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COORDINATORE Prof. Bernardi Francesco

*Epstein-Barr virus and Multiple Sclerosis:
in-depth analysis of the virus-specific
antibody response at the time of diagnosis
and during therapy*

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Dottorando

Dott. Castellazzi Massimiliano

Tutore

Prof. Bellini Tiziana

Co-Tutore

Dott. Fainardi Enrico

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Qual vaghezza di lauro, qual di mirto?

Povera et nuda vai philosophia,
dice la turba al vil guadagno intesa.

Such desire for laurel, and for myrtle?

*'Poor and naked goes philosophy',
say the crowd intent on base profit.*

Francesco Petrarca, Il Canzoniere (Canto 7 vv. 9-11)

Summary

INTRODUCTION	5
IMMUNE RESPONSES WITHIN THE CENTRAL NERVOUS SYSTEM	5
CNS AS AN IMMUNOLOGICALLY SPECIALIZED SITE	5
IMMUNE SURVEILLANCE IN THE CNS	7
IMMUNE SENTINELS OF THE CNS	9
ROLE OF B-CELLS	11
MULTIPLE SCLEROSIS	12
DEVELOPMENT OF AUTOIMMUNITY IN MS	16
PROGRESSION OF THE DISABILITY IN MS	18
INFECTION THEORY IN MS	19
EPSTEIN-BARR VIRUS	21
EPSTEIN-BARR VIRUS AND MULTIPLE SCLEROSIS	22
BIOMARKERS AND MULTIPLE SCLEROSIS	23
CEREBROSPINAL FLUID ANALYSIS IN THE DIAGNOSIS OF MULTIPLE SCLEROSIS	24
INTRATHECAL SYNTHESIS OF ANTIGEN-SPECIFIC ANTIBODY (QUANTITATIVE APPROACH)	26
INTRATHECAL SYNTHESIS OF ANTIGEN-SPECIFIC ANTIBODY (QUALITATIVE APPROACH)	27
ANTIBODY AFFINITY	27
TREATMENT WITH TYSABRI®	28
OBJECTIVES OF THE STUDY	30
1) THE ASSOCIATION BETWEEN EPSTEIN-BARR VIRUS AND MULTIPLE SCLEROSIS AT THE TIME OF DIAGNOSIS.	30
2) THE ROLE OF EBV-SPECIFIC ANTIBODIES AS BIOMARKERS DURING DISEASE MODIFYING THERAPY.	30
MATERIALS AND METHODS	31
STUDY DESIGN	31
MAGNETIC RESONANCE IMAGING ANALYSIS	33
SERUM AND CSF LEVELS OF ANTI-EBV ANTIBODIES	34
CALCULATION OF EBNA-1 AND ANTI-VCA IGG -SPECIFIC ANTIBODY INDEX	35
ANTIGEN-SPECIFIC IMMUNOBLOTTING	35
DETERMINATION OF AFFINITY DISTRIBUTIONS OF EBV-SPECIFIC ANTIBODIES	36
DETERMINATION OF EBV-SPECIFIC IGG OCB AFFINITY	38
STATISTICS	39

RESULTS (1)	40
CSF AND SERUM LEVELS AND INTRATHECAL SYNTHESIS OF ANTI-EBV IGG IN RRMS PATIENTS AND CONTROLS	40
EBV-SPECIFIC IGG OCB IN RRMS PATIENTS	42
AFFINITY DISTRIBUTIONS OF ANTI-EBNA-1 AND ANTI-VCA IGG IN RRMS PATIENTS AND CONTROLS	44
AFFINITY DISTRIBUTIONS OF EBV-SPECIFIC OLIGOCLONAL ANTIBODIES	47
DISCUSSION (1)	49
RESULTS (2)	52
SERUM LEVELS OF ANTI-EBNA-1 AND ANTI-VCA IGG IN TYSABRI®-TREATED RRMS PATIENTS	52
SERUM LEVELS OF ANTI-EBNA-1 AND ANTI-VCA IGG IN TYSABRI®-TREATED RRMS PATIENTS GROUPED INTO RESPONDERS AND NONRESPONDERS	53
DISCUSSION (2)	55
CONCLUSIONS	56
GRANTS	57
REFERENCES	58
ACKNOWLEDGMENTS	70

INTRODUCTION

Immune responses within the central nervous system

The Central Nervous System (CNS) has traditionally been considered as an immunologically privileged site in which the immune surveillance is lacking and where the development of an immune response is more limited compared to other non-CNS organs. This view was based on the results obtained in earlier transplantation studies demonstrating that a relative tolerance to grafts is present in the brain and on the following complementary observations (Medawar 1948; Beckmann 2005; Carson et al, 2006):

- 1) the existence of a blood-brain barrier (BBB), a mechanical diffusion barrier for hydrophilic molecules, immune cells and mediators which is formed by specialized endothelial cells with tight junctions located at the level of brain capillaries and by the surrounding basement membrane and astroglial end-feet (glia limitans);
- 2) the absence of a lymphatic drainage of the brain parenchyma;
- 3) the lack of a constitutive expression of Major Histocompatibility Complex (MHC) class I and class II antigens on neural cells;
- 4) no occurrence of professional antigen presenting cells (APCs) in the CNS.

However, a growing body of evidence coming from experimental and human investigations now suggests that this paradigm should be modified.

CNS as an immunologically specialized site

The immune privilege of the CNS has recently been challenged by several findings showing that:

- 1) rejection of tissue grafts (Mason et al, 1986) can be observed in the CNS;
- 2) activated lymphocytes are able to enter the brain trafficking across the BBB in the non-inflamed CNS (Hickey et al, 1991);

- 3) brain antigens are efficiently drained into cervical lymph nodes via the cribroid plate and perineural sheaths of cranial nerves (Kida et al, 1993);
- 4) CNS-associated cells acting as APCs are detectable (McMenamin, 1999);
- 5) all brain cell types can express MHC class I and II molecules after activation in the inflamed CNS (Hemmer et al, 2004).

In particular, it has been documented that foreign tissue grafts are rejected when injected into the ventricular system, whereas bystander demyelination and axonal loss are triggered by a delayed type hypersensitivity response after intraventricular bacterial injection (Galea et al, 2007). In addition, migration of activated T cells from the intravascular compartment into the CNS can occur by using different routes of entry (Ransohoff et al, 2003):

- a) from blood to cerebrospinal fluid (CSF) across the choroid plexus;
- b) from blood to subarachnoid space;
- c) from blood to parenchyma.

In this setting, it is important to note that, in absence of ongoing CNS inflammation, only activated T cells travel into the brain since resting T lymphocytes fail to transit across the BBB. On the other hand, the subarachnoid and perivascular spaces of the nasal-olfactory artery are connected, via the cribriform plate, with nasal lymphatics and cervical lymph nodes, thus allowing CSF drainage into the cervical lymphatics (Ransohoff et al, 2003; Galea et al, 2007). In this way, after their migration in CSF from white matter through the ependyma and from grey matter along perivascular spaces, brain soluble proteins can be transported to local peripheral lymph nodes where they can trigger priming and activation of naïve T lymphocytes. Nevertheless, these interactions require local APCs capable of expressing specific antigens associated to MHC molecules on cell surface after engulfment. Resident APCs of the CNS include a variety of myeloid-lineage cells such as perivascular cells (macrophages), meningeal macrophages and dendritic cells, intraventricular macrophages and choroid plexus macrophages and dendritic cells (Ransohoff et al, 2003). Moreover, also microglial cells acquire APC properties in the course of CNS inflammation (Aloisi

et al, 2000). In this regard, of relevance is the presence of meningeal and choroid plexus dendritic cells which are the most effective APCs for initiating T cell responses. In fact, these cells could capture CSF soluble proteins coming from brain parenchyma and transport them to draining cervical lymph nodes. Furthermore, dendritic cells may present such antigens to naïve T cells at the level of local lymph nodes (Galea et al, 2007). In normal brain, a constitutive expression of MHC antigens is present on endothelial cells, perivascular, meningeal and choroid plexus macrophages and some microglial cells for MHC class I molecules (Hoftberger et al, 2004). Conversely, MHC class II molecules result constitutively expressed only on perivascular, meningeal and choroid plexus cells since their expression on resting microglia still remains a controversial issue (Aloisi et al, 2000; Hemmer et al., 2004). During intrathecal inflammatory responses, microglial cells and astrocytes become MHC-I and MHC-II positive, whereas oligodendrocytes and neurons upregulate MHC class I molecules (Aloisi, 2001). However, in absence of pathologic conditions, the interactions between the immune system and the CNS occur within the CSF, whereas brain parenchyma maintains a relative immune privilege. For this reason, the immune specialization of the CNS should be assumed to be a dynamic process regulated by functional characteristics of the intrathecal compartment (Galea et al, 2007).

Immune surveillance in the CNS

Under physiologic circumstances, it is widely accepted that immune surveillance is performed at the level of perivascular spaces (Ransohoff et al, 2003; Becher et al, 2006). In fact, the intrathecal compartment is constantly patrolled by T cells which have already been activated by the primary encounter with neural antigens in cervical lymph nodes. These cells penetrate the CSF across the choroid plexus and, to a lesser extent, the vessel wall of postcapillary venules located in Virchow-Robin spaces and then accumulate principally in the perivascular spaces where they interact with the corresponding local APCs. At this point, if perivascular cells do not present the cognate antigen to T lymphocytes, these activated immunocompetent cells do not progress across the glia limitans

and recirculate into the blood stream or undergo apoptotic death. On the contrary, if T cells recognize the related antigen presented by perivascular macrophages, they cross the glia limitans, invade the CNS parenchyma and promote the activation of microglial cells which release several soluble factors leading to the development of an inflammatory response. In both these cases, the mechanisms of lymphocyte recruitment are largely unknown, although it has been hypothesized that the egress of T cells into the CSF is regulated by chemokines and adhesion molecules such as selectins (Rebenko-Moll et al, 2006), whereas the migration of T cells into the brain could be due to proteolytic enzymatic activity of matrix metalloproteinases (MMPs) (Bechmann et al, 2007). Of relevance, not only T cells, but also B cells can contribute to CNS immune surveillance since their entry into the CSF has been described (Uccelli et al, 2005). The presence of immune mechanisms which provide a continuous monitoring of CNS microenvironment plays a fundamental role in protecting the brain. In fact, immune responses contribute to host defense against pathogens and preservation of tissue homeostasis since they aim to eliminate of dangerous infectious agents invading the CNS, remove irreversible damaged cells and their products and promote tissue repair (Becher et al, 2006). Moreover, immune reactions to foreign antigens are self-limited because, after the eradication of the antigens, the immune system returns to its basal resting state due to apoptotic deletion of activated T cells (Jiang and Chess, 2006). However, when the antigen is difficult to clear from the CNS or a self brain protein is recognized as non-self, there is a persistent antigenic stimulation of the immune system which favours the development of a chronic intrathecal inflammatory response leading to tissue destruction. Thus, immune surveillance can exert not only beneficial but also detrimental effects (Becher et al, 2006). Therefore, in the course of CNS immune surveillance, two distinct phases can be identified (Bechmann et al, 2007). The first step implies the migration of activated T cells from blood to perivascular spaces through choroid plexus and postcapillary vessels which is not necessarily associated to pathological conditions involving the brain since it can occur when the appearance of a strong immune response in the body promotes the priming of T cells at the level of the secondary lymphoid organs (Hickey, 2001). The second step is

characterized by the penetration of activated T cells from perivascular spaces to brain parenchyma across the glia limitans which is a restricted phenomenon because it depends on antigen presentation performed by perivascular cells. In fact, activated T are able to invade the CNS only when they re-encounter their cognate antigen in the context of appropriate MHC molecules associated to perivascular APCs. Table 1 summarizes the mechanisms of CNS immune surveillance.

Table 1. The biphasis nature of immune surveillance in the CNS (modified from Fainardi and Catellazzi, 2010).

Phases	Location	Mechanisms
Migration of activated T cells from blood to perivascular spaces (step 1)	Choroid plexus and postcapillary vessel wall	Activation of T cells in the secondary lymphoid organs due to a strong immune response in the body
Migration of activated T cells from perivascular spaces to brain parenchyma (step 2)	Glia limitans (astroglial end-feet)	Recognition of cognate antigens by activated T cells after presentation in the context of appropriate MHC molecules expressed on perivascular cells

Immune sentinels of the CNS

Given their ability to act as resident APCs for T cells in normal brain, perivascular cells can be viewed as sentinels at the gate of the CNS parenchyma (Becher et al, 2006).

Considering their importance in CNS immune surveillance, perivascular cells and the other resident APCs are persistently repopulated by bone-marrow-derived monocytes (Becher et al, 2006).

Although this peculiarity is absent in microglial cells and astrocytes, during intrathecal inflammation these cells may exert APC functions and can, therefore, be considered as sentinels within the CNS parenchyma (Aloisi et al, 2000; Aloisi, 2001; Becher et al, 2006).

In the inflamed CNS, there is an activation of microglial cells which upregulate MHC class I and class II molecules and co-stimulatory molecules at their cell surface and then acquire the ability to

present antigen to previously primed CD8⁺ and CD4⁺ T lymphocytes. Therefore, like meningeal and choroid plexus dendritic cells and perivascular cells, also microglial cells are resident APCs. However, while dendritic cells are professional APCs which are able to initiate a primary immune response by the presentation of brain antigens to naïve T cells in the secondary lymphoid organs, perivascular and microglial cells are non-professional APCs which trigger a secondary immune reaction by the presentation of neural antigens to already activated T cells in the Virchow-Robin space and within the brain, respectively (Aloisiet al, 2000; Aloisi, 2001; Becher et al, 2006). Astrocytes are cells of neuroectodermal origin which are fundamental for brain homeostasis and neuronal function since they contribute to the induction and maintenance of BBB by their foot processes, induce scar formation and tissue repair by astrogliosis, produce neurotrophic factors and regulate neuronal functions by providing metabolic support and uptake of neurotransmitters (Dong and Benveniste, 2001). During inflammation, astroglia become MHC class I-positive and can express low levels of MHC class II and co-stimulatory molecules (Aloisi et al, 2000; Dong and Benveniste, 2001; Hemmer et al, 2004; Becher et al, 2006). Therefore, the effective involvement of these cells in intrathecal antigen presentation still remains uncertain and, at present, is believed to be restricted to CD4⁺ T helper with Th2 phenotype (Aloisi et al, 2000). On the other hand, the activation of microglia and astrocytes due to the presence of an inflammatory response within the brain is associated to increased cellular expression of pattern-recognition receptors (PRPs) which can identify a broad spectrum of microbial proteins and pathogenic insults (Farina et al, 2007). In addition, microglial cells and astroglia share with neurons and endothelial cells the ability to eliminate T cells invading the CNS through Fas (CD95)/Fas ligand (FasL or CD95L)-dependent apoptosis under both physiologic and pathologic circumstances (Bechmann et al, 1999). The interaction between FasL expressed by resident brain cells and Fas expressed by immune cells trafficking across the BBB can induce apoptotic deletion of T cells migrating into the CNS. Consequently, microglia, astroglia, neurons and endothelial cells form an immunological brain barrier that preserves the brain against the infiltration of immunocompetent cells by the

maintenance of a state of immune suppression within the CNS (Bechmann et al, 1999). The characteristics of CNS immune sentinels are reported in Table 2.

