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A Toll-Like Receptor 3-Agonist as Promising Candidate in Multiple Sclerosis Treatment

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Abstract

Perturbations in immune processes play an important role in multiple sclerosis (MS), an autoimmune disorder where specific innate immune pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) have recently been shown to play a major role in the initiation disease, the triggering of relapses, and regulation of CNS damage. The abnormal immune response in MS has been shown to be dependent on genetic background, despite environmental factors, including pathogens capable of overstimulating innate immune response through TLRs, appear to contribute to the development of autoreactive T cells that in turn cause myelin damage. However, whereas the upregulation of most TLRs plays a detrimental role in MS pathogenesis, recent studies suggest an ameliorative role of TLR3 in the onset and progression of MS and experimental autoimmune encephalomyelitis (EAE), a murine model of MS. TLR3 activation appears to protect from the disease, mainly through induction of interferon (IFN)β. Therefore, TLR3 stimulation with synthetic immunomodulators could represent a potential alternative approach in MS therapy. Among the investigational compounds TLR3-targeting, the mismatched double-stranded RNA molecule Ampligen®, can offer promise in the treatment of relapsing MS patients. Ampligen® is currently in phase III clinical trial in the treatment of chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME), an illness that shows remarkable levels of similarity with MS. The aim of this paper is to provide a brief overview about Ampligen® historical development, clinical pharmacology, clinical trials, and safety data, and to discuss about its potential role in MS treatment in the context of existing therapeutic options.

Keywords: Multiple sclerosis; Innate immunity; Toll-like receptors; Toll-like receptor 3-agonist; Interferon-beta.

Abbreviations

CFS/ME: Chronic Fatigue Syndrome/Myalgic Encephalomyelitis; CNS: Central Nervous System; DMAs: Disease-Modifying Agents; dsRNA: double-stranded Ribo Nucleic Acid; EAE: Experimental Autoimmune Encephalomyelitis; FDA: Food and Drug Administration; IFN: Inferferon; IKKε: IκB Kinase Epsilon; IRF: Interferon Regulatory Factor; JNK: c-Jun Amino-Terminal Kinase; MAP: Mitogen-Activated Protein; MS: Multiple Sclerosis; PAMPs: Pathogen-Associated Molecular Patterns; PRRs: Pattern-Recognition Receptors; TBK: TANK-Binding Kinase; TIR: Toll/IL-1 Receptor; TLRs: Toll-Like Receptors; TRAF: TNF Receptor Associated Factor; TRIF: TIR-Domain-Containing Adapter-Inducing IFNβ

Introduction

Multiple sclerosis (MS) is an autoimmune disorder characterized by demyelination, chronic inflammation and neuronal damage, mainly induced by activation of auto-reactive immune cells directed against myelin [1]. This abnormal immune activation appears to be triggered by a combination of genetic and environmental factors, including pathogens [2]. Several pathogens, that are likely involved in MS development, have shown to be capable of overstimulating innate immune response, leading to the development of autoreactive T cells, which play important roles in mediating demyelination and axonal damage [3]. More specifically, immune cells, including monocytes, dendritic cells, NK cells, $CD4^+$ and $CD8^+$ T cells, and B cells, are activated and migrate into the central nervous system (CNS), where mediate myelin destruction, axon damage and neuronal apoptosis [4]. These immunological phenomena have been recently shown to be the result of an initial activation of the innate immune response mediated by specific pattern-recognition receptors (PRRs), among which the Toll-like receptor (TLR) family represents a major component [2,4]. Pathogens able to induce innate immune responses TLR-mediated in the CNS seem to be responsible for the development of autoreactive T cells due to antigen spread, in a process known as bystander activation [2,5]. TLRs can be stimulated both by conserved structures of microbial agents, also known as pathogen-associated molecular patterns (PAMPs) [6-9], and/or by host-derived ligands, also known as damage-associated molecular patterns (DAMPs) [10]. Both PAMPs and DAMPs include various compounds, such as proteins, glycosaminoglycans, glycoproteins, RNA, and DNA. TLRs play then a crucial role in danger recognition and immune response induction, and the activation of TLR-mediated pathway modulates inflammatory responses and primes antigen-specific adaptive immunity [4]. In the CNS, TLRs play a key role in the development and regulation of inflammation, neuronal degeneration, and brain trauma, but they also provide to neurogenesis and neurite outgrowth [11]. In addition, they contribute to defence against hamful pathogens, and neurotoxic compounds, such as the "altered self" molecules and apoptotic cells [11]. This important and dual role of TLRs has been

