against 2, 3, or 4 AI in 17, 4, and 2%), but remains monospecific (one elevated AI against M, R, Z, or H) in 22% [39]. The number of elevated AI strongly correlates with both age at spinal tap and disease duration and slightly changes over time [39, 190]. Moreover, there is a trend to a higher proportion of elevated AI in SP-MS than in RR-MS patients [191]. Immunosuppressive treatments including natalizumab are ineffective to prevent the persistence of the MRZ pattern [38, 103, 189]. Unlike in controls, no decline in AI levels occurs with age in MS patients considering either serum or CSF titers, and there is even a slight increase [192, 193].

11.2. Broadening the "MRZ pattern" to all encountered infectious agents?

A more comprehensive analysis of ITS with a larger range of infectious agents demonstrated that the MRZ pattern is an interesting concept that could be broadened. In fact, ITS against many other infectious agents has been confirmed in MS: rotavirus, HHV6 (20–30%), mumps, influenza, parainfluenza, adenovirus, respiratory syncytial virus, distemper virus, Coxsackie virus B4, vaccinia, JC virus (3%), CMV, poliovirus, toxoplasmosis (10%), *Borrelia burgdorferi* (26%), and mycoplasma (complete review in reference [194]). A previous silent CNS infection can be ruled out since the seroprevalence for each infection was the same in both MS and controls, whereas the latter had no specific reaction. Moreover, an ITS against tetanus and diphtheria toxoids was also demonstrated in vaccinated patients whose CNS was never exposed to native toxins [195, 196]. In a study of ITS against 17 infectious agents, 57% of MS patients had ITS against ≥5 infectious agents, with an increasing number over time [197], and virtually all patients demonstrated a reaction against at least one of nine viruses [198].

The pattern of reaction mirrors the individual's history of previous infections and the immunization level of the population: reaction against rubella is more observed in MS from Germany than in Cuban patients (lower incidence and immunization campaign in Cuban females) [199].

The rate of intrathecal reaction against a given infectious agent correlates with the rate of seroprevalence of this agent in the population.

Some major consequences can be drawn from these findings as follows:

1. ITS against an infectious agent in MS considered in isolation should not be considered as a clue for a chronic infection in MS, since the coexistence of reaction against many infectious agents is the rule in MS.
2. The concept of "MRZ pattern" should be extended to a "polyspecific infectious pattern."
3. The hypothesis of the antigenic mimicry of a single infectious agent should always be considered in the light of a broad reaction against various infectious agents, whose presence depends mostly on epidemiological variations.

Polyspecific ITS against all infectious agents is just a component of polyspecific ITS, which is thought to be characteristic of MS, and could throw light on the pathophysiology of MS.

11.3. Nonspecific synthesis against infectious agents is lower in MS than in CNS infections

The fraction of a specific antibody response against an infectious agent within the complete intrathecal IgG response is called the specific fraction (F_s) [200]. For example, an F_s value for measles of 2% means that 2% of the total intrathecal IgG response is directed against measles. Neuroinfections are thought to be associated with very high F_s against viruses: F_s are 8–45% for viral encephalitis (HSV, measles, VZV, subacute sclerosing panencephalitis). In other words, about 55–92% of the intrathecal Ig in infectious pathologies are directed against non-causative antigens [184, 200, 201], that is, a nonspecific ITS is very common even in neuroinfections. In MS, each specificity in the MRZH reaction typically retains a very low median F_s of 0.2–1.3% (ranging from 0.03 to 5.3) [200, 202, 203] and comparable results are obtained for F_s anti-EBV [203]. These specific F_s results are about 40-fold lower in MS patients than those found in neuroinfections without overlap.