Table 2. Features of the CNS cells acting as immune sentinels in the normal and inflamed brain (modified from Fainardi and Castellazzi, 2010)

Cell type	Functions	Mechanisms
<i>CNS-associated cells</i> (meningeal and choroid plexus macrophages and dendritic cells, perivascular cells)	Immune sentinels at the gate of the CNS parenchyma	Expression of MHC class I and II antigens, co-stimulatory molecules and pattern-recognition receptors
<i>Microglia</i>	Immune sentinels within the CNS parenchyma	Expression of MHC class I and II antigens, co-stimulatory molecules, pattern-recognition receptors and, along with neurons and endothelial cells, Fas ligand
<i>Astroglia</i>	Immune sentinels within the CNS parenchyma	Expression of MHC class I and II antigens, co-stimulatory molecules at low levels, pattern-recognition receptors and, along with neurons and endothelial cells, Fas ligand

Role of B-cells

CD4⁺ Th1 cells help the differentiation of B cells into plasma cells which produce opsonizing and complement-binding IgG1 and IgG3 (Hemmer et al, 2004). These antibodies cause demyelination via opsonisation, consisting in the stimulation of macrophage-mediated phagocytosis by binding to Fc receptors expressed on the surface of phagocytes, and complement activation, in which they act as chemoattractants for lymphocytes and macrophages. In addition, antibodies are myelinotoxic also by means of antibody-dependent cell-mediated cytotoxicity (ADCC) exerted by NK cells, due to interactions between antibodies and Fc receptors expressed on NK cells, and by the production of proteolytic enzymes such as MMPs (Archelos et al, 2000; Archelos and Hartung, 2000). The intense release of antibodies restricted to the CNS by B cells confined within the brain is referred to

as intrathecal IgG synthesis which is a hallmark of multiple sclerosis (MS) since such antibodies can be identified in the CSF of MS patients as oligoclonal bands (Correale and Bassani-Molinas, 2002). Of note, in this phase of the CNS inflammatory response, activate astrocytes also contribute to intrathecal production of antibodies by the secretion of B-cell activating factor of the TNF family (BAFF) that is an important survival factor during B-cell maturation (Farina et al, 2007). The intrathecal release of antibodies is further facilitated by the development of ectopic lymphoid follicles in the inflamed meninges, a phenomenon indicated as lymphoid neogenesis or tertiary lymphoid organ formation (Uccelli et al., 2005). During CNS inflammation, the persistent antigen stimulation leads to a continual activation of B cells infiltrating the CNS which increase their expression of cytokines, such as linfoxin- $\alpha_1\beta_2$, and homeostatic chemokines, migrate into and colonize the meningeal layers where these activated B cells organize themselves forming ectopic lymphoid tissue, undergo the same recapitulation occurring in the secondary lymphoid organ and differentiate into memory B cells and plasma cells. The evidence of an accumulation of memory B cells and short-lived plasma cells in the CSF during neuroinflammation seems to corroborate the assumption that B cells play a significant role as effector cells of immune responses taking place in inflamed brain (Cepok et al, 2005; Cepok et al, 2006).

Multiple Sclerosis

Multiple sclerosis (MS) is currently postulated to be an autoimmune chronic inflammatory disease of the CNS of unclear etiology in which both demyelination and axonal loss occur (Sospedra and Martin, 2005; Hauser and Oksenberg, 2006). Worldwide distribution of MS is reported in Figure 1. The disease is more common among people in Europe, the United States, Canada, New Zealand, and sections of Australia and less common among people in Asia and the tropics.

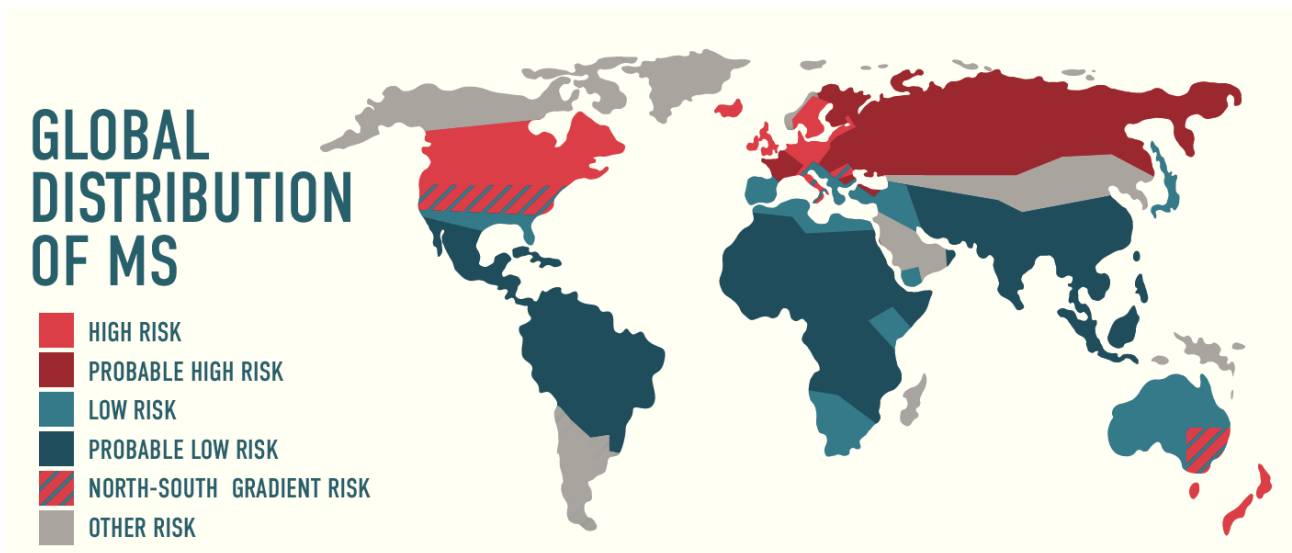


Figure 1. Worldwide distribution of MS (<http://multiplesclerosis.net/what-is-ms/statistics/>).

MS commonly affects young adults and women more frequently than men and is clinically characterized by the dissemination in space and time of relapses, also called clinical attacks or exacerbations, which consist in the occurrence of neurological symptoms and signs (Figure 2). In fact, in MS, relapses typically affect different CNS functional systems (dissemination in space) in different periods of time separated by phases of recovery and remission (dissemination in time) (Compston and Coles, 2002). Clinical expression of the disease is highly variable, but three main courses of MS are generally recognized (Noseworthy et al, 2000; Compston and Coles, 2002). About 80% of MS patients begin with an initial relapsing-remitting (RR) course characterized by self-limited acute exacerbations followed by periods of clinical stability which, in many patients, evolves into a secondary-progressive (SP) phase characterized by a steady worsening in neurological function unrelated to acute attacks. Less often (20%), a primary progressive (PP) form with a slow and inexorable deterioration of clinical condition without acute attacks represents the onset of the disease.

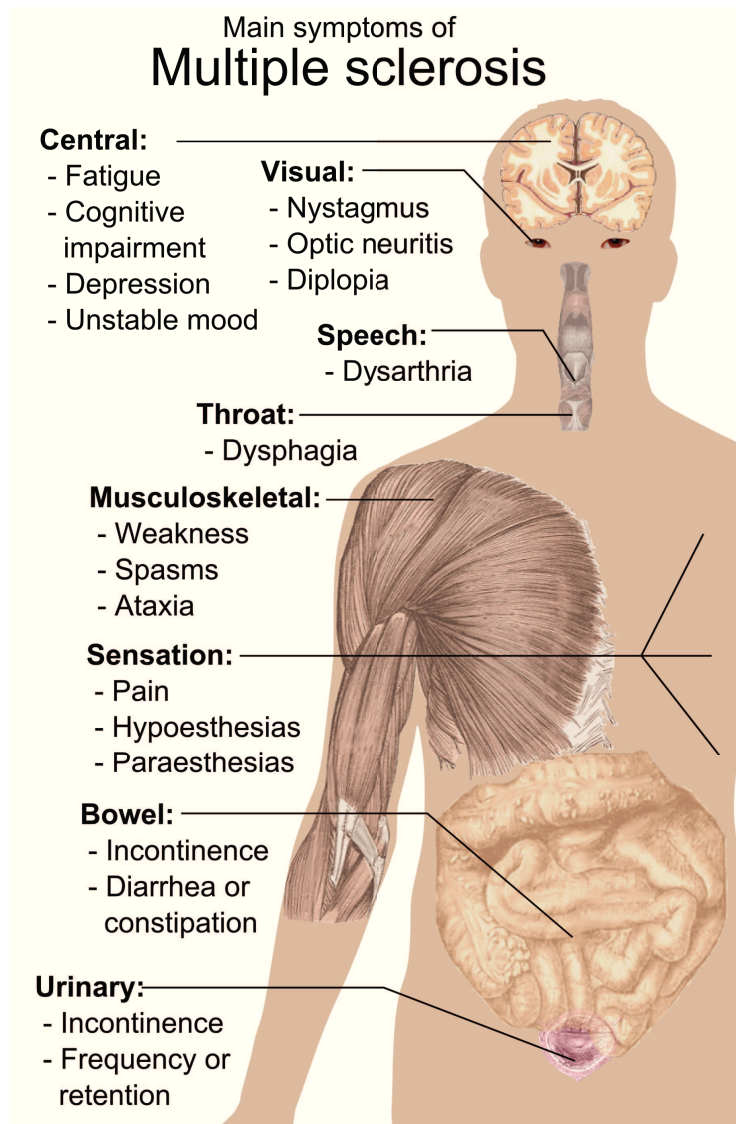


Figure 2. MS signs and symptoms: autonomic, visual, motor, and sensory problems are the most common.
(http://upload.wikimedia.org/wikipedia/commons/a/a3/Symptoms_of_multiple_sclerosis.png)

However, according to the recently proposed criteria (McDonald et al, 2001; Polman et al, 2011) the diagnosis of MS requires additional radiological and laboratory findings. In particular, more than 95% of MS patients show multi-focal lesions in the periventricular white matter on T2-weighted Magnetic Resonance Imaging (MRI) scans with or without Gadolinium (Gd) enhancement on T1-weighted MRI scans (Figure 3, *panel A and B*), which are able to demonstrate dissemination in space and time. On the other hand, in more than 90% of cases isoelectric focusing (IEF) identifies oligoclonal IgG bands only in CSF and not in the corresponding serum reflecting an

intrathecal synthesis of IgG sustained by few clones of antibody-secreting B cells sequestered into the CNS (Figure 3, *panel C*).

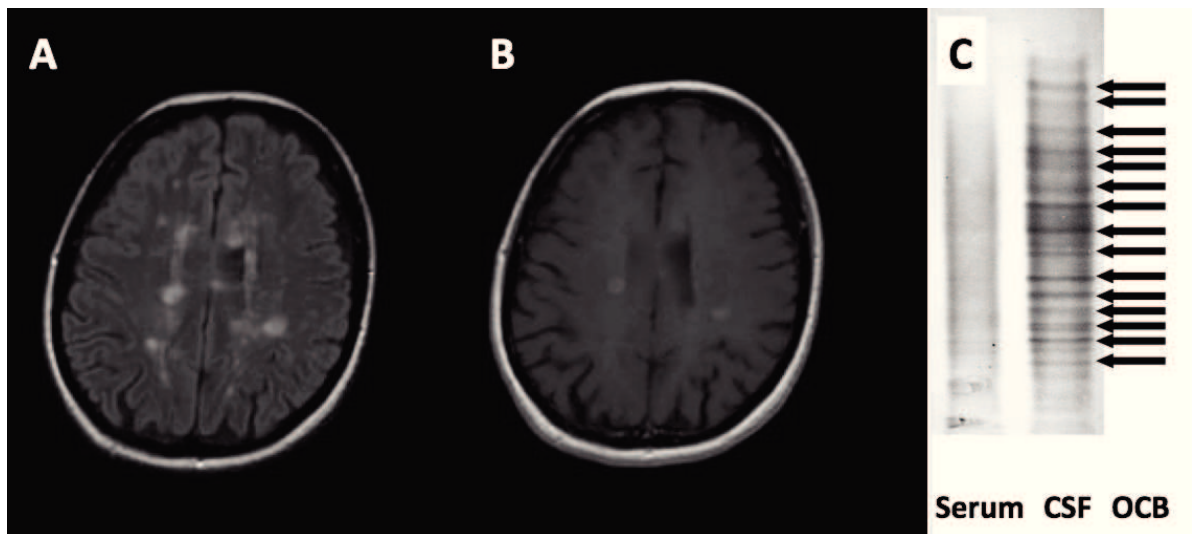


Figure 3. Magnetic Resonance Imaging (MRI) appearance of multiple sclerosis (MS) brain lesions disseminating the periventricular white matter as hyperintense foci on T2-weighted Fluid Attenuated Inversion Recovery (FLAIR) scans (panel A) and as Gadolinium (Gd)-enhanced small areas on post-contrast T1-weighted images (panel B). Panel C shows oligoclonal IgG bands (OCB) only in CSF and not in the corresponding serum reflecting an intrathecal synthesis of IgG as detected by isoelectric focusing (IEF) (modified from Fainardi and Castellazzi, 2010).

Clinical dissemination in space is defined as the occurrence of neurological symptoms and signs (relapses) involving different CNS functional systems. Clinical dissemination in time is considered as the appearance of neurological symptoms and signs (relapses) in different periods of time separated by phases of recovery and remission. MRI dissemination in space is designated as the presence of at least three of the following criteria: 1) one Gd-enhancing brain lesion or nine T2-weighted hyperintense brain lesions; 2) one infratentorial lesion; 3) one juxtacortical lesion; 4) three periventricular lesions. Notably, one spinal cord lesion can replace one brain lesion. MRI dissemination in time is regarded as the occurrence of at least one of the following criteria: 1) a Gd-enhancing lesion demonstrated in a scan done at least three months after the onset of a relapse at a site different from attack; 2) a Gd-enhancing lesion or a new T2 lesion identified in a follow-up scan done after additional three months.

Development of autoimmunity in MS

The complex approach adopted for the diagnosis of MS reflects the uncertainty about disease pathogenesis. MS is currently hypothesized to be an autoimmune disease directed by autoreactive CD4+ Th1 cells which traffick across the BBB and migrate into the CNS after activation (Hemmer et al, 2004; Sospedra and Martin, 2005; Hauser and Oksenberg, 2006). These cells seem to regulate a coordinated attack of both innate and acquired immune responses directed against myelin proteins which includes monocytes, macrophages, NK cells, B cells and CD8+ T cells and results in CNS inflammation promoting myelin damage and axonal injury. In this context, it is generally believed that the initiation of MS autoimmunity takes place in the periphery due to failure of self-tolerance since T and B cells are primed in the peripheral lymphoid tissue after the presentation of neural antigens released from the CNS performed by meningeal and choroid plexus-associated dendritic cells which provide for transfer of these proteins from the brain to the cervical lymph nodes via the nasal lymphatics of the cribriform plate (Hemmer et al, 2004; Sospedra and Martin, 2005; Hauser and Oksenberg, 2006). Under physiologic circumstances, myelin-specific autoreactive T cells are detectable in peripheral blood of healthy individuals since they are part of normal T cell repertoire (Sospedra and Martin, 2005). These autoaggressive T cells are usually eliminated or inactivated through the mechanisms of peripheral immunologic tolerance by which autoreactive T cells that recognize self antigens become incapable of responding to these proteins. Therefore, brain antigens can be recognized as non-self by a dysfunction of regulatory immune cells (“autoimmune hypothesis”) or by a reaction with proteins released from the CNS after primary degeneration (“degeneration hypothesis”) or infection (“infection hypothesis”) (Hemmer et al., 2004; Sospedra and Martin, 2005). The current hypotheses for MS pathogenesis are described in Figure 4.