also outlined in several neurodegenerative disorders, including MS [4]. Results from recent studies showed that in this disabling disease the activation of specific TLRs, including TLR2 and TLR4, induces the differentiation of autoreactive T cells and the production of proinflammatory cytokines, so contributing to CNS damage [12]. In contrast, the upregulation of TLR3 has been shown to play a beneficial role through the production of endogenous IFNβ, that is capable of inhibiting the production of harmful cytokines, therefore improving MS severity [13-16]. The evidence for a neuroprotective role played by this receptor is further supported by studies showing that TLR3 activation and the downstream processes are capable of inducing a functional inhibition of neuronal cell death in human cell cultures, and this evidence also suggests additional roles for TLR3-mediated signaling in the CNS [17-18]. The possibility to modulate immune response with specific TLR agonists or antagonists and by inhibiting intracellular proteins involved in the signaling cascade has sparked great interest as alternative approach to treat immunological disorders, including MS [13]. Among the immunotherapeutic compounds TLR-targeting that are in preclinical or clinical studies for other diseases, specific TLR3 agonists could have beneficial effects in MS treatment [16]. According to recent studies the TLR3 agonist Rintatolimod (tradename Ampligen®, also known as poly(I):poly(C_{12}U) (Hemispherx, Biopharma Inc., PA, USA), has shown promising results in the treatment of cancer, HIV, influenza, hepatitis B and C infection, and chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME), an illness that shows remarkable levels of similarity in phenomenology and neuroimmune characteristics with MS [19-22]. Therefore, this drug could represent a safe and effective treatment for MS patients, and could be considered a valuable adjunct to multiple different immunotherapies currently in use or in development[13]. This article reviews the potential therapeutic role of Ampligen® in the context of current and emerging options for the treatment of MS, by mainly focusing on the relevant findings that could promote its use in MS therapy, and discussing about the development, pharmacological properties, safety and tolerability of the new molecular formula.

TLR Signaling Pathways and the Role of Tlr3 in MS

TLRs are classically defined as a large family of PRRs that represent an important component of the innate immune response and form a primary danger signal response to the presence of microbial pathogens detected both internally and externally in the tissues and cells of higher organisms. TLRs are expressed on many types of innate immune cells [23,24], as well as non-immune cells, such as epithelial and endothelial cells [25-30]. In the brain TLRs are constitutively expressed by both infiltrating [4] and resident immune cells [31], including T cells, microglia [32] and astrocytes [33], that represent the key sentinels of innate CNS immunity [34]. TLRs are also expressed by other brain cells not strictly involved in the CNS immunity, such as oligodendrocytes [35], neurons and neuronal progenitor cells [36]. These receptors are characterized by a leucine-rich repeat (LRR) ectodomain, which mediates the ligand binding, a transmembrane region, and a Toll/IL-1 receptor (TIR) endodomain, with a highly conserved structure, required for downstream signal transduction [37]. Among the 10 human TLRs described in the literature, TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are expressed on the cell surface, where they can be stimulated by molecular components, including proteic and lipidic PAMPs and DAMPs. In contrast, TLR3, TLR7/8, and TLR9, that are located in endosomal/lysosomal compartments and endoplasmic reticulum, have evolved to detect dsRNA, ssRNA, and ssDNA respectively [38-42]. The TLR pathways have been extensively reviewed elsewhere [43]. TLR stimulation in the CNS results in the production of cytokines, such as IL-6, IL-1β, and TNF-α, that promote the destruction of blood brain barrier and the recruitment of lymphocytes into sites of inflammation, where they can further promote inflammatory response and induce adaptive autoimmunity [44]. However, there is an increasing body of evidence that TLR signaling can also mediate beneficial effects, since they are also capable of influencing multiple dynamic processes in the developing and adult CNS, including neurogenesis, axonal growth and structural plasticity [45]. Altogether, the functional outcome of TLR-induced activation in the CNS is related to a correct balance between protective and harmful effects [45]. The subcellular distribution of TLRs in CNS is almost similar to others cell types [46], although there are some tissue-specific differences [47]. All TLRs signal through MyD88 downstream adaptor, except TLR3 and a subset of TLR4 signaling events, which are mediated by the exclusive use of the TIR-domain-containing adapter-inducing IFNβ (TRIF) [39]. Analysis of TLR gene expression in the CNS detected high expression of TLR3. More specifically, high levels of TLR3 were shown to be expressed on the endoplasm of astrocytes and a broad range of antigen-processing cells, tissue dendritic cells, mast cells, monocytes, natural killer, and other immune cells [48], in addition to cerebral endotelial cells, neurons, microglia, astrocytes and oligodendrocytes [49-51]. The MyD88-independent and TRIF-dependent signaling pathway TLR3-mediated leads to the production of type I IFNα/β as result of NF-κB, interferon-regulatory factor (IRF)-3, and mitogen-activated protein (MAP) kinase signaling, that is in turn mediated by p38 and c-Jun amino-terminal kinase (JNK) [52]. Type I IFNα/β are best known for their effects in viral infections, but they are also capable of inhibiting inflammatory responses [53,54], and this supports the hypothesis that specific TLR3 agonists could play a beneficial role, IFNβ-mediated, in several inflammatory diseases, as well as MS [4,13,55,56]. In addition, TLR3/TRIF-dependent signaling pathway has been shown to play a further positive roles, being capable both of triggering neuroprotective responses in astrocytes and controlling the growth of axons and neuronal progenitor cells [18]. TLR3 is triggered by dsRNA with a minimum size of at least 50 base pairs and by specific endogenous ligands, as well as the endogenous microtubule regulator stathmin, taken up into the endosome [57]. The dsRNA molecules can originate from the genome of specific viruses, can be also intermediates generated during the viral RNA replication, or dsRNA produced intracellularly by stem-loop forming or with siRNA-aligned mRNAs [58]. The size requirement or discrimination of dsRNA by TLR3 prevent responses to non-microbial sources of dsRNA, including micro or transfer RNA. Above 50 base pairs, binding affinity is a function of size with a progressive enhancement in binding affinity directly correlated to increased lenght in linear non-branched dsRNA [59]. The extracellular domain of TLR3 dimerizes when it binds dsRNA, and this dimeric complex appears to be composed of two dsRNA binding domains located near the N-terminus and the C-terminus domain, and when combined with dsRNA, a sole dsRNA molecule associates two TLR3 molecules through four dsRNA binding sites, to form an “m” shaped dimer” [60-62]. The TIR domain of TLR3 activated associates directly with the TIR domain of TRIF protein, that in turn activates the TNF receptor associated factor (TRAF)3. TRAF3 induces the activation of TANK-binding kinase (TBK)1, which then phosphorylates IRF3, leading to its dimerization and translocation to the nucleus, where it stimulates the transcription of the gene encoding IFNβ [61,63]. Peli1, a member of Pellino family, plays also an important role in the TLR3-TRIF-TBK1 signaling pathway, driving the positive feedback loop [64]. More specifically, Peli1, activated by TBK1