The amount of intrathecally secreted specific antibodies should be proportional to the number of intrathecal plasma cells. If one considers that circulating plasma cells are nonspecifically and randomly selected from blood to home to CNS TLO, the relative proportion of each specific IgG synthesis in CSF should grossly parallel to their proportion in blood [197]. The ranking of specific antibody concentrations in blood and CSF differs in 67% of MS patients, confirming that CSF IgG secretion does not simply mirror blood secretion. ITS of the specific antibodies occurs independently from each other [200]. We propose two non-mutually exclusive explanations. A first hypothesis involves the differential intrathecal proliferation of specific B-cells after being recruited from blood but before being committed to terminal differentiation into plasma cells, owing to a favorable intrathecal lymphoid environment. A second hypothesis posits a nonrandom brain homing of circulating plasmablasts. It seems unlikely that plasmablast homing is driven by IgG specificity. Rather, the critical intensity of plasmablast/plasma cell recruitment to the brain owing to the intensity of the peripheral immune response to infection could be involved.

11.4. The paradox of low intrathecal anti-EBV reaction

Seroprevalence for EBV is virtually complete in MS patients, so the probability of ITS should be at least equal to or higher than other nonspecific viruses. However, anti-EBV intrathecal values have shown that an unexpectedly small proportion of patients have ITS (in the range of <20%) [203–211]. Comparing CSF OCB against HHV6 and EBV, OCB occurred twice more frequently against HHV6 than against EBV [211]. Moreover, AI against EBV is sometimes twofold lower than AI against each MRZ component [207]. A difference in seroprevalence can be ruled out. Measles and rubella seroprevalence resulting from either natural infection or vaccination exceed 90% in most populations and vaccination campaigns started decades before [39]. Furthermore, varicella seroprevalence exceeds 90% in all European populations and EBV seroprevalence is virtually complete in MS patients.

The lower-than-expected intrathecal response against EBV is not consistent with the strong correlation linking high serum anti-EBV levels and MS activity. In the light of this finding, such an extreme discrepancy can be interpreted as a strong clue for EBV infection preceding MS clinical onset [203]. Possible pathophysiological explanations have been developed elsewhere in the light of TLO [194]. The peculiar relationship between EBV and MS pathology is reinforced by the demonstration of a high intrathecal EBV-specific CD8+ cytotoxic activity only early in MS patients, without recruitment of CD8+ cells against different targets (CMV-specific CD8+ cells) [212] and the clearance of ITS in a case receiving autologous CD8+ T-cells activated against EBV [109].

11.5. Nonspecific synthesis may be a simple property of meningeal tertiary lymphoid organs

Chronic nonspecific synthesis is constant in MS, but may not strictly indicate a bystanding reaction since they are almost absent in other mostly acute CNS inflammations [194]. These unspecific reactions rather indicate a dedicated specific function suggestive of the persistence of TLO in CNS. During immune activation in the periphery, naïve B-cells undergo hypermutation of the Ig genes driven by germinal centers in the spleen and ganglia, and surviving cells are committed to plasmablasts released in the blood for a few days on their way to survival niches, where they differentiate into long-lived plasma cells. Most of the niches are situated in the bone marrow but they are also to be found in secondary and tertiary lymphoid organs, where they display the ability to retain the newly formed plasma cells. Cultures of synovial fragments (containing TLO) from rheumatoid arthritis retain the ability to secrete non-disease-specific antibodies, such as anti-tetanus toxin IgG [213]. Three weeks after, lupus-prone mice were vaccinated against OVA, and the same number of antibody-secreting cells against OVA was recovered in inflamed kidneys as in the bone marrow [214]. Therefore, nonspecific homing of plasma cells may be a common feature of inflammatory tissues and lead to local nonspecific synthesis.

The prolonged survival of retained plasma cells necessitates an anti-apoptotic environment provided by cell niches. For example, CXCL12, which is a major determinant of PC retention in niches, is elevated in MS CSF, expressed by astrocytes and in the vicinity of lymphoid infiltrates [215]. Therefore, we previously suggested that nonspecific Ig synthesis in MS might simply be a common function of TLO harvesting Ig-secreting cells, as is observed in most

inflammatory disorders [194, 213]. An experimental demonstration was given by a series of MS patients who developed CSF IgG against TT after tetanus toxoid vaccination [196]. According to this assumption, the recruitment of intrathecal antibody-secreting cells should be progressive and the complete MRZ pattern might take years or even decades to achieve. This was observed in clinical studies where the mean age at spinal tap and disease duration correlated with the number of elevated antibody indices [39]. Half of the patients having fewer than 5 years of disease duration had no elevated AI (MRZ), whereas 70% of those with more than 10 years duration had AI ≥2 [39]. A similar trend was observed in a study based on 17 antigens [197].