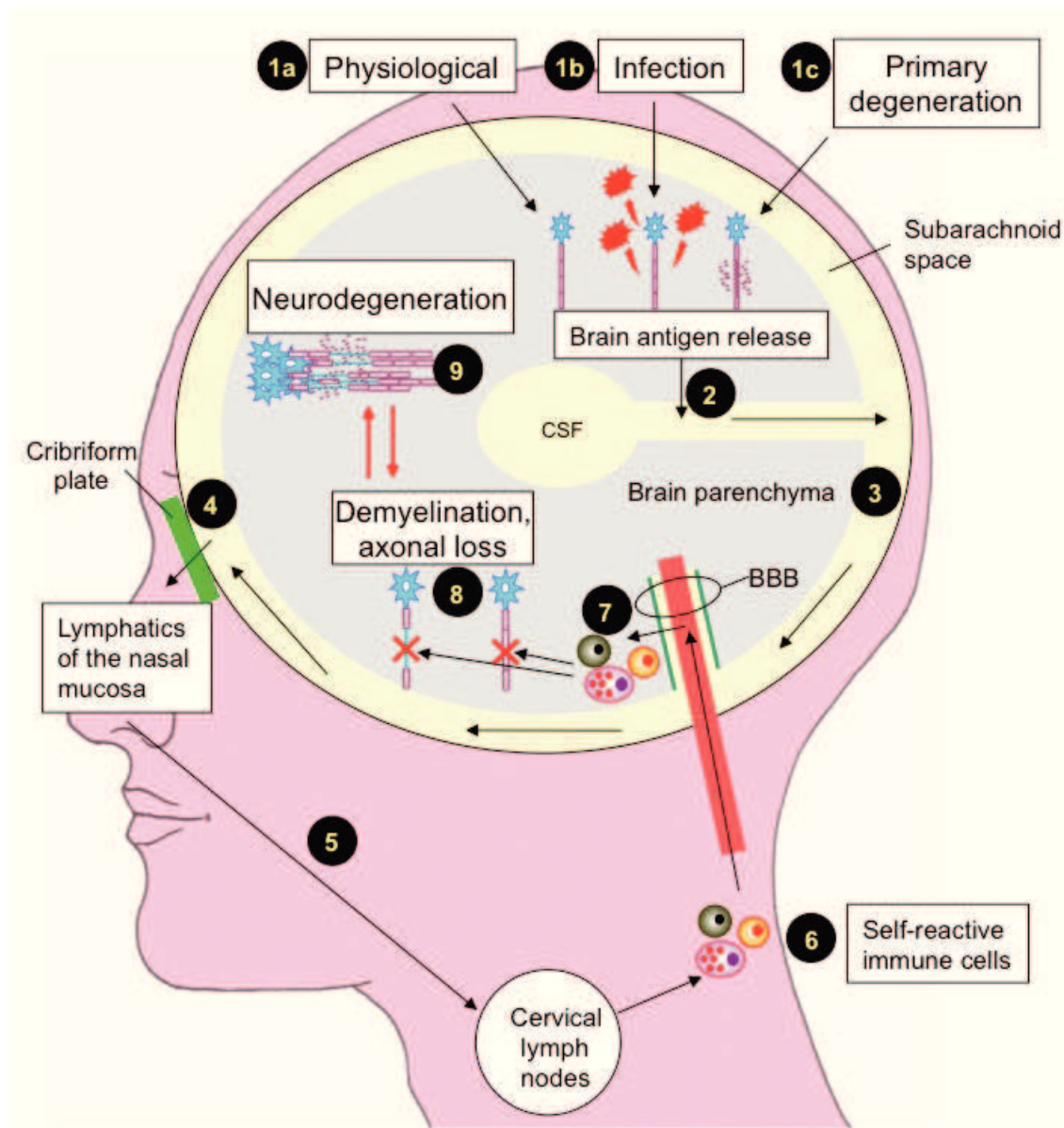


Figure 4. A schematic view of the current hypotheses on the mechanisms responsible for the initiation of multiple sclerosis (MS) autoimmunity. Under normal conditions, brain proteins are released in the cerebrospinal fluid (CSF) as the result of physiological processes of remodeling and tissue repair (1a). In MS, tissue injury due to infection (1b) or degeneration (1c) increases the shedding of central nervous system (CNS) antigens in the CSF including cryptic epitopes that are unknown for the immune system. These neural CSF proteins are captured by meningeal and choroid plexus dendritic cells (2) that transport them (3) to perivascular spaces of the nasal olfactory artery (4) and then, via nasal lymphatics of the cribriform plate, to peripheral cervical lymph nodes (5). Within the secondary lymphoid organs, brain protein coming from the CNS can be recognized as non-self by infection-mediated interactions (molecular mimicry, epitope spreading, bystander activation) or a dysfunction of regulatory immune cells. It follows the priming of naive autoreactive T and B cells that become activated and undergo clonal expansion (6). Subsequently, these cells recirculate into the CNS where the re-encounter and the recognition of the cognate antigen lead to the development of an intrathecal inflammatory response (7) that produces demyelination and axonal loss (8). Over time, inflammatory microenvironment promotes the progression of neurodegeneration, generating irreversible disability. Conversely, cumulative axonal destruction can occur independently of neuroinflammation and may cause irreversible disability (9) (modified from Fainardi and Castellazzi, 2010).

Progression of the disability in MS

Whatever the mechanisms promoting the initiation of the disease are unknown, two different temporally distinct stages can classically be identified in MS (Steinman, 2001):

- 1) an early inflammatory phase due to autoimmune-mediated demyelination leading to clinical recurrence of relapses and remissions (RR MS form);
- 2) a late degenerative phase due to axonal loss leading to clinical chronic progression (SP and PP MS forms).

This model assumes that, in early RR MS clinical course, Th1-mediated inflammatory responses induce clinical relapses promoting demyelination mainly through the release of toxic mediators by activated macrophages and microglia and the production of antibodies by B cells. As axonal injury is present early in the disease, neurodegeneration begins in the same period due to Th1-related inflammatory mechanisms such as cytotoxic activity of CD8⁺ T cells and Ca⁺⁺-dependent glutamate excitotoxicity driven by activated macrophages and microglial cells. On the contrary, the resolution of neuroinflammation is followed by clinical remissions. Over time, the recurrence of several inflammatory events creates a persistent pro-inflammatory intrathecal microenvironment maintaining a permanent axonal loss in association with the reduced support for the axons and the destabilization of axon membrane potentials which follow myelin damage. When the compensatory immunoregulation fails, the cumulative axonal injury leads to the irreversible progression of neurological disability (Sospedra and Martin, 2005; Hauser and Oksenberg, 2006). The traditional view of MS as a “two-stage disease” is further challenged by radiological studies which confirm the heterogeneity of MS, due to the occurrence of different lesional patterns underlying distinct mechanisms of tissue injury, and show that inflammatory and degenerative phases can coexist (Charil and Filippi, 2007). For this reason, it has been proposed that MS may be a “simultaneous two-component disease” (Charil and Filippi, 2007) in which neuroinflammation and neurodegeneration could represent two distinct events occurring separately. From the clinical point of view, MS disease severity and progression could be scored by a numerical scale, the Kurtzke’s

Expanded Disability Status Scale (Kurtzke, 1983) in which zero corresponds to a normal neurological examination and 10 corresponds to death due to MS. The EDSS quantifies disability in eight functional systems: pyramidal, cerebellar, brainstem, sensory, bowel and bladder, visual, cerebral, other. Neurologists assign a score in each of these.

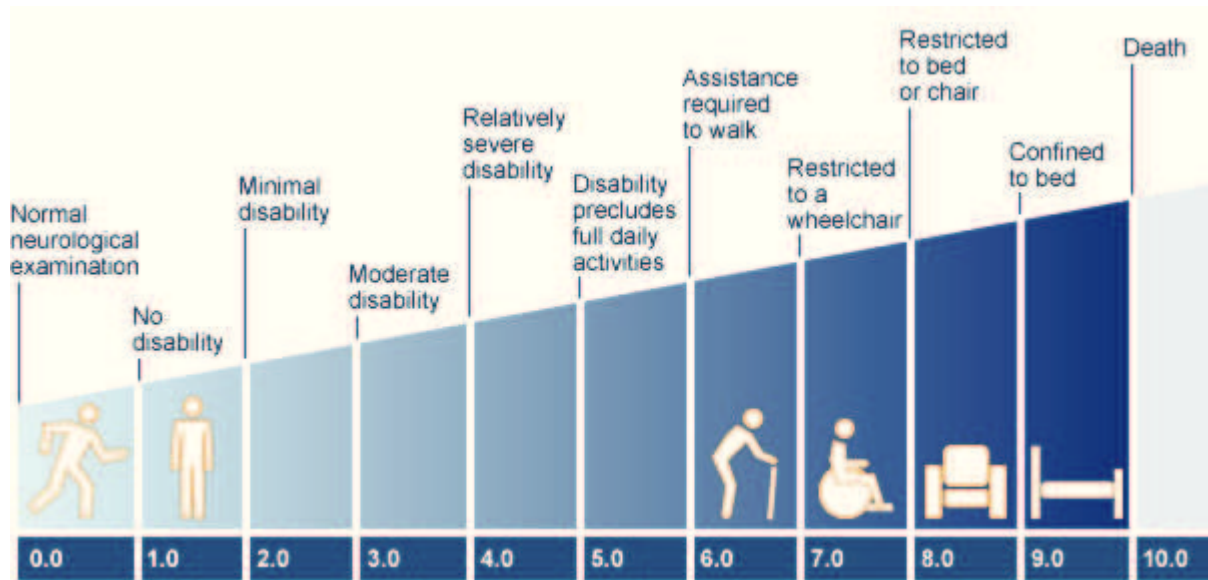


Figure 5. Kurtzke's expanded disability status scale (EDSS) (<http://www.msdecisions.org.uk>).

Infection theory in MS

Epidemiological studies indicate that exposure to an environmental factor, such as an infectious agent, in combination with genetic predisposition could be implicated in MS pathogenesis (Casetta and Granieri, 2000; Sospedra and Martin, 2005, Ascherio and Munger, 2007). The risk of MS is enhanced by the presence of specific genes on chromosome 6 in the area of MHC, HLA in humans. In particular, HLA-DR and HLA-DQ genes, which are involved in antigen presentation, are strongly associated to the development of the disease. However, although the risk of the disease is higher in monozygotic than in dizygotic twins (about 30% and 5%, respectively), the low concordance rate obtained in identical twins suggests that non-genetic factors can contribute to the initiation of the disease. In this setting, the potential role for an infectious agent in MS pathogenesis is supported by descriptive epidemiological studies showing a non homogeneous geographical

distribution, a variation in trend in some areas of the world, the evidence of possible clusters and a change of risk in migrants. A primary encounter with this microbial agent could occur in young genetically susceptible adults, who subsequently develop the disease. This antecedent infection is believed to trigger autoimmune events operating in MS after reactivation. There is also substantial clinical and experimental evidence supporting the possible involvement of an infectious agent in the pathogenesis of MS:

- non specific systemic infections, particularly those affecting the upper respiratory tract, represent a risk factor for relapse in MS patients (Correale et al, 2006);
- CSF oligoclonal bands are present not only in MS, but also in chronic bacterial, fungal, parasite and viral CNS infections in which this intrathecal oligoclonal antibody response is directed against the causative agent (Contini et al, 1998; Fainardi et al, 2001);
- CNS viral infection are able to induce inflammation and demyelination in humans and in MS animal models, as demonstrated by JC papovavirus-mediated multifocal leukoencephalopathy and measles-induced subacute sclerosing panencephalitis (Sospedra and Martin, 2005; Lipton et al, 2007);
- MHC class I-restricted CD8⁺ T cell response, usually triggered by viruses, takes part in MS immune deregulation (Skulina et al, 2004).

Infectious agents could generate an autoimmune response within the CNS by various mechanisms including antigen-specific and non antigen-specific pathways such as:

- a) molecular mimicry;
- b) epitope spreading;
- c) bystander activation;
- d) cryptic epitopes;
- e) superantigens (Scarbrick and Rodriguez, 2003; Sospedra and Martin, 2005).

Molecular mimicry is a cross-reactive T cell immune response between microbial and CNS self-antigens, due to their sequence homology, and is antigen-specific. Epitope spreading describes a

spreading of an antigen-specific T cell immune response from infectious antigens to multiple CNS self-epitopes which are released as a consequence of microbial-mediated brain inflammation. Bystander activation consists of a non antigen-specific T cell immune reaction targeting CNS self-antigens promoted by infected T cells secreting pro-inflammatory cytokines and chemokines. Cryptic epitopes are antigens usually sequestered in the brain tissue which are unveiled and recognized as non-self by antigen-specific T cells after direct infection of target cells. Superantigens are microbial molecules originating primarily from bacteria or viruses which stimulate the activation of T cells cross-reacting with CNS self-antigens in an antigen-independent manner.

Epstein-Barr virus

Epstein-Barr virus (EBV) is a ubiquitous human γ -herpesvirus that has the unique ability to infect, activate, and latently persist in B lymphocytes for the lifetime of the infected host. EBV infects naive B cells in the tonsils during primary infection and drives the infected B cell out of the resting state to become an activated B blast, and then exploits the normal pathways of B cell differentiation so that the B blast can become a latently infected resting memory B cell (Thorley-Lawson and Gross, 2004). To achieve this, the virus deploys a series of different latency transcription programmes (Thorley-Lawson and Gross, 2004). In the latency III or “growth” programme all viral proteins are expressed: Epstein-Barr nuclear antigens (EBNA) 1, 2, 3A, 3B, 3C, and LP and the latent membrane proteins (LMP) 1, 2A, and 2B. This activates the naive B cell to become a B blast, which then enters a germinal centre in the tonsils where it down-regulates the expression of the EBNA proteins 2, 3A, 3B, 3C, and LP. The continuing expression of EBNA1, LMP1, and LMP2 (latency II or “default” programme) allows the infected B cell to progress through a germinal centre reaction to become a memory B cell. The EBV-infected memory B cell expresses no viral proteins except during cell division, when it expresses only EBNA1 (latency I). The lack of viral protein expression allows the virus to persist in memory B cells notwithstanding the immune surveillance. Latently infected memory B cells can return to the tonsils and then differentiate into plasma cells,

which initiates the lytic (replicative) transcription programme with the production of infectious virus (Laichalk and Thorley-Lawson, 2005). It is remarkable that, while in normal B cell differentiation naive B cells are activated by antigens through the B cell receptor (BCR) and by T cell help through the CD40 receptor, in EBV-infected naive B cell LMP2A and LMP1 mimic the antigen-activated BCR and the activated CD40 receptor, respectively (Pender, 2011).

Epstein-Barr virus and multiple sclerosis

Seroepidemiological studies have shown that there could be a strong association between MS and EBV (Handel et al, 2010). In a meta-analysis of case-control observational studies published before January 2009 (Santiago et al, 2010) the authors found an association between MS and an exposure to EBV particularly sustained by the levels of anti-viral capsid antigen (VCA) IgG (odds ratio [OR] = 5.5; 95% confidence interval [CI] = 3.37–8.81; $p < 0.0001$) and anti-EBNA-1 IgG (OR = 12.1; 95% CI = 3.13–46.89; $p < 0.0001$). No significant association were found when studying anti-early antigen (EA) IgG (OR = 1.3; 95% CI = 0.68–2.35; $p = 0.457$). A past infectious mononucleosis (IM) was found to be more frequent and the seroprevalence of anti-EBNA-1 and anti-VCA IgG higher in MS patients than in controls (Handel et al, 2010; Santiago et al, 2010; Almohmeed et al, 2012). High serum levels of anti-EBNA-1 IgG increased the risk of developing MS (Ascherio and Munger 2010), correlated with disease activity (Farrell et al, 2009), predicted the conversion from clinical isolated syndrome (CIS) to definite MS and could be used as a prognostic marker for disease conversion and disability progression (Lünemann et al, 2010). Elevated serum concentrations of anti-VCA IgG were related to grey matter atrophy (Zivadnov et al, 2010). The role of EBV in MS pathogenesis was in part supported by the experimental demonstration that EBV proteins and myelin-basic protein epitopes share structural similarity (Lang et al, 2002). However, conflicting results have been obtained in cellular, molecular and neuropathological studies since, in MS patients, blood EBV-specific CD8⁺ T cell response was found increased, decreased or absent, cerebrospinal fluid (CSF) and blood EBV DNA load was high or not measurable and the detection

of EBV-infected B cells in brain lesions was inconsistent (Santiago et al, 2010; Ascherio and Munger, 2010; Lünemann, 2012). Controversial findings also emerged from the quantitative and qualitative analysis of intrathecal synthesis of anti-EBV IgG in MS. In particular, an intrathecal synthesis of anti-EBV IgG determined by the calculation of the specific antibody index (AI) was increased (Pohl 2010) or not significantly augmented (Castellazzi et al, 2010; Sargsyan et al, 2010; Villegas et al, 2011) in MS patients. Moreover, intrathecally synthesized EBV-specific IgG were not associated to MS after normalization with total IgG (Jafari et al, 2010) and were considered as a part of the polyspecific immune response that occur in MS in another study (Otto et al, 2011). EBV-specific oligoclonal IgG bands (OCB) were found in almost 100% of MS postmortem CSF (Serafini et al, 2007), in a third of the MS CSF (Rand et al, 2000) and in some patients after screening in a protein library (Cepok et al, 2005b). However, when EBV-specific IgG OCB were determined in paired CSF and serum samples only 13% of MS patients showed an intrathecal synthesis (Virtanen et al, 2013) or, on the contrary, almost half of the patients had a systemic EBV-specific immune response without intrathecal production (Franciotta et al, 2011). Nevertheless, none of the previous studies investigated the affinity distributions of intrathecally released anti-EBV antibodies.