12. Deciphering a potentially disease-related IgG ITS

12.1. MS-specific targets? A puzzling problem

From an historical point of view, the quest for an Ig target in MS has followed the methodology successfully used to solve the paradigmatic problem of subacute sclerosing panencephalitis (SSPE), where it was demonstrated that the majority of intrathecal IgG was virus-specific. Unfortunately, MS CSF demonstrated weak and inconsistent interaction with CNS antigens in numerous earlier studies. It was thereafter proposed that IgG production in MS may be due to nonsense antibodies [71]. These over-hasty conclusions should now be mitigated.

CSF Igs constitute a private repertoire that may harbor toxic properties [216]. Functional electrophysiological modifications of neurotransmission after CSF application were observed long ago. Patterns of demyelinating lesions containing Ig and complement deposition in and around macrophages are typical of pattern 2 observed in MS [148, 217, 218]. The application of CSF to cultured cells (rat cerebellar granule neurons) significantly labels the axonal surface with IgM dots [118] and purified antibodies against myelin/oligodendrocyte glycoprotein (MOG) from MS serum bind to intact myelin in rat [219]. Various recombinant antibodies (rAb) synthesized from CD138+ CSF B-cells of MS patients stain most of the glial components [220, 221] and many specific antibodies have been shown to react against cultured oligodendrocytes or human CNS tissue [222]. In animal models, anti-MOG antibodies purified from human MS serum strongly enhance lesions without increasing inflammation [219, 223]. In a model of focal implantation of hybridoma cells secreting Ig against myelin in rat spinal cord, demyelination occurred around the implantation site [224]. Moreover, the implantation of anti-GalC hybridoma cells may inhibit myelination in a complement-dependent pathway both in vivo and in vitro [225, 226]. These latter models elegantly combine focal IgG synthesis with focal anatomical lesions and are reminiscent of the infiltration by plasma cells inside non-remyelinated plaques.

In conclusion, the hypothesis that CSF Ig could play an active role in brain dysfunction, both at functional and at anatomical levels, is largely justified.

12.2. Does the microheterogeneity of ITS translate into disease heterogeneity?

Although the bulk of ITS targets unknown antigens, some of the antibody clones are thought to play an active biological role that could be either deleterious or beneficial. For example, occasional cases of MS may display anti-N-methyl-D-aspartate receptor (NMDAR) (<1%) or anti-MOG (12%) antibodies, which are associated with cognitive and demyelinating features [227]. However, until now and unlike the anti-aquaporin4 (AQP4) observed in neuromyelitis optica, none of the hundred or so identified antibodies has definitely been associated with the clinical (i.e., epilepsy, amnesia) or pathological distinctive features of MS (i.e., ability to remyelinate). The pathogenicity of these antibodies may be characterized in vitro by the extent of the demyelination or axonal loss obtained in myelinated cultures [216], by specific cell losses such as oligodendrocyte progenitors [228] or by functional alteration of synaptic transmission [89, 228]. For example, IgGs directed against MOG or sulfatide produce a complete myelin loss whereas the axons are preserved. On the other hand, IgG against neurofascine produces both myelin loss and loss of 15–40% of axons [216]. Therefore, at the global level, subsets of patients harbor variable toxicity against myelin or axons owing to one or more different antibody specificities against the targets. In the seminal work of Elliott et al. [216], a third of patients' sera were able to destroy myelin and only 6% targeted axons, but this was probably massively underestimated owing to the limited number of targets examined, fewer than 10. Heterogeneity is also present at the clonal level since different IgG clones targeting the same antigens may display different pathogenicity ranging from null to high. This clonal microheterogeneity is driven by the ability of each clone to fix complement, and, for example, among the many MOG-specific clones generated in EAE, only some of them also recognize the myelin sheath, and their pathogenicity is driven by complement fixation [187]. On the other end of the Ig spectrum, anti-MBP IgMs are associated with a more benign course. Interestingly, these Ig target a shared epitope antigen between MBP and the extracellular loop of CD64, which is one of the IgG Fc receptors responsible for the immunologic properties of macrophages [229].

Microarrays using a small subset of autoantigens discriminated immune signatures in the sera of RR, SP, and PP MS patients [84]. Interestingly, immune signatures based on 13 IgG and one IgM also discriminated pathologic patterns I and II. These results assign a main role to Ig patterns in the pathophysiology of axonal loss, and replication in the CSF may be even more informative since paired serum and CSF samples display different patterns [93]. The large-scale collection of targeted antigens would help to define the functional state of the intrathecal immune system. Moreover, the availability of techniques able to profile ITS IgG diversity and pathogenicity might help to decipher the crucial problem of predicting the severity of MS.

12.3. Putative target antigens—what do OCBs target inside the brain?

The targets are often considered to be largely unknown [230] and studies have yielded contradictory results. CSF antibodies were often considered to be nonspecific owing to their lack of specificity against the three major myelin proteins, but this does not preclude any other specificity and studies devoid of a priori may reveal unknown specificities against other membrane proteins, lipids, or glycolipids. The failure to find a major antigen for intra-BBB-synthesized Ig may not relate to a nonsense antibody production but instead may reflect the

molecular complexity of the CNS and the presumed antigenic target [71]. By analogy with infectious diseases where the antigenic response is driven by pathogen antigenicity, most strategies have concentrated upon the identification of relevant antigenic targets. Paradoxically, from these efforts emerged the unexpected problem of the immense multiplicity and variability of targets.

12.3.1. A priori methods

In the last 30 years, the paradigmatic representation came by analogy with EAE models triggered by a single antigen vaccination, in line with Koch's postulates. Therefore, it was initially thought that ITS was directed against a (single) putative antigen. Unfortunately, intensive investigations of a wide range of potential targets were based on this misleading conception and failed to demonstrate any definitive association apart from more than 100 protein targets [231–233] and roughly 300 epitopes (6% being non-peptidic) [234]. Unlike in many other autoimmune disorders (e.g., myasthenia gravis), no study has succeeded in demonstrating any single antibody specificity for MS but few candidate epitopes (i.e., GAGA4) are still awaiting confirmation of their specificity by replication studies and the availability of commercial kits. Similar results have been obtained in SLE where large-scale clusters of antibodies capture significant information regarding diagnosis and prognosis, whereas limited information is obtained from routinely measured antibodies [235].

12.3.2. Non-a priori methods

12.3.2.1. Phage display

The phage display random peptide library was used to try to characterize unknown epitopes. Phagotopes identified from a CSF test pool of 10 MS patients were not recognized by the CSF of 55 other MS patients [76], which was interpreted as the demonstration that isolated phagotopes are patient- but not disease-specific epitopes [76]. In a study using a similar design, 6 clones were selected from a CSF of 4 CIS, but panning to 187 MS patients found 0–22% positivity depending on the clone and MS type [236]. In the seminal work by Yu et al. [72], one dominant peptide was isolated from each of five MS CSF. Each peptide was patient-specific, and in a given CSF, multiple OCBs (but not all) reacted against the peptide under study, confirming a restricted clonal IgG expression in CSF [72]. The specificity of CSF Ig against peptides was 10-fold higher than from serum, suggesting a local affinity maturation of antibodies.