Biomarkers and multiple sclerosis

According to literature the following working definitions were adopted in this study (Bielekova and Martin, 2004):

- a “biomarker” is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention;
- a “type 0 biomarker” is a marker of the natural history of a disease and correlates longitudinally with known clinical indices;
- a “type I biomarker” captures the effects of a therapeutic intervention in accordance with its mechanism of action.

The requirements for a biomarker, i.e. validity, specificity and ease of measurement, will be quite different depending on its intended use (e.g. preclinical versus early clinical and late clinical stages) and on the body compartment in which we intend to measure the biomarker.

- Blood is relatively simple to collect. The amount collected depends on the assay used and mainly ranges in few millilitres. The main disadvantage is the diurnal variation of many soluble markers and invasiveness of the collection.

- CSF may better reflect the relevant inflammatory process due to its proximity to inflammatory lesions in the CNS. Moreover CSF collection does prevent biological degradation of excreted markers by the liver or by renal excretion. However CSF collection is an invasive, although relatively benign, procedure that is widely used mainly for diagnostic purpose.

Cerebrospinal fluid analysis in the diagnosis of multiple sclerosis

In 1994, the Committee of the European Concerted Action for Multiple Sclerosis (Charcot Foundation) organised five workshops to discuss CSF analytical standards in the diagnosis of multiple sclerosis. This consensus report from 12 European countries summarises the results of those workshops (Andersson et al, 1994).

Briefly, CSF must be analysed in parallel with corresponding serum. Lumbar puncture, whenever possible, is performed in the morning, together with blood sampling. All samples are collected in sterile conditions, glass/siliconized/polypropylene tubes. Blood samples should not be hemolysed, or lipemic and CSF samples should be analysed from a non-traumatic lumbar puncture. CSF samples should be evaluated for appearance and colour, before and after centrifugation. Appearance and colour are expressed with qualitative scales (for example, “crystal-clear”, “turbid”, “cloudy” for appearance, and “colorless”, “xanthochromic”, “erythrochromic” for color) and CSF cells should be counted and processed within 2 h of sampling. Biochemical analysis are performed on supernatants of centrifuged CSF, and on paired serum samples. Glucose, which is measured with colorimetric methods, should be reported as a percentage ratio or as concentration. The

determination of serum and CSF albumin with the calculation of albumin ratio, or albumin quotient, is the most accurate index for blood-brain-barrier damage, and should replace the determination of CSF total protein (Tibbling et al, 1977). Intrathecal IgG synthesis should be expressed with the use of non-linear functions, such as Reiber and Felgenhauer's formulae (Clin Chim Acta 1987), which take into account blood-brain-barrier functionality. To decrease analytical imprecision, serum and CSF albumin and IgG must be determined on the same analytical session. Methods allowed for albumin and IgG determination are nephelometry, turbidimetry and radial immunodiffusion. There is complete agreement that isoelectric focusing (IEF) on agarose gels followed by immunoblotting for detecting the presence of oligoclonal IgG bands should be the "gold standard" method for the determination of an intrathecal synthesis of immunoglobulin (Anderson et al, 1994, Freedman et al 2005, Franciotta et al, 2005). The European consensus report, which described 5 patterns, represented a cornerstone for the interpretation of IEF results (Andersson et al, 1994) (Figure 6):

- a) "Normal CSF", characterized by the presence of a diffuse polyclonal IgG background;
- b) "Local synthesis", characterized by the presence of at least two OB restricted to CSF; it suggests an intrathecal IgG-specific immune response.
- c) "Mixed", characterized by the presence of restricted OB with additional, identical bands in CSF and serum; it is an index of both intrathecal and systemic immune response.
- d) "Mirror", with identical OB in CSF and serum; It suggests a general immune system activation extended to CNS due to the presence of BBB dysfunction.
- e) "Paraprotein bands", that is monoclonal bands in CSF and serum; this appears only in monoclonal gammopathy.

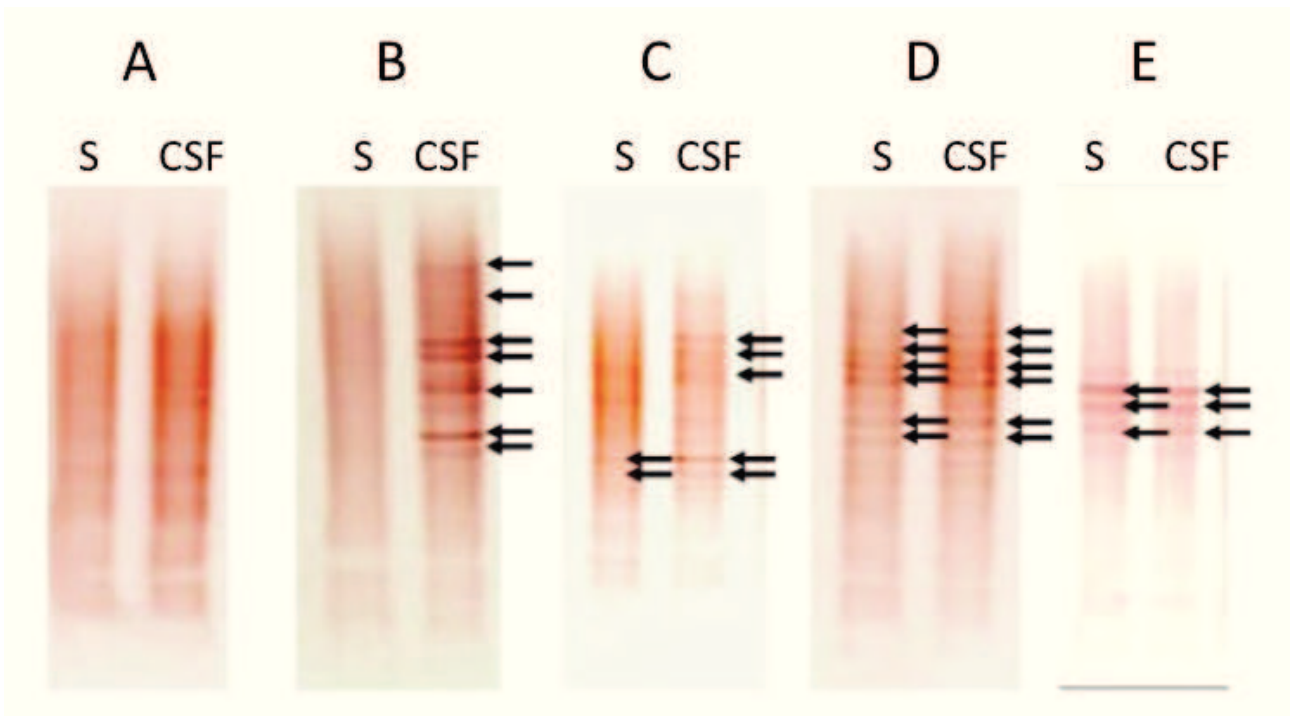


Figure 6. Serum-CSF oligoclonal IgG patterns. A) “Normal” (polyclonal); B) “intrathecal synthesis”; C) “mixed”; D) “mirror”; E) “paraproteinemic”. S = serum; CSF = cerebrospinal fluid.

Intrathecal synthesis of antigen-specific antibody (quantitative approach)

An intrathecal synthesis of antigen-specific antibody could be quantified by combining immunoassay with a sophisticated evaluation method that involves calculating the ratio between CSF/serum quotient for specific antibodies (Q_{spec}) and total IgG (Q_{IgG}) (Reiber and Lange, 1991). This antibody Index ($AI = Q_{\text{spec}}/Q_{\text{IgG}}$) discriminates between blood-derived and brain-derived pathological antibodies taking into account the permeability for the BBB. A simple approach to the topic is as follows: a) total IgG concentration in serum and CSF is determined; b) serum sample is diluted to the same CSF total IgG concentration; c) both CSF and serum samples are tested with immunoassay for the specific antigen and results reported in OD units; d) antibody concentrations can be determined by using a reference standard curve and plotting the OD value to obtain the respective arbitrary unit (AU) value. See materials and methods section for details.

Intrathecal synthesis of antigen-specific antibody (qualitative approach)

It has been recognized for a number of years that patients with MS produce locally synthesized IgG within the CNS (Anderson et al, 1994). A small proportion of these antibodies react against specific viral or bacterial antigens (Reiber and Lange, 1991). However, the target of this oligoclonal immune response is still unknown or controversial (Serafini et al, 2007; Fainardi et al, 2009). Besides, during CNS infection, it has been shown that CSF IgG OB are specific for the causative antigen (Fainardi et al, 2001). Qualitative methods provide the most sensitive means of identifying an intrathecal immune response (Anderson et al, 1994) and these can be adapted to detect antigen-specific IgG (Fainardi et al, 2001; Fainardi et al, 2009). Intrathecal synthesis of specific antibodies can be demonstrated by antigen specific immunoblotting (ASI). Briefly, after isoelectric separation of CSF and serum protein into an agarose gel, the gel is onto a nitrocellulose membrane precoated with the antigen of interest. See material and methods section for details. Four antigen-specific IgG oligoclonal bands can be identified considering paired CSF and serum samples: normal CSF, local synthesis, mixed and mirror (Fainardi et al, 2001; Fainardi et al, 2009). The paraproteinemic pattern is here absent.

Antibody affinity

The assessment of the virus-specific antibody production in the brain is crucial to understand the effective relevance of a pathogen in MS because it is well known that, while in MS a polyspecific intrathecal immune response composed by anti-viral low-affinity antibodies occurs, in infectious diseases intrathecally synthesized high-affinity antibodies, of which at least 30% specifically directed against the causative agents, predominate (Sindic et al, 1994; Conrad et al, 1994; Luxton et al, 1995). It has been demonstrated that in the course of an infection, the process of immune maturation requires a progressive increase in antibody affinity that leads to an intensive production of high-affinity antibodies specifically directed against the causative pathogen (Luxton et al, 1995). This consist in a kind of molecular Darwinism in which the best “fit” of various antibodies will

“survive” and/or proliferate over the other antibodies and thus along with the classical increase in titres of antibody, there is a parallel increase in affinity. Since the bonds which hold the antigen in the “cleft” on the antibody (between the heavy and light chain variable regions) are primarily hydrogen bonds, various molecules which can “chelate” these protons are used in successively increasing concentrations to effectively dissociate the bonds between antigen and antibody. Examples include negatively charged anions (such as the iodide ion in NaI) or sodium thiocyanate (NaSCN) where the thiocyanate anion is also effective in “chelating” the protons of the hydrogen bonds.

Treatment with TYSABRI®

Natalizumab (Tysabri, Biogen Idec Inc, Cambridge, Massachusetts, USA) is a selective adhesion molecule inhibitor that blocks $\alpha 4$ integrin, which is expressed on the surface of lymphocytes and is required for endothelial adhesion, facilitating migration of peripheral blood lymphocytes into the central nervous system (Polman et al, NEJM 2006). In a 2-year, phase 3 study of patients with relapsing-remitting multiple sclerosis (RRMS), natalizumab monotherapy demonstrated consistent efficacy in the overall study population and across multiple subgroups of patients predefined on the basis of demographic and baseline disease characteristics, including age, sex, number of brain MRI lesions, disability status and number of relapses in the prior year. With Determination dated 7 December 2006 (GU n. 292 del 16.12.06), the Italian Drug Agency (AIFA) has implemented a centralized European approval (Determination/C no. 115/2006) of the medicinal product TYSABRI® (Natalizumab) and established the system of dispensation and the class of reimbursement paid by the national health system. TYSABRI® is licensed as disease modifying monotherapy in relapsing-remitting MS to prevent relapses and slow the progression of disability. For safety reasons, the treatment is restricted to specific groups of patients:

1. Patients with relapsing-remitting MS (RRMS) who have not responded to a full and adequate course of therapy with immunomodulatory therapies currently approved for RRMS. The patient must meet the following characteristics:

- a) diagnosis of relapsing-remitting MS;
- b) treatment with immunomodulatory therapy for at least twelve months (the period may be shorter if the lack of response to treatment is clearly documented in a shorter period of time);
- c) the presence of at least two relapses in the last year in therapy, or;
- d) the presence of a relapse in the last year in therapy with incomplete recovery and residual disability of not less than 2 on the EDSS;
- e) the presence of at least nine T2 lesions on MRI, or;
- f) presence of at least one gadolinium-positive lesions on MRI.

2. Patients with severe relapsing-remitting MS with rapidly evolving. The patient must meet the following characteristics:

- a) diagnosis of relapsing-remitting MS;
- b) the presence of at least two relapses in the last year with incomplete recovery and residual disability of not less than 2 on the EDSS;
- c) the appearance of new T2 lesions (refer to changes in both numerical and volumetric) with respect to a MRI examination performed no later than twelve months ago, or the appearance of gadolinium-positive lesions compared to a MRI done no more than twelve months earlier.

TYSABRI® 300 mg should be administered by intravenous infusion once every 4 weeks. The use of TYSABRI® has been associated with an increased risk of progressive multifocal leukoencephalopathy (PML), an opportunistic infection caused by the JC virus, which can be fatal or cause serious disability.

OBJECTIVES OF THE STUDY

The present study focused on two distinct aspects.

1) The association between Epstein-Barr virus and multiple sclerosis at the time of diagnosis.

The first part of the study aimed to investigate the association between MS and EBV through an in-depth analysis of the virus specific antibody response in a large cohort of relapsing-remitting MS patients, categorized according to clinical and magnetic resonance imaging disease activity, and, as controls, in patients with other neurological inflammatory diseases (OIND) and non-inflammatory neurological disorders (NIND). In these different groups, we determined: a) CSF and serum levels of anti-EBNA-1 and anti-VCA IgG, considered as latent and lytic markers, respectively, by using enzyme-linked immunosorbent assay (ELISA); b) intrathecal synthesis of anti-EBV IgG by means of Antibody Index (AI): quantitative determination; c) intrathecal synthesis of anti-EBV oligoclonal IgG, employing an antigen specific immunoblotting (ASI) protocol: qualitative determination; d) affinity distributions of anti-EBV antibodies utilizing a modified ELISA method; e) affinity distributions of CSF EBV-specific oligoclonal IgG bands using a modified ASI method.

2) The role of EBV-specific antibodies as biomarkers during disease modifying therapy.

The second goal of the research was to verify the use of serum EBV-specific antibodies as a biomarker for the response to treatment with TYSABRI® in a cohort of relapsing-remitting MS patients during the first 15 months of therapy.

MATERIALS AND METHODS

Study design

The first part of the study included 100 consecutive patients with relapsing-remitting definite MS (RRMS) according to the currently accepted criteria (Polman et al. 2011) (Table 3).

Table 3. Demographic, clinical, radiological and CSF characteristics in 100 patients with relapsing-remitting multiple sclerosis (RRMS).

Sex: F/M	70/30
Age, years: mean \pm SD	37.3 \pm 10.6
Disease duration, months: mean \pm SD	30.5 \pm 46.6
Disease severity, EDSS: mean \pm SD	2.0 \pm 1.3
CA MS: n/total (%)	77/100 (77%)
CS MS: n/total (%)	23/100 (23%)
Gd+ MS: n/total (%)	37/100 (37%)
Gd- MS: n/total (%)	63/100 (63%)
Blood-CSF-barrier dysfunction, QAlb: positive/total (%)	18/100 (18%)
Intrathecal IgG synthesis, IgG Index: positive/total (%)	71/100 (71%)
CSF-restricted oligoclonal IgG bands: positive/total (%)	84/100 (84%)

EDSS = Expanded disability status scale; CA = Clinically Active (presence of relapse at entry); CS = Clinically Stable (absence of relapse at entry); Gd+ = MRI appearance of gadolinium enhancing lesions; Gd- = no MRI evidence of gadolinium enhancing; QAlb = CSF/serum albumin quotient; IEF = isoelectric focusing followed by IgG-specific immunoblotting.