12.3.2.2. Generation of recombinant antibodies (rAbs) from clonally expanded plasma cells from CSF

The major advantages of this technique are as follows: (a) absence of a priori knowledge of the putative targets, (b) revelation of the most representative targeted peptides, and (c) precise identification of the candidate protein. Its main drawbacks are that it samples very few clones from each CSF. The generated rAbs clones bound to neuronal or astrocyte targets but myelin array failed to identify any precise target in most of them [237]. Interestingly, the aligned

protein candidates identified among the multiple CSF analysis from the different groups did not overlap, thus suggesting a broad range of specificities [72, 238].

12.3.2.3. CSF analysis with protein microarrays

Basically, protein microarray chips are high-throughput parallelized enzyme-linked immunosorbent assay (ELISA) enabling extensive analysis of the breadth of antibody response. Sera from 17 RR-MS patients were compared to those from 15 controls on a protein microarray printed with more than 3101 candidate antigens that represented multiple antigen families [231]. Although numerous candidate proteins were identified, no clue was obtained about individual pattern variability. A larger microarray displaying 37,000 tagged proteins was created in which patterns of immunoreactivity differed between individual patients [232]. The main drawback of this screening method is the biased antibody profiling approach, since the technique does not account for posttranslational modifications of proteins and non-proteic targets.

12.4. Screening new targets associated with chronic CNS inflammation

Comparison of CSF Ig recognition patterns against normal or MS brain tissue lysates revealed a profile of 16 bands highly predictive of MS, suggesting that new antigens are specifically expressed in inflamed brain [239]. Chronic inflammation was associated with the aberrant expression of carbohydrate markers, which were associated with a high level of specific antibodies in CSF [240].

Development of microarrays targeting myelin proteome or brain lipids allowed the confirmation of a diverse polyclonal reaction against these non-protidic targets both in humans and in EAE [84, 113, 241, 242]. Unconventional lipid targeting may be common since the proportion of CSF B-cells targeting lipids was as high as 27% in a study based on rAb [243] and injection of anti-sulfatid monoclonal antibody causes more severe EAE [241]. Unfortunately, only a limited number of potential targets were printed on these pioneering microarrays and only a minute number of rAb clones were obtained from a single patient.

Sugar moieties are an integral part of normal glycoproteins and glycolipids. Glycosylation occurs in the intracellular compartment where the first added sugar moieties are further masked in the mature-released glycoprotein. Such "cryptic" carbohydrate structures are potentially immunogenic as demonstrated in human immunodeficiency virus (HIV)-1 and cancer models [240]. Finally, an antigen microarray platform was constructed to display a wide range of autoantigens (proteins of the myelin sheath, liposomes of varying lipid composition, and cryptic glycan epitopes) and confirmed that CSF antibodies directed against various non-proteinic structures are common in EAE and MS [240]. Although simultaneously targeting a limited number of structures, these pioneering studies opened perspectives for subsequent work based on larger microarrays [244, 245], thereby acknowledging the necessity to include various natural targets such as peptides, lipids, and glycans of both human and foreign origin and their posttranslational modifications. Interestingly, microbe-derived polysaccharides are targeted by the host immune response and may share cross-reactivity with normal human

glycan structures [245]. In-depth screening of foreign antigens including viral particles might be fruitful for deciphering a potential complex network of cross-reactivity (**Figure 6**).

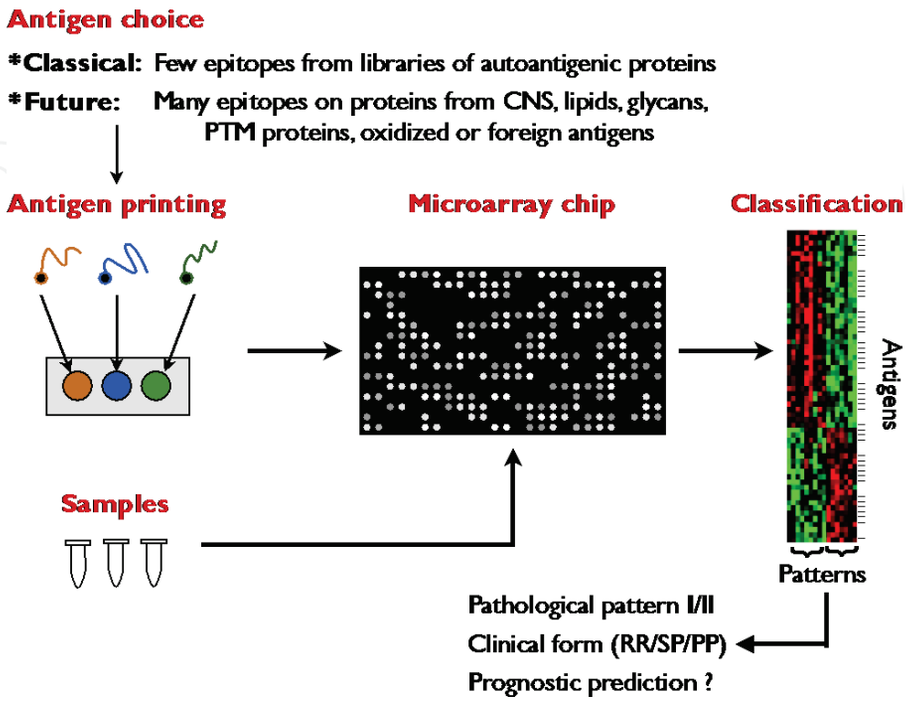

Figure 6. Overview of antigen microarray principles. Interesting antigens are selected from libraries of autoantigen peptides or foreign peptides (i.e., EBV proteins), but post-translationaly modified proteins, lipids, or glycans are suitable. Dense arrays of antigens are spotted on prepared glass slides. Patient samples (serum, CSF) are dropped on the chip, relevant antibodies hybridize to target antigens, and fluorescent-labeled antibodies are applied for detection. The specific antibody signature results in a pattern informative of disease (MS vs other) and stages (RR vs SP) and reveals the early dynamics of the antibody response [42, 93]. Future chips may include a large variety of antigens including atypical (i.e., lipids, glycans, oxidized) and foreign antigens (i.e., viral proteins).

12.5. Posttranslational modifications

About 200 posttranslational modifications for proteins and sharing common properties have been described to date: PTMs target protein hotspots, mostly exposed on the protein surface, they may occur in physiologic life or in reaction to pathology, and protein modifications are irreversible. Although PTM-targeting antibodies occurring in vivo have been rarely described until now in autoimmune disorders, they are being increasingly recognized and all of them might exist since most PTMs are targeted by commercially available monoclonal antibodies raised for research purposes. “Normal” antigens may be masked by PTM and may generate new self-antigens. A growing body of evidence suggests that PTM may play a central role in some inflammatory pathologies such as rheumatoid arthritis [246], and in inflammatory bowel diseases, type I diabetes, and atherosclerosis. Tolerance to self-antigens may be broken when self-antigens undergo protein modifications, whereby they are recognized by antibodies and T-cells as foreign antigens. This kind of molecular (self-)mimicry might trigger inflammation. Therefore, PTM may also be key players for specific autoantibody recognition in MS. This

major hypothesis could account for the failure to uncover specific targets of the intrathecally synthesized antibodies screened using native protein microarrays. Screening of libraries of synthetically posttranslational-modified peptides mimicking antigenic epitopes may allow the characterization of new targets.

Citrullination is increased during EAE and MS and the targeted proteins are catabolized faster. Antibodies in rheumatoid arthritis mainly target citrullinated peptides. Methylation of arginine and lysine residues occurs in EAE brains and these PTMs are targeted in lupus erythematosus. Acetylation of intracellular proteins increases as MS evolves and acetylated MBP produces an immunodominant T-cell epitope in mice that may trigger EAE [247].

Enzymatic glycosylation of protein is a common PTM and polymorphism of N-glycan-branching enzyme (MGAT5) is associated with the risk of MS and spontaneous EAE. The MOG fragment (aa30-50) bears an N-glycosylated asparagine at position 31 that is responsible for its antigenic properties and this PTM peptide is mimicked by the synthetic peptide CSF114(Glc) [248]. Another interesting candidate is the glucose-based antigen GAGA4 [Glc(α1,4)Glc(α)] [249]. Both peptides are associated with specific serum antibodies in many MS patients.