At the time of sample collection: a) disease severity was scored using Kurtzke's Expanded Disability Status Scale (EDSS) (Kurtzke 1983); b) disease duration was scored and expressed in months; c) presence of relapse was recorded as clinical activity; d) lesions showing Gd-enhancement on T1-weighted scans were defined as MRI activity.

Table 4. Demographic and clinical features in other inflammatory neurological disorders (OIND) and non-inflammatory neurological disorders (NIND).

Patients	n	Sex		Age (years)	Type of disease
		F	M		
OIND	100	70	30	37.5 ± 10.7	
	29				Chronic inflammatory demyelinating polyneuropathy
	18				Viral encephalomyelitis
	18				Acute inflammatory demyelinating polyneuropathy
	11				Bacterial meningitis
	10				Optic neuritis
	6				HIV encephalopathy
	3				Neurolupus
	3				NeuroSjogren
	3				NeuroBechet
NIND	100	70	30	38.1 ± 11.1	
	14				Transient ischemic attack
	13				Epilepsy
	12				Headache
	10				Cervical spondylosis
	10				Hereditary ataxia
	9				Vascular dementia
	9				Migraine
	8				Amyotrophic lateral sclerosis
	7				Compression neuropathy
	4				Paresthesias
	4				Alzheimer disease

OIND = Other Inflammatory Neurological Diseases; NIND = Non-Inflammatory Neurological Diseases.

One hundred patients with other inflammatory neurological disorders (OIND) and 100 subjects with other non-inflammatory neurological disorders (NIND) were selected as neurological controls (Table 4) who were age and sex matched to RRMS. Paired CSF and serum samples were prospectively collected at the Section of Neurology, University of Ferrara, and at the Operative Unit of Neurology, Azienda Ospedaliero-Universitaria di Ferrara, during the period from January 2007 to December 2011 from RRMS, OIND and NIND patients. Samples were obtained for purposes of diagnosis and measured under exactly the same conditions. MS OIND and NIND patients were free of immunosuppressant drugs, including steroids, at the time of sample collection. CSF and serum IgG and albumin levels were measured by immunochemical nephelometry with the Beckman Image 800 system (Beckman Instruments, Fullerton, CA, USA). In all patients, Blood-CSF-barrier (B-CSF-B) dysfunction was determined by CSF/serum albumin quotient (QAlb) (Tibbling et al, 1977) and total IgG OCB by isoelectric focusing (IEF) (Franciotta et al, 2005).

The second part of the study was focused on a cohort of MS patients treated with TYSABRI®. Patients were enrolled at the “Fondazione Istituto Neurologico C. Mondino” in Pavia:

- 17 female: Age (mean \pm SD)= 24.2 \pm 7.1 years; EDSS (mean \pm SD)= 1.5 \pm 1.6;
- 3 male: Age (mean \pm SD)= 29.0 \pm 1.7 years; EDSS (mean \pm SD) = 0.5 \pm 0.9.

Serum samples were collected at baseline and consecutively after 3, 6, 9, 12, 15, 18 and 21 months after the beginning TYSABRI® therapy. To exclude short-term drug effects, all samples were taken at least 12–36 h after TYSABRI® injection. None of the patients had been receiving corticosteroids when these samples were collected. The studies were both approved by the Regional Committee for Medical Ethics in Research.

Magnetic Resonance Imaging analysis

All MS patients underwent brain Magnetic Resonance Imaging (MRI) scans at entry on a 1-Tesla MRI unit (GE Signa Horizon, General Electric Medical Systems, Milwaukee, WI). Routinely used T1- weighted axial spin echo images were obtained approximately 10 min after intravenous

injection of 0.1 mmol/kg of Gd-DTPA in each patient. All brain MRI scans were evaluated by one investigator blinded to clinical and sample data.

Serum and CSF levels of anti-EBV antibodies

CSF and serum concentrations of anti-EBNA-1 and anti-VCA IgG were measured by enzyme-linked immunosorbent assay (ELISA) and expressed as arbitrary Units (AU) as published before (Castellazzi et al, 2010). The methods were based on the use of commercially available ELISA kits (Novagnost™ EBV-EBNA1 IgG and EBV-VCA IgG, cod. num. EBVG0580DB and EBVG0150DB, respectively). Briefly, a reference curve was generated in each assay using six serial dilutions of pooled high-positive serum samples ranging between 0.1 and 2.0 OD. CSF and corresponding serum prediluted with a range of 1:2-1:6 and 1:100-1:1200, respectively, were dispensed in duplicate into two microtiter plates, one precoated with highly purified EBV-EBNA-1 and the other precoated with EBV-VCA. Reference curve was generated in each assay using the standard serial dilutions by plotting the standard well concentrations, expressed as AU, versus the relative OD values. The lowest and the upper standard values of each plate were considered as 3.125 and 100 AU, respectively. The linear relationship between the AU values and the OD titers provides for easy extrapolation of concentration values. For each sample anti-EBNA-1 and anti-VCA IgG concentrations were obtained by multiplying AU value for the corresponding dilution factor. Within-assay and between-assay precisions were determined after 10 repeated measured into the same plate and by repetition of the same sample in 10 consecutive plates, respectively. Both intra and inter-assay variations, expressed as coefficient of variations (CV)%, were less than 8% for both anti-EBNA-1 and anti-VCA IgG.

Calculation of EBNA-1 and anti-VCA IgG -specific Antibody Index

According to literature (Reiber and Lange, 1991), Antibody Index (AI) means the ratio between CSF and serum AU values (Q_{Spec}) and CSF/serum total IgG levels expressed as mg/dl (Q_{IgG}) in accordance to the formula:

$$\text{AI} = Q_{\text{Spec}}/Q_{\text{IgG}} \quad (1)$$

with $Q_{\text{Spec}} = \text{AU}_{\text{CSF}}/\text{AU}_{\text{serum}}$ and $Q_{\text{IgG}} = \text{IgG}_{\text{CSF}}/\text{IgG}_{\text{serum}}$

In the case of intrathecal synthesis of total IgG, Q_{IgG} appeared more elevated than Reiber's hyperbolic discrimination line (Q_{Lim}). Q_{Lim} represented the completely blood-derived CSF total IgG fraction calculated from the individual Q_{Alb} of a single patient. Therefore, owing to the introduction of this barrier-related correction of the Q_{IgG} , the ratio became between Q_{Spec} and Q_{Lim} in agreement with the formula:

$$\text{AI} = Q_{\text{Spec}}/Q_{\text{Lim}} \quad (2)$$

with $Q_{\text{Lim}} = 0.93 \sqrt{(Q_{\text{Alb}})^2 + 6 \cdot 10^{-6}} - 1.7 \cdot 10^{-3}$

Therefore, to avoid false-negative results Equation 1 ($Q_{\text{IgG}} < Q_{\text{Lim}}$) is used when no significant intrathecal total IgG synthesis occurs, while Equation 2 ($Q_{\text{IgG}} > Q_{\text{Lim}}$) was preferred in case of intense brain-derived total IgG production in CSF. EBV-specific intrathecal IgG synthesis was assumed for values of AI greater than 1.5 (Reiber and Lange, 1991; Castellazzi et al, 2010).

Antigen-specific immunoblotting

EBV-specific IgG OCB were investigated by antigen-specific immunoblotting (ASI) as reported elsewhere (Fainardi et al, 2009) using as target antigen a crude viral lysate containing a high concentration of EBV antigens, including VCA, EBNA, EA-D and EA-R. Briefly, equal amounts of CSF and serum samples, at the same IgG concentrations, were applied to the agarose gel. After IEF run, the gel was blotted onto a nitrocellulose sheet previously coated overnight at room temperature with EBV antigens (EBV antigen, cod. 11-511-248315-1, Genway, US) diluted in phosphate buffer (PBS) at the concentration of 100 $\mu\text{g}/\text{ml}$. The nitrocellulose membrane was then washed with

distilled water and subsequently saturated by incubation in physiologic solution containing 2% bovine serum albumine (BSA) for 30 minutes at room temperature, in constant agitation. The antigen-specific blotting was then performed according to the manufacturer's instructions. Aspecific binding sites were further blocked in saline buffer containing 2% BSA for 30 minutes. The immunoblot was then incubated for 30 min at room temperature with peroxidase conjugated rabbit anti-human IgG diluted in 0.2% BSA saline. After another washing cycle with PBS, the blots were stained using peroxidase conjugated substrate of the kit according to the manufacturer's instructions. The immunoblotting specificity was evaluated by testing CSF from a patient with subacute sclerosing panencephalitis that served as control antigen to exclude non-specific binding. No cross-reactivity to EBV was detected. As described for total OCB patterns, antigen-specific immunoblotting IgG banding patterns were categorized by two independent investigators.

Determination of affinity distributions of EBV-specific antibodies

CSF and serum affinity distributions of anti-EBNA-1 and anti-VCA IgG were measured at the same conditions by performing a ELISA protocol based on the employment of increasing concentrations of sodium thiocyanate (NaSCN) using the above-mentioned ELISA kits (Luxton and Thompson, 1990; Fainardi et al, 2004) Briefly, 100 µl of each CSF or serum sample were added to 4 adjacent wells of each antigen-specific precoated ELISA microplate. After 1 h incubation at 37°C and three washing cycles, 4 consecutive dilutions of NaSCN (range: 0M, 1.25M, 2.5M, 5M, where 0M corresponded to 0.4% saline buffer) were applied into the wells corresponding to each patient for 10 min at 37°C. After incubation, NaSCN was removed and after three washing cycles wells were blocked by adding 100µl per well of 2% BSA for 30 min at 37°C. After incubation, BSA was discarded and detection antibody reaction, colour development and absorbance recordings were performed as described in ELISA assay for serum and CSF levels of anti-EBNA-1 and anti-VCA IgG. Positive and negative controls and appropriate background wells (blanks) were included in each test. The average optical density between the blanks values was subtracted from all readings.

The affinity distribution histograms for EBNA-1 and VCA-specific IgG were obtained assuming that, for each CSF and serum sample, the results observed in the first well, in which NaSCN were absent, represented the total antigen-specific IgG. In each series of 4 subsequent NaSCN dilutions per row, we divided the difference in OD values between two successive wells (e.g. well 1 and well 2) by OD values of the total antigen-specific IgG, and multiplied by 100. In this way, the variation in OD values between two subsequent wells was expressed as percentage of OD values of the total antigen-specific IgG measured in the first well. According to Luxton and coworkers (Luxton et al, 1995) the difference in OD values between two consecutive wells reflected the proportion in which the bounded IgG molecules were reduced by the higher concentration of NaSCN dispensed in the two wells, and the corresponding percentage was considered as the Relative Affinity (RA) of antibodies. When OD values measured in the well with higher levels of NaSCN were slightly greater compared to those detected in the adjacent well with lower levels of NaSCN, then it was assumed to be equivalent and RA thus assumed to be zero. When positive OD values were still measurable in the wells with highest concentration of NaSCN (5M in the fourth well), we compared these OD values with those of a simulated following well which was considered to be zero. Based on this principle, we obtained an RA value showing the proportion of antigen-specific IgG which was not removed by the highest concentration (5M) of NaSCN, and then the highest antibody affinity. On this principle, for each sample, we determined:

- RA1: percentage of IgG removed by 1.25M NaSCN;
- RA2: percentage of IgG removed by 2.5M NaSCN;
- RA3: percentage of IgG removed by 5M NaSCN;
- RA4: percentage of IgG remaining after treatment with 5M NaSCN.

CSF and serum samples with RA value more than 2.5% from well number four containing the highest concentration (5M) of NaSCN (RA4) were considered as having high-affinity anti-EBNA-1 and anti-VCA IgG, whereas CSF and serum samples with RA4 value equal or less than 2.5% were regarded as showing low-affinity anti-EBNA-1 and anti-VCA IgG. In addition, to increase the

sensitivity in the detection of antibody binding affinity, the affinity of anti-EBNA-1 and anti-VCA IgG was also defined in all the samples by Affinity Ratio (AR), a single numerical value which relates the percentages of high affinity antibodies (RA4) to the percentages of low affinity antibodies (RA1):

$$\text{Affinity Ratio (AR)} = \text{RA4/RA1} \times 100$$

Determination of EBV-specific IgG OCB affinity

Affinity of EBV-specific IgG OCB was evaluated following a previously published immunoblotting protocol that employed increasing concentrations of NaSCN (Chapman et al, 2006). CSF samples were loaded in two parallel agarose gels (two groups of four samples per gel). IEF were performed for both gels at the same time and conditions in order to reduce errors due to inter-assay variation. After focusing, the gels were blotted onto the EBV precoated Ultrabind 450 membrane (Gelman, UK) for 45 minutes under a 1 kg weight. Antigen precoating was performed overnight as illustrated in ASI protocol. Ultrabind membrane was used due to its ability to bind covalently antigens which could be stripped by NaSCN from the nitrocellulose membrane, where their bind is usually non-covalent. Each membrane was then divided into two identical parts each with four samples. Each part was incubated with four different concentrations of NaSCN (0M, 1.25M, 2.5M, 5M, respectively) for 30 min at room temperature. The membranes were then washed in running tap water to remove any residual NaSCN and reincubated in blocking solution (BSA) for 30 minutes to ensure that any binding sites uncovered by the NaSCN incubation were blocked. Conjugated rabbit anti-human IgG diluted in 0.2% BSA saline was then added and after another washing cycle with PBS, the blots were stained using peroxidase conjugated substrate (ASI protocol). Afterwards, the affinity of EBV-specific IgG OCB was judged qualitatively based on the intensity of the bands detected by visual inspection. A persistence of EBV-specific IgG OCB at 5M NaSCN was interpreted as suggestive of high affinity.

Statistics

Statistic analysis was performed with GraphPad Prism®. After checking data for normality by using the Kolmogorov–Smirnov test, normality of data distribution was rejected in several variables. Therefore, statistical analysis was performed by a non-parametric approach. More precisely, continuous variables were compared using the Kruskal–Wallis test followed by the Mann–Whitney U test, whereas categorical variables were compared by means of Chi-square test (χ^2). Correlation between continuous variables were assessed by Spearman rank correlation coefficient test. Bonferroni correction was utilized for multiple comparisons. A value of $p < 0.05$ was accepted as statistically significant.

RESULTS (1)

CSF and serum levels and intrathecal synthesis of anti-EBV IgG in RRMS patients and controls

Detectable CSF levels of anti-EBNA-1 IgG were statistically more frequent in RRMS than in NIND ($p < 0.05$), whereas measurable CSF amounts of anti-VCA IgG were more frequent in OIND than in RRMS ($p < 0.001$) without any significant differences among RRMS, OIND and NIND for serum quantifiable levels of anti-EBNA-1 and anti-VCA IgG (Table 5).

Table 5. Frequency of CSF and serum samples with detectable levels of anti-EBNA-1 and anti-VCA IgG in patients with relapsing-remitting multiple sclerosis (RRMS), other inflammatory neurological disorders (OIND) and non-inflammatory neurological disorders (NIND).

	anti-EBNA-1 IgG		anti-VCA IgG	
	CSF	serum	CSF	serum
RRMS (n=100)	96/100 (96%)*	99/100 (99%)	65/100 (65%)	90/100 (90%)
OIND (n=100)	91/100 (91%)	96/100 (96%)	86/100 (86%)^	94/100 (94%)
NIND (n=100)	86/100 (86%)	94/100 (94%)	74/100 (74%)	90/100 (90%)

CSF anti-EBNA-1 IgG (Chi-square with Bonferroni correction): *RRMS vs. NIND ($p < 0.05$); CSF anti-VCA IgG (Chi-square with Bonferroni correction): ^OIND vs. RRMS ($p < 0.001$).

As listed in Figure 7, CSF levels of anti-EBNA-1 and anti-VCA IgG were statistically different among RRMS, OIND and NIND (Kruskal-Wallis: $p < 0.0001$ and $p < 0.01$, respectively). Significantly different levels of anti-EBNA-1 and anti-VCA IgG among RRMS, OIND and NIND were also observed in serum samples (Kruskal-Wallis: $p < 0.001$ and $p < 0.05$, respectively). Specifically, CSF anti-EBNA-1 IgG mean levels were more elevated and in RRMS ($p < 0.01$) and in OIND ($p < 0.05$) than in NIND (Panel A). CSF mean levels of anti-VCA IgG were higher in OIND than in RRMS and NIND groups ($p < 0.01$ and $p < 0.0001$, respectively) (Panel B). Serum anti-

EBNA-1 IgG mean levels were more elevated in RRMS than in OIND and NIND ($p < 0.0001$) (Panel C). Conversely, no significant differences were found for serum anti-VCA IgG mean levels among the groups examined (Panel D). When RRMS patients were grouped according to clinical and MRI activity, no statistical differences were observed between RRMS patients with and without clinical and MRI evidence of disease activity for CSF and serum mean concentrations of anti-EBNA-1 and anti-VCA IgG.

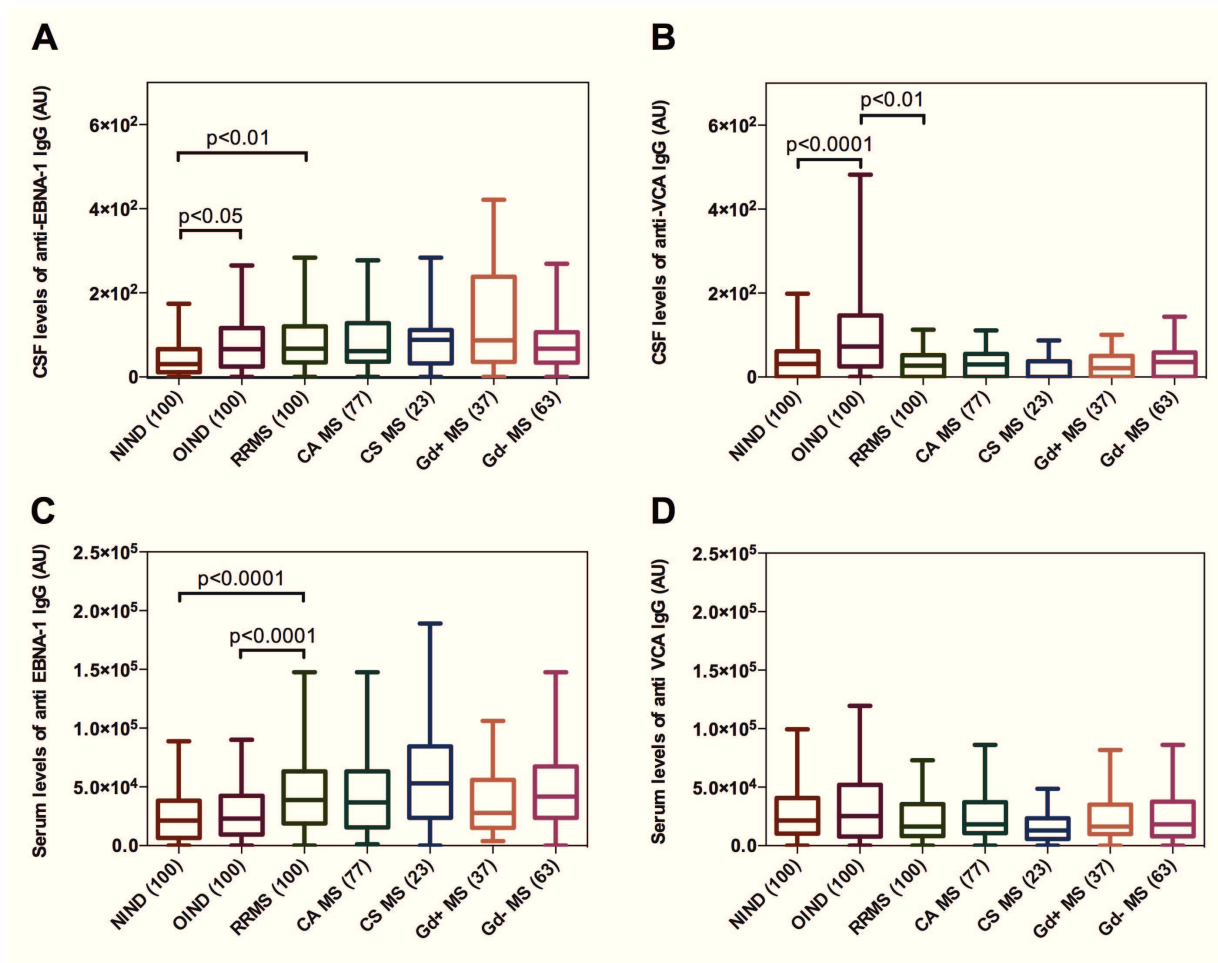


Figure 7. CSF and serum levels of anti-EBNA-1 and anti-VCA IgG in patients with relapsing-remitting multiple sclerosis (RRMS), other inflammatory neurological disorders (OIND) and non-inflammatory neurological disorders (NIND). AU = arbitrary units; CA = Clinically Active (presence of relapse at entry); CS = Clinically Stable (absence of relapse at entry); Gd+ = MRI appearance of gadolinium enhancing lesions; Gd- = no MRI evidence of gadolinium enhancing. CSF anti-EBNA-1 IgG levels (Mann–Whitney with Bonferroni correction): MS vs. NIND ($p < 0.01$); OIND vs. NIND ($p < 0.05$) (**Panel A**). CSF anti-VCA IgG levels (Mann–Whitney): OIND vs. MS ($p < 0.0001$); OIND vs. NIND ($p < 0.01$) (**Panel B**). Serum anti-EBNA-1 IgG levels (Mann–Whitney with Bonferroni correction): MS vs. OIND ($p < 0.0001$); MS vs. NIND ($p < 0.0001$) (**Panel C**). No differences were found for serum anti-VCA IgG mean levels (**Panel D**). The boundaries of the boxes represent the 25th–75th quartile. The line within the box indicates the median. The vertical lines above and below the box correspond to the highest and lowest values, excluding outliers.

An inverse correlation (Spearman: $r=-0.336$) was found between serum anti-EBNA-1 IgG levels and EDSS. We did not observe further definite relationships between disease severity and duration and CSF and serum concentrations of anti-EBNA-1 and anti-VCA IgG in RRMS patients (Table 6).

Table 6. Correlations between CSF and serum anti-EBNA-1 and anti-VCA IgG and severity of the disease, expressed by EDSS, and the disease duration, expressed in months.

	EDSS	Disease duration (months)
CSF anti-EBNA-1 IgG levels (AU)	$r = - 0.1642$	$r = 0.1181$
CSF anti-VCA IgG levels (AU)	$r = 0.2294$	$r = 0.1184$
Serum anti-EBNA-1 IgG levels (AU)	$r = - 0.336^*$	$r = 0.0963$
Serum anti-VCA IgG levels (AU)	$r = 0.1901$	$r = 0.1443$

EDSS = Expanded disability status scale; AU = arbitrary units. Spearman: $*p<0.001$

An intrathecal synthesis of anti-EBNA-1 IgG and anti-VCA IgG, as indicated by AI values greater than 1.5, was present in a small percentage of RRMS patients and controls (RRMS=6%, OIND=7%, NIND=2% for EBNA-1-specific AI; RRMS=2%, OIND=5%, NIND=1% for VCA-specific AI), without any statistical differences among the groups.

EBV-specific IgG OCB in RRMS patients

EBV-specific IgG OCB were detected in 25/100 (25%) RRMS patients (Table 7). Four different profiles were recognized (“normal”; “local synthesis”; “mixed” and “mirror). Among these, “local synthesis” and “mixed” patterns indicated an intrathecal synthesis of anti-EBV IgG OCB, whereas “mirror” pattern revealed an EBV-specific systemic oligoclonal response. Twenty-one RRMS patients had “local synthesis”, 3 had “mixed” and 1 had “mirror” patterns. Two illustrative cases describing paired total and EBV-specific IgG OCB from RRMS patients are reported in Figure 8. RRMS patients with and without intrathecally synthesized EBV-specific IgG OCB did not differ for

disease duration and severity, clinical and MRI activity, and CSF and serum anti-EBNA-1 and anti-VCA IgG concentrations, with the exception of anti-VCA IgG concentrations in serum which were greater in ASI negative than in ASI positive RRMS (Mann-Whitney: $p < 0.05$).

Table 7. Demographic and clinical features, and CSF and serum and intrathecal synthesis of anti-EBNA-1 and anti-VCA IgG levels in 100 relapsing-remitting multiple sclerosis (RRMS) patients divided according to antigen-specific immunoblotting (ASI) findings.

	ASI positive RRMS (n=24)	ASI negative RRMS (n=76)
Sex: F/M	17/7	53/23
Age, years: mean \pm SD	36.7 \pm 9.1	37.5 \pm 11.1
Disease duration, months: mean \pm SD	28.7 \pm 40.2	30.8 \pm 48.5
Disease severity, EDSS: mean \pm SD	2.2 \pm 1.5	2.0 \pm 1.2
CA MS: n/total (%)	20/24 (83.3%)	57/76 (75.0%)
CA MS: n/total (%)	4/24 (16.7%)	19/76 (25.0%)
Gd+ MS: n/total (%)	13/24 (54.2%)	24/76 (31.6%)
Gd- MS: n/total (%)	11/24 (45.8%)	52/76 (68.4%)
CSF anti-EBNA-1 IgG (AU): mean \pm SD	193.0 \pm 266.0	155.2 \pm 232.1
CSF anti-VCA IgG (AU): mean \pm SD	41.6 \pm 56.9	81.5 \pm 243.4
Serum anti-EBNA-1 IgG (AU): mean \pm SD	126502 \pm 205623	132114 \pm 198699
Serum anti-VCA IgG (AU): mean \pm SD	21572 \pm 24497	50122 \pm 75151 ^a
EBNA-1 AI > 1.5: n/total (%)	3/21 (12.5%)	3/76 (4.0%)
VCA AI > 1.5: n/total (%)	1/21 (4.2%)	1/76 (1.3%)

ASI positive = presence of EBV-specific IgG oligoclonal bands; ASI negative = absence of EBV-specific IgG oligoclonal bands; EDSS = Expanded disability status scale; CA = Clinically Active (presence of relapse at entry); CS = Clinically Stable (absence of relapse at entry); Gd+ = MRI appearance of gadolinium enhancing lesions; Gd- = no MRI evidence of gadolinium enhancing; SD = standard deviation. AI > 1.5 = Antibody Index abnormal values suggestive of EBV-specific

intrathecal synthesis; Serum anti-EBNA-1 IgG levels (Mann–Whitney): ^aASI negative vs. ASI positive ($p < 0.05$).

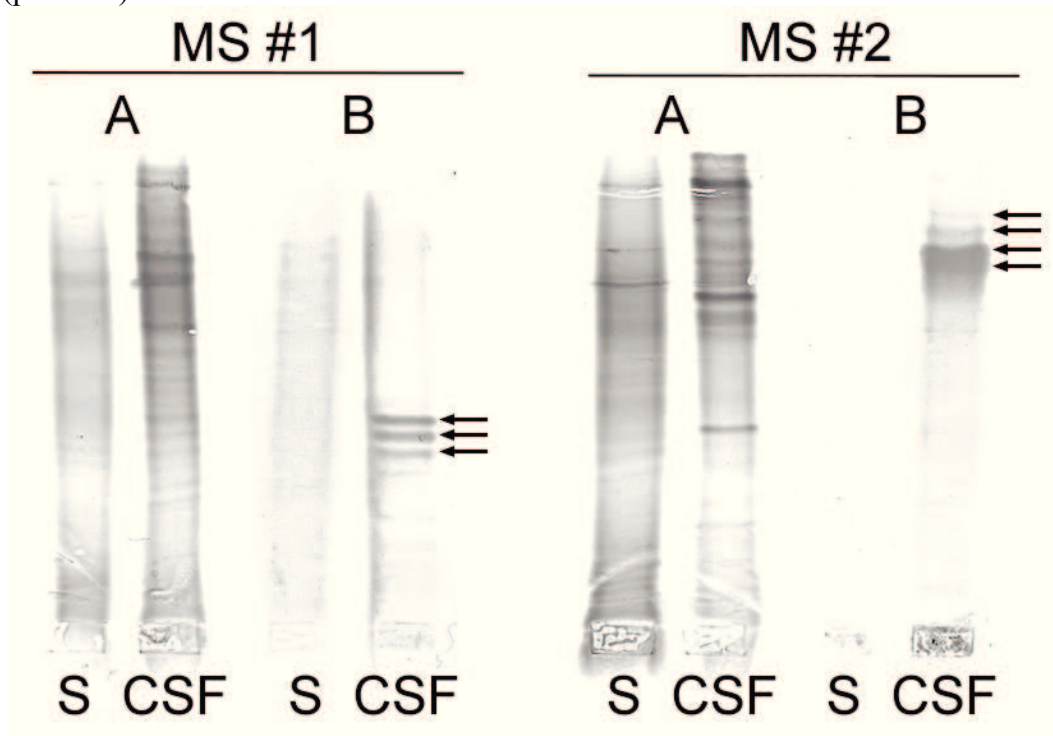


Figure 8. Comparison between total (A) and EBV-specific (B) IgG OCB profiles obtained with isoelectric focusing and antigen-specific immunoblotting, respectively, in paired cerebrospinal fluid (CSF) and serum samples of two relapsing-remitting multiple sclerosis (RRMS) patients. Corresponding bands are indicated by arrows. Among RRMS patients with EBV-specific IgG OCB, CSF-restricted total IgG OCB were absent only in a RRMS patient with a “mirror” pattern. In the remaining 24 RRMS patients with an EBV-specific intrathecal oligoclonal IgG synthesis there was a little overlap between total and virus-specific IgG OCB unique-to-CSF due to their high individual variability. More precisely, EBV-specific numbered less (mean \pm SD = 3.6 ± 1.9 ; range from 2 to 9) than total IgG OCB (mean \pm SD = 9.9 ± 4.6 ; range from 2 to 20), and did not always correspond to them because of the presence of additional bands in antigen-specific immunoblotting which were not visible in IEF.

None of the patients with OIND and NIND showed OCB specifically directed against EBV, not even those with total IgG OCB (9 OIND and 2 NIND with “local synthesis”, 1 OIND and 1 NIND with “mixed”, 8 OIND and 7 NIND with “mirror” and 3 OIND with “monoclonal” patterns).

Affinity distributions of anti-EBNA-1 and anti-VCA IgG in RRMS patients and controls

Affinity distributions of anti-EBNA-1 and anti-VCA IgG were quantitatively evaluated in CSF and serum from a representative subpopulation of 50 RRMS (34 female and 16 male, mean age= 34.9 ± 8.2) and 50 OIND (34 female and 16 male, mean age= 35.5 ± 8.8), 50 NIND patients (34

female and 16 male, mean age=36.1±8.3) with high titers of both these EBV-specific antibodies. All RRMS, OIND and NIND patients with AI abnormal values and all RRMS patients with EBV-specific IgG OCB were included. CSF and serum Relative Affinity (RA) values obtained with increasing concentrations of NaSCN were significantly greater for anti-EBNA-1 IgG than for anti-VCA IgG at the lowest concentration of NaSCN while, on the contrary, for anti-VCA IgG than for anti-EBNA-1 IgG at higher NaSCN concentration, whereas no differences were found at the highest concentration of NaSCN in all the three groups analyzed (Figure 9, Panels A, B, C, D, E and F).