Oxidization generates a large range of by-products since reactive oxygen species (ROS) are able to oxidize nearly all the biological components according to their oxidation potential as follows:

1. Oxidized proteins. Nitration of tyrosine residues, which normally occurs during brain aging and neurodegenerative processes, is specifically targeted by antibodies already identified during rheumatoid arthritis [246]. Malondialdehyde-acetaldehyde adducts (MAA) are generated by the reaction of products of ROS-mediated lipid peroxidation with peptidyl-lysine. MAA adducts are immunogenic in the absence of adjuvant, abundant in synovial lesions of RA, and anti-MAAs are elevated in rheumatoid arthritis and atherosclerosis patients [250]. Antibodies directed against oxidized proteins were demonstrated in the sera of EAE and SP-MS patients [251].

2. Oxidized sugars may form a stable advanced glycation end product that dramatically affects protein antigenicity since antibodies against the native form are usually non-cross reactive with the PTM form [246].

3. Oxidization of lipids may also create autoreactive antibodies. A high level of oxidation could be a distinctive feature of MS pathology in models of CNS inflammation and may be targeted by antibodies. Anti-oxidized-low-density lipoprotein (LDL) antibodies have been described in the serum of MS patients. Oxidized phosphatidylcholine is more abundant in MS brain extracts and anti-oxPCs are synthesized in the CSF of MS patients and EAE mice [252]. These results were confirmed in human MS and mice EAE with various lipid targets [241, 253]. The level of oxidative protein products in CSF does not influence the ITS level [254].

Considering the wide range of possibilities to modify self-antigens by oxidation, this process is an attractive candidate to initiate an "oxidative PTM intolerance" [255].

12.6. Designing microarrays of PTM proteins for multiplex screening with antibodies from CSF of MS patients

Most of the PTM described above are induced by enzymatic catalysis or a chemical reaction occurring under stringent conditions, and their biologic synthesis is not facilitated by the large range of parameters involved. The question is whether it is possible to print large microarrays of antigens able to express all or most of the possible combinations of PTM. Technical considerations and successful experimentation on a small scale suggest that various PTMs may be successfully applied on immobilized proteins [255, 256] and that the development of commercial PTM protein microarrays holds much promise for the future.

13. Conclusion

A distinctive feature of MS is a long-lasting intrathecal inflammatory response, unlike chronic CNS infections in which intrathecal inflammation abates after antibiotherapy. The next step will be to decipher the factors promoting the sustainment of the intrathecal inflammatory response and to identify specific antibody targets. Understanding the role of long-lived plasma cells that produce OCB during the chronic CNS surge could open up new prospects for progressive MS therapies. As Tourtellotte stated long ago, one of the goals of an effective MS treatment could be the eradication of ITS. Therapies targeting CNS B-cells to disrupt B-cell traffic on both sides of the BBB are under development and eradication of resident CNS plasma cells remains an ultimate goal.

14. Abbreviations

AI, activity index.

AID, activation-induced cytidine deaminase enzyme

BBB, blood-brain barrier

CDR, complementary-determining region

CIS, clinically isolated syndrome

CLN, cervical lymph node

CMV, cytomegalovirus

CNS, central nervous system

CSF, cerebrospinal fluid

EAE, experimental allergic encephalomyelitis

EBV, Epstein-Barr virus

EDSS, expanded disability status scale

FLC, free light chain

HSV, herpes simplex virus

IEF, isoelectric focusing

Ig, immunoglobulin

ITS, intrathecal (Ig) synthesis

MS, multiple sclerosis

OCB, oligoclonal bands

PP, primary progressive MS

PTM, posttranslational modification

rAb, recombinant antibodies

RR, relapsing-remitting MS

SHM, somatic hypermutation

SP, secondary progressive MS

TLO, tertiary lymphoid organs

VZV, varicella zoster virus.

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