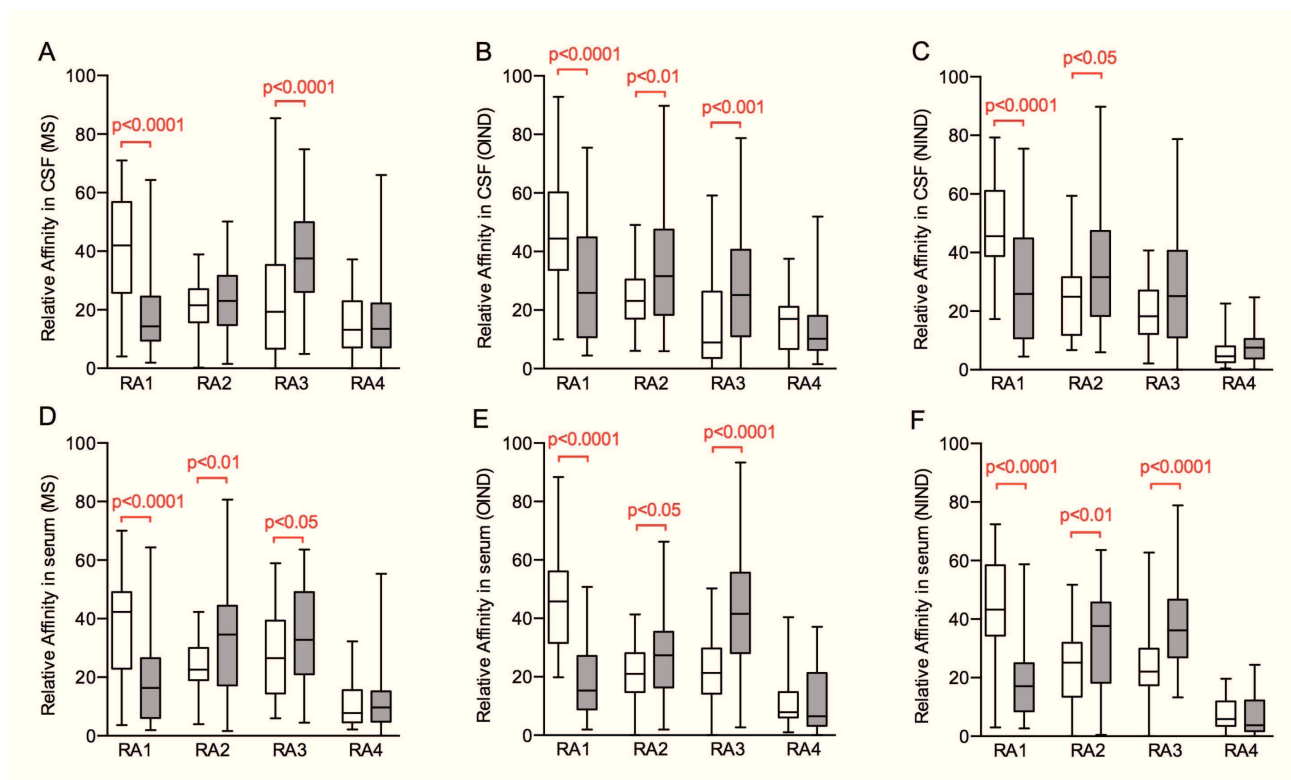


Figure 9. Relative affinity (RA) of anti-EBNA-1 IgG (white boxes) and of anti-VCA IgG (grey boxes) values obtained with increasing concentrations of sodium thiocyanate (NaSCN) in CSF and serum from 50 RRMS, 50 OIND and 50 NIND patients. CSF and serum RA1 values were higher for anti-EBNA-1 IgG than for anti-VCA IgG in RRMS (**panels A and D**; Mann-Whitney; $p < 0.0001$), OIND (**panels B and E**; Mann-Whitney; $p < 0.0001$) and NIND (**panels C and F**; Mann-Whitney; $p < 0.0001$). CSF RA2 values were more elevated for anti-VCA IgG than for anti-EBNA-1 IgG in OIND (**panel B**; Mann-Whitney; $p < 0.01$) and NIND (**panel C**; Mann-Whitney; $p < 0.05$). Serum RA2 values were greater for anti-VCA IgG than for anti-EBNA-1 IgG in RRMS (**panel D**; Mann-Whitney; $p < 0.01$), OIND (**panel E**; Mann-Whitney; $p < 0.05$) and NIND (**panel F**; Mann-Whitney; $p < 0.01$). CSF RA3 values were higher for anti-VCA IgG than for anti-EBNA-1 IgG in RRMS (**panel A**; Mann-Whitney; $p < 0.001$) and OIND (**panel B**; Mann-Whitney; $p < 0.001$). Serum RA3 values were more increased for anti-VCA IgG than for anti-EBNA-1 IgG in RRMS (**panel D**; Mann-Whitney; $p < 0.05$), OIND (**panel E**; Mann-Whitney; $p < 0.0001$) and NIND (**panel F**; Mann-Whitney; $p < 0.001$). The boundaries of the boxes represent the 25th–75th quartile. The line within the box indicates the median. The vertical lines above and below the box correspond to the highest

and lowest values, excluding outliers. MS: relapsing-remitting multiple sclerosis; OIND: other inflammatory neurological disorders; NIND: non-inflammatory neurological disorders; RA1: percentage of IgG removed by 1.25M NaSCN; RA2: percentage of IgG removed by 2.5M NaSCN; RA3: percentage of IgG removed by 5M NaSCN; RA4: percentage of IgG remaining after treatment with 5M NaSCN.

As illustrated in Table 8, CSF high-affinity anti-EBNA-1 IgG, as indicated by RA4 value at 5M NaSCN > 2.5%, were significantly more frequent in RRMS ($p < 0.05$) than in NIND, while CSF high-affinity anti-VCA IgG were statistically more represented in RRMS and OIND ($p < 0.05$) than in NIND. On the other hand, serum high-affinity anti-VCA IgG were significantly more common in RRMS ($p < 0.01$) than in NIND.

Table 8. CSF and serum distributions of high-affinity anti-EBNA-1 and anti-VCA IgG in a subgroup of RRMS, OIND and NIND patients.

	RRMS (n = 50)	OIND (n = 50)	NIND (n = 50)
CSF anti-EBNA-1 IgG: AR4 > 2.5, n/total (%)	48/50 (96%) ^a	45/50 (90%)	40/50 (80%)
CSF anti-VCA IgG: AR4 > 2.5, n/total (%)	48/50 (96%) ^b	47/50 (94%) ^c	40/50 (80%)
serum anti-EBNA-1 IgG: AR4 > 2.5, n/total (%)	47/50 (94%)	47/50 (94%)	48/50 (96%)
serum anti-VCA IgG: AR4 > 2.5, n/total (%)	44/50 (88%) ^d	40/50 (80%)	32/50 (64%)

RRMS = relapsing-remitting multiple sclerosis; OIND = other inflammatory neurological disorders; NIND = non-inflammatory neurological disorders; AR4 = affinity ratio 4, percentage of IgG remaining after treatment with 5 M NaSCN. CSF anti-EBNA-1, AR4 > 2.5 (Chi-square with Bonferroni correction): ^aRRMS vs. NIND ($p < 0.05$); CSF anti-VCA, AR4 > 2.5 (Chi-square with Bonferroni correction): ^bRRMS vs. NIND ($p < 0.05$) and ^cOIND vs. NIND ($p < 0.05$); serum anti-VCA: AR4 > 2.5 (Chi-square with Bonferroni correction): ^dMS vs. NIND ($p < 0.01$).

No other statistical differences were found among the groups evaluated and between RRMS patients with and without clinical and MRI active disease. When we considered affinity ratio (AR), CSF for anti-EBNA-1 and anti-VCA IgG were statistically different among RRMS, OIND and NIND (Kruskal-Wallis: $p < 0.0001$). More precisely, CSF AR values were significantly higher in RRMS and OIND than in NIND for anti-EBNA-1 and anti-VCA IgG ($p < 0.0001$) (Figure 10,

panels A and B), without any further statistical differences between RRMS and controls for anti-EBNA-1 and anti-VCA IgG serum AR values (Figure 10, panels C and D).

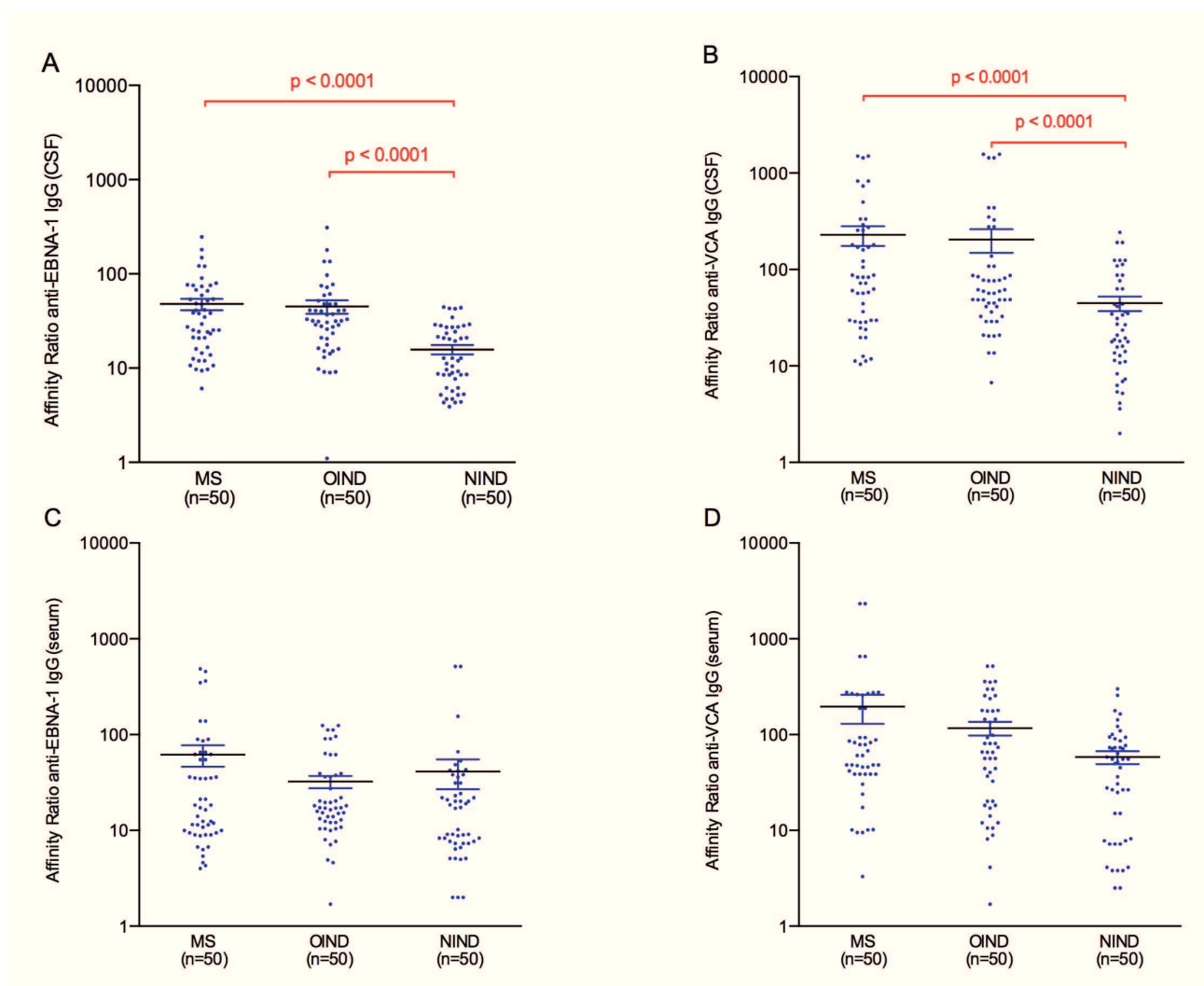


Figure 10. Affinity Ratio (AR) values of anti-EBNA-1 IgG and of anti-VCA IgG in CSF and serum from 50 RRMS, 50 OIND and 50 NIND patients. Anti-EBNA-1 IgG and anti-VCA IgG CSF AR values were significantly higher in MS and OIND than in NIND (Mann-Whitney with Bonferroni correction; $p < 0.0001$ in all cases) (**Panels A and B**), without any further statistical differences between MS and controls for anti-EBNA-1 IgG and anti-VCA IgG serum AR values (**Panels C and D**). Each point represents a single observation, excluding outliers. Horizontal bars indicate medians and error bars correspond to standard error.

Affinity distributions of EBV-specific oligoclonal antibodies

Affinity distributions of EBV-specific IgG OCB were qualitatively assessed in CSF from all 24 RRMS patients with EBV-specific CSF-restricted oligoclonal IgG response. In all CSF samples analyzed, EBV-specific IgG OCB were found to have low affinity since they completely disappeared after treatment with the highest concentration (5M) of NaSCN. Particularly, in only 4

CSF samples EBV-specific IgG OCB persisted at a concentration of 2.5M NaSCN, whereas in the remaining 20 CSF specimens EBV-specific IgG OCB were lost after treatment with 1.25M NaSCN concentration. Figure 11 depicts three explicative cases showing the effects on intensity of EBV-specific OCB IgG of increasing concentrations of NaSCN in RRMS patients.

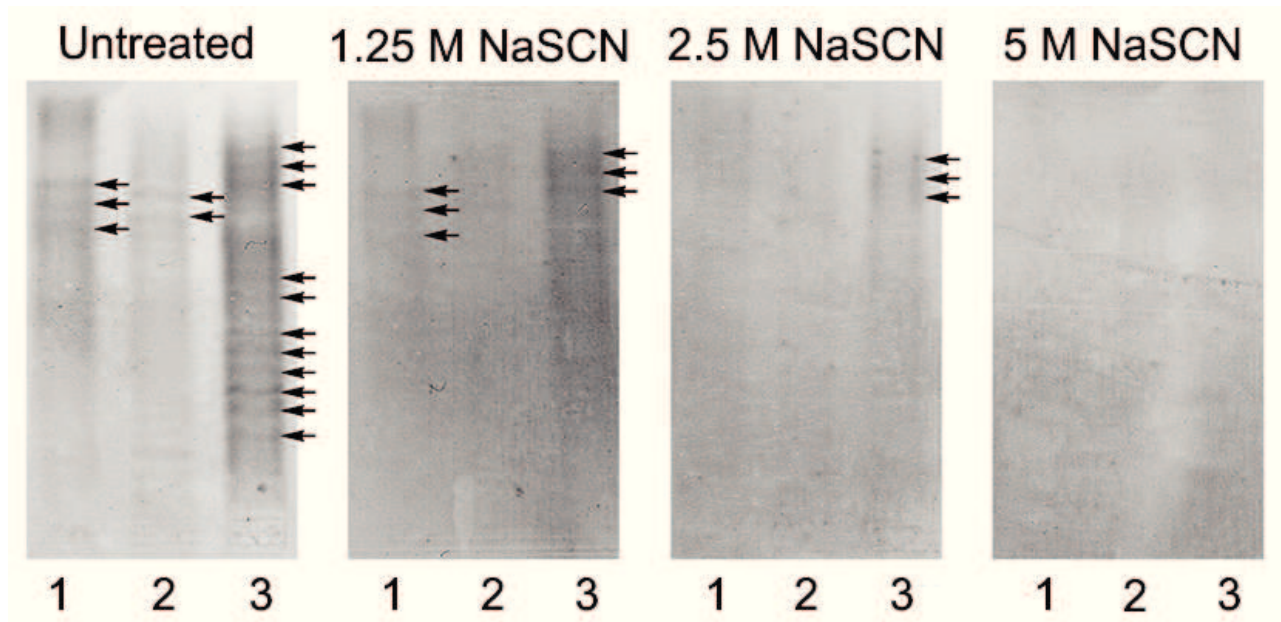


Figure 11. Changes in intensity of EBV-specific IgG OCB (arrows) detected by Antigen-mediated immunoblots (AMI) specific for EBV performed with employment of different concentration of NaSCN. A) Untreated: 0M NaSCN (saline buffer); B) 1.25 M NaSCN; C) 2.5 M NaSCN; D) 5 M NaSCN.

DISCUSSION (1)

In this study, we investigated different aspects of CSF and serum EBV-specific humoral immune response in RRMS focusing on intrathecal production and affinity distributions of EBV-specific IgG. We here showed that concentrations of anti-EBNA-1 IgG were higher in RRMS and OIND than in NIND in CSF and in RRMS than in OIND and NIND in serum. Interestingly, an inverse correlation between serum levels of anti-EBNA-1 IgG and EDSS was observed in RRMS. On the contrary, CSF anti-VCA IgG levels were higher in OIND than in RRMS and NIND. No relationships were found between anti-EBNA-1 and anti-VCA IgG and clinical and MRI MS activity, and an intrathecal synthesis of anti-EBNA-1 and anti-VCA IgG were observed in a minority of RRMS patients and controls. These results confirm that, while serum levels of anti-EBNA-1 IgG can be considered as a hallmark of MS (Santiago et al, 2010; Almohmeed et al, 2012) elevated CSF amounts of anti-EBNA-1 IgG do not seem to be selectively associated with MS, but are shared by several inflammatory neurological conditions (Castellazzi et al, 2010). In accordance with our recent publication (Castellazzi et al, 2010), but in contrast with other previous studies (Farrell et al, 2009; Lünemann et al, 2010) our results do not support the value of serum titers of anti-EBNA-1 IgG as biomarker for MS disease activity and progression but indicate that an increase in disability may be linked to a decline in systemic humoral reaction to EBNA-1. In addition, our findings further pointed out the poor significance of CSF and serum levels of anti-VCA IgG, although serum concentrations of anti-VCA IgG were repeatedly related to MS (Santiago et al, 2010; Almohmeed et al, 2012) and an association between them and MRI cortical atrophy was recently described (Zivadinov et al, 2009). On the other hand, the low rate of detection for an intrathecal release of anti-EBNA-1 and anti-VCA IgG documented in RRMS and controls was concordant with all (Pohl et al, 2010; Castellazzi et al, 2010; Jafari et al, 2010, Sargsyan et al, 2010; Villegas et al, 2011; Otto et al, 2011) but two (Jaquièry et al, 2010; Rand et al, 2000) prior investigations, providing additional evidence that EBV-specific intrathecally produced antibodies are innocent bystanders reflecting a polyspecific humoral reactivity directed against many different

pathogens not related to the cause of the disease and promoted by the overactive chronic immune stimulation associated with MS brain inflammation (Pohl et al, 2010; Otto et al, 2011) The divergences which emerged with previous studies (Rand et al, 2000; Farrell et al, 2009, Zivadinov et al, 2009; Lünemann et al, 2010; Jaquière et al, 2010) may derive from differences in patient selection and determination techniques, highlighting, moreover, the need for standardized protocols. The methodological issues could also explain the discrepancies found between our and some earlier investigations for CSF-restricted EBV-specific OCB which were recognized in MS patients (Rand et al, 2000; Cepok et al, 2005b; Serafini et al, 2007; Franciotta et al, 2011; Virtanen et al, 2014). We exclusively detected EBV-specific OCB restricted to CSF in 24% RRMS patients suggesting that, as qualitative analysis is more sensitive than quantitative in the determination of an intrathecal IgG synthesis (Thompson, 2004) an EBV targeted intrathecal humoral immune response implying that an EBV persistent chronic brain infection may occur in a subset MS patients. However, our affinity qualitative studies demonstrated that CSF-restricted EBV-specific OCB had low affinity in all RRMS patients. These findings indicate that EBV-specific intrathecal oligoclonal high-affinity antibodies are absent in MS. Therefore, as the process of immune maturation promoted by an infection leads to a gradual increase in antibody affinity resulting in a strong production of high-affinity antibodies specifically directed against the causative pathogen (Luxton and Thompson, 1990) our observations collectively argue against the possibility that an EBV persistent brain chronic infection may exist in MS. This suggests that the widely accepted association between anti-EBNA-1 IgG and MS may not always correspond to causation (Virgin et al, 2009). Conversely, quantitative analysis based on RA4 value at 5M NaSCN > 2.5% and AR collectively demonstrated that CSF high-affinity anti-EBNA-1 and anti-VCA IgG were more frequent in RRMS and OIND than in NIND and equally represented in RRMS and OIND. In absence of an oligoclonal intrathecal production of high affinity antibodies, these results suggest the presence of an EBV-specific polyclonal humoral immune response operating in CSF compartment that is not limited to MS, but represents a common feature of inflammatory CNS disorders, which is

predominantly blood-derived, and, thus, likely reflects an antecedent EBV infection during puberty (Hangartner et al, 2006). In addition, this reaction may be in part induced by a polyclonal activation of memory B cells and long-lived plasma cells which predominate within the inflammatory brain microenvironment (Meinl et al, 2006). Intriguingly, we observed, in both RRMS and controls, the occurrence of higher affinity anti-EBV viral surface protein (VCA) antibodies in comparison with those specific for nuclear viral proteins released from dying cells (EBNA-1). These results prove that EBV acts as an intermittently cytopathic virus (Hangartner et al, 2006). In summary, this study has shown that, at intrathecal level, MS is generally marked by polyclonal high-affinity anti-EBV antibodies which passively transudate from serum. In a subset of patients (24%), there are additional EBV-specific oligoclonal low-affinity antibodies which are locally synthesized within the CNS. These data do not justify the hypothesis that EBV persistence leading to a productive infection in the brain can play a role in MS. Nevertheless, the presumed EBV persistent CNS infection may occur in a latent form that, unlike the chronic form, is characterized by an intermittent and not a continual antigen stimulation without a significant production of specific CSF and serum antibodies (Lipton et al, 2007). Thus, the possibility that EBV is a cofactor in the development and maintenance of the disease, sustaining the polyspecific intrathecal antibody synthesis typical of MS (Franciotta et al, 2008) cannot be completely excluded. In addition, in this study we did not explore other potential mechanisms which can trigger MS autoimmunity such as molecular mimicry consisting of a cross-reaction between EBV and CNS self-antigens (Lang et al, 2002), bystander damage due to a dysregulation of EBV infection within the brain (Serafini et al, 2007) and accumulation of EBV infected autoreactive B cells in the CNS related to a decrease in systemic CD8 T cell control of EBV infection (Lucas et al, 2011). Finally, our results do not exclude the hypothesis that a dysregulated EBV infection of B cells may play a role in MS autoimmunity (Pender, 2003; Pender, 2011).

RESULTS (2)

Serum levels of anti-EBNA-1 and anti-VCA IgG in TYSABRI®-treated RRMS patients

Only for eleven patients was it possible to perform the analysis at each time point, on the contrary for nine patients the sample collection was not carried out at all the various time points. This could be the main bias of the study. However, we decided to analyse the samples all together at each time point. Detectable levels of anti-EBNA-1 IgG were present in 100% of analyzed samples. The distribution of data was verified for each time point by the Kolmogorov-Smirnov test. As shown in Table 9, mean levels of anti-EBNA-1 IgG had a normal distribution at all times with the exception of the twelfth month (T12, P value <0.05). Despite the increase in the mean levels from the ninth month of therapy, the statistical analysis of the data with the Kruskal-Wallis test did not reveal any significant differences between the concentrations analyzed.

Table 9. Longitudinal fluctuations of anti-EBNA-1 IgG levels in 20 RRMS patients during 15 months of TYSABRI® treatment. *p < 0.05; SD = standard deviation; AU = arbitrary units; KS = Kolmogorov-Smirnov; T0 = baseline; T3 = 3rd month; T6 = 6th month; T9 = 9th month; T12 = 12th month; T15 = 15th month.

Time point	Serum anti-EBNA-1 IgG concentrations (AU)		KS normality test
	mean ± SD	Range	P value
T0 (n=16)	28710 ± 15513	3424 – 58213	> 0.10
T3 (n=19)	27479 ± 15022	2115 – 56053	> 0.10
T6 (n=19)	28772 ± 15498	2965 – 64063	> 0.10
T9 (n=19)	34909 ± 30986	1672 – 143774	0.0974
T12 (n=20)	37735 ± 37214	2744 – 164574	0.0012*
T15 (n=18)	37650 ± 26581	2184 – 101307	> 0.10

Detectable levels of anti-VCA IgG were present in all the samples tested. The distribution of antibody levels at every time point was verified by the Kolmogorov-Smirnov test (Table 10). Comparison of the mean concentrations obtained at every time point with the Kruskal-Wallis test did not reveal any significant differences.

Tabel 10. Longitudinal fluctuations of anti-VCA IgG levels in 20 RRMS patients during 15 months of TYSABRI® treatment. *p < 0.05; SD = standard deviation; AU = arbitrary units; KS = Kolmogorov-Smirnov; T0 = baseline; T3 = 3rd month; T6 = 6th month; T9 = 9th month; T12 = 12th month; T15 = 15th month.

Time point	Serum anti-VCA IgG concentrations (AU)		KS normality test
	mean ± SD	Range	P value
T0 (n=16)	103019 ± 56996	14911 – 199402	> 0.10
T3 (n=19)	97027 ± 54339	17730 – 203687	> 0.10
T6 (n=19)	99606 ± 53866	19985 – 190493	> 0.10
T9 (n=19)	119322 ± 74596	29345 – 343112	> 0.10
T12 (n=20)	125215 ± 86778	8708 – 364044	> 0.10
T15 (n=18)	139741 ± 75789	38082 – 286458	0.0414*

Serum levels of anti-EBNA-1 and anti-VCA IgG in TYSABRI®-treated RRMS patients grouped into responders and nonresponders

According to very stringent criteria (Fainardi et al, 2004), 15 RRMS patients who experienced complete disappearance of clinical attacks were classified as “responders”, while 5 RRMS patients having no cessation of relapses were indicated as “nonresponders”. Despite the low sample size, statistical analysis with Student’s t test showed no difference between responders and

nonresponders both for anti-EBNA-1 and anti-VCA IgG at each time point (Figure 12, panels A and B).

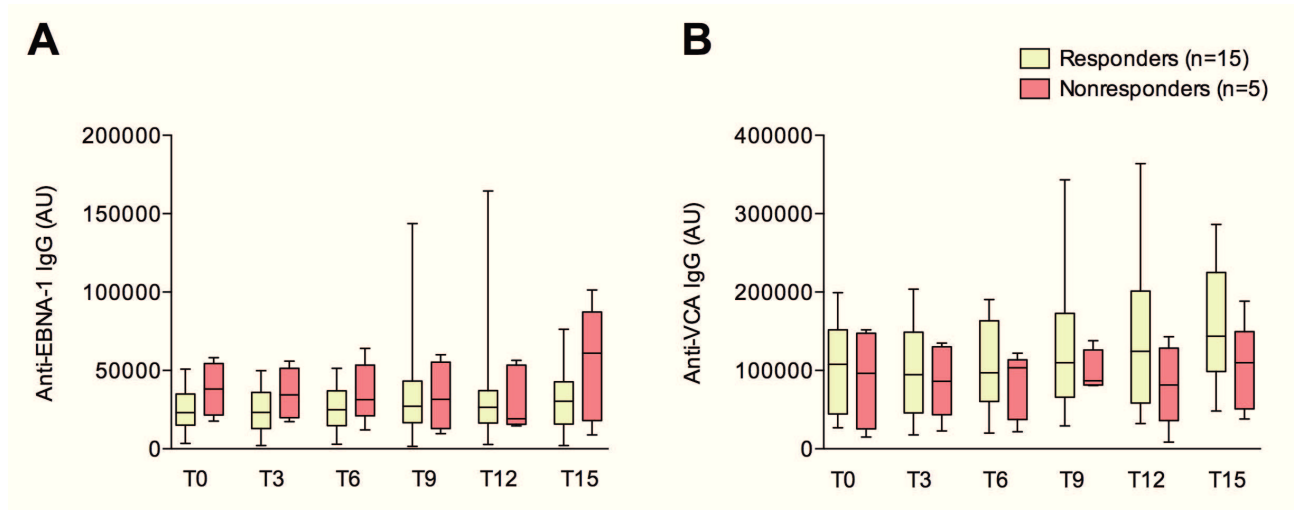


Figure 12. Temporal fluctuations of anti-EBNA-1 and anti-VCA IgG in RRMS patients grouped according to therapy response. **Panel A)** Anti-EBNA-1 IgG: T0, Resp Vs NoResp (t test; $p = 0.1696$); T3, Resp Vs NoResp (t test; $p = 0.1738$); T6, Resp Vs NoResp (t test; $p = 0.2295$); T9, Resp Vs NoResp (t test; $p = 0.9077$); T12, Resp Vs NoResp (t test; $p = 0.7934$); T15, Resp Vs NoResp (t test; $p = 0.0999$). **Panel B)** Anti-VCA IgG: T0: Resp Vs NoResp (t test; $p = 0.6108$); T3, Resp Vs NoResp (t test; $p = 0.6328$); T6, Resp Vs NoResp (t test; $p = 0.3885$); T9, Resp Vs NoResp (t test; $p = 0.5397$); T12, Resp Vs NoResp (t test; $p = 0.2076$); T15, Resp Vs NoResp (t test; $p = 0.2006$). The boundaries of the boxes represent the 25th–75th quartile. The line within the box indicates the median. The vertical lines above and below the box correspond to the highest and lowest values, excluding outliers. Responders: patients who experienced complete disappearance of clinical attacks; Non-responders: patients having no cessation of relapses.

DISCUSSION (2)

At the best of our knowledge, temporal fluctuations of serum levels of EBV-specific IgG in RRMS patients receiving TYSABRI® have never been investigated before. In recent decades, several studies have shown correlations between antibodies specific for certain antigens of EBV, in particular antigens EBNA-1 and VCA, and some clinical features of MS, such as onset, progression, activity and severity (Farrell et al, 2009; Lünemann et al, 2010; Ascherio and Munger, 2010; Almohmeed et al, 2013). For these reasons, these antibodies should be considered serological markers of the natural history of the disease, or “type 0 biomarkers” (Bielekova and Martin, 2004). However, our data seem to exclude the role of EBV-specific antibody response as a biomarker for the evaluation of therapeutic response, “type 1 biomarkers”, to TYSABRI®. In fact, during 15 months of therapy, temporal fluctuations of EBV-specific antibodies were not affected by drug treatment. This lack of statistical significance was also confirmed by the comparison between patients who responded to treatment and patients who had, instead, relapses during the study period. This is in contrast with previous studies in which the levels of anti-EBNA-1 IgG correlated with disease activity (Farrell et al, 2009) and anti-VCA IgG correlated with the progression of the disease (Zivadnov et al, 2009). The main limitations of this study were certainly the low number of enrolled subjects and the fact that all serum samples were not available for some patients. Another limiting factor is the lack of samples collected at the time of relapse which could have provided better guidance on correlations with clinical activity. Taken together, these data argue against the use of EBV-specific antibody levels as biomarkers for monitoring therapeutic response to TYSABRI® in the course of relapsing-remitting multiple sclerosis. However, future studies are needed to verify the actual significance of anti-EBV antibodies in MS patients who are undergoing TYSABRI® therapy.

CONCLUSIONS

In this study we deeply investigated the relationship between EBV and MS by evaluating the affinity distribution of the virus-specific antibodies at the time of diagnosis and the temporal fluctuations of these antibodies during disease modifying therapy. The main results are summarized as follow:

- a) multiple sclerosis is associated to elevated serum titers of anti-EBV antibodies, in particular of anti-EBNA-1 IgG, reflecting a past EBV infection;
- b) the occurrence of higher affinity anti-EBV viral surface protein (VCA) antibodies in comparison with those specific for nuclear viral proteins released from dying cells (EBNA-1) is a shared condition in both MS and CNS inflammatory diseases, and confirms that EBV can act as an intermittently cytopathic virus;
- c) in a subset of MS patients (24%) an intrathecal synthesis of EBV-specific oligoclonal low-affinity antibodies was observed, and these data do not justify the hypothesis that EBV persistence leads to a productive infection in the brain which can play a role in MS;
- d) the in-depth analysis of the virus-specific antibody response suggests that the widely accepted association between anti-EBNA-1 IgG and MS may not always correspond to causation;
- e) although serum titers of anti-EBNA-1 IgG are considered a biomarker for the natural history of MS, the study of the EBV-specific antibody response in patients treated with TYSABRI® seems to exclude the use of these antibodies as biomarkers of therapeutic intervention.

Taken together, our results do not argue against the hypothesis that a dysregulated EBV infection of B cells may play a role in MS pathogenesis.

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