and IκB kinase epsilon (IKKε) by induction of its covalent modification, presumably phosphorylation, induces the interaction of IRF3 with the IFNβ promoter. Peli 1 not only stimulates the initial phase of IFNβ formation, but also drives the positive feedback loop by which the small amounts of IFNβ formed initially amplify IFNβ production. In this pathway, IFNβ stimulates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, leading to STAT1 and STAT2 phosphorylation [65,66], which is followed by the formation of a complex between STAT1/2 heterodimer and IRF9, to form the IFN-stimulated transcription factor (ISGF)3. This multimeric regulator of transcription stimulates IRF7, that in turn can activate the IFNβ promoter either directly or as heterodimeric complex with IRF3, or by stimulating the transcription of other proteins of this positive feedback loop, such as IFNα. Altogether, dsRNA, acting as agonist of TLR3, is capable of inducing the stabilization of IFNβ mRNA by a TRIF-dependent mechanism, so leading to ehnanced IFNβ levels [67] (Figure 1).

Recent evidence also supports the positive role of TLR3/TRIF-mediated pathway in experimental autoimmune encephalomyelitis (EAE) mouse models, that represents the most commonly studied animal of MS [68]. In EAE mice TLR3 stimulation leads to the release of IL-27, that in turn mediates the inhibition of Th17 cells, that are known to play a critical role in the disease [69-71]. Other studies supporting this evidence showed that EAE mice, inoculated intraperitoneally with the synthetic TLR3 agonist poly(I):poly(C) acid, produced high levels of IFNβ, that significantly reduced disease severity [16]. Normal adult human astrocytes have shown to increase the production of anti-inflammatory cytokines such IL-10, and to downregulate proinflammatory cytokines such as IL-12 (p40) and IL-23, in response to TLR3 ligation [48]. Recent studies have also shown that the endogenous TLR3 ligand stathmin, identified in astrocytes, microglia, and neurons from human MS-affected brains, is capable of stimulating the same set of neuroprotective factors induced by poly(I):poly(C) [18].

Development of Ampligen

The invention of Ampligen® poly(I):poly(C_{12}U) [72], relates to a novel synthetic mismatched dsRNA with an high specificity of binding to TLR3, that results in a selective activation of TLR3-mediated immune responses maybe depending on transient expression of multiple genes and subsequent activation of a signal transduction cascade. The genes induced by TLR3 activation include IFNs, 2-5' adenylate synthetase, and protein kinase (p68) [73,74]. As reported by historical overviews, "this synthetic dsRNA molecule, first developed in the 1960's by Merck and Co. to reduce tumor formation, resulting effective but too toxic for use, languished, until Dr. William Carter, working with other Johns Hopkins University researchers in the 1970's, was able to reduce its toxicity" by adding uridylic acid molecules at specific intervals along the dsRNA molecule, so obtaining a particular dsRNA, denoted poly(I):poly(C_{12}U), wherein one of the two polyribonucleotides is polyriboinosinic acid and the other is polyribocytidyl C12, uridylic acid. The new compound, called Ampligen® (for AMPLIfied GENetic activity) was capable of stimulating IFN production like poly (I): poly(C), but resulted less toxic [75]. Its smaller and rugged molecular structure, as measured by physico-chemical techniques, appeared more resistant to molecular unfolding, including denaturation and branching, and this led to an increase in bioactivity, due to higher ability to bind TLR3. Ampligen® was synthesized acting on the hypothesis that nucleotide sequence requirements for beneficial and adverse effects are different. More specifically, it was obtained by preserving the RNA double helical structure, that is required for TLR3 activation and type I IFN production [76], and by modifying the molecular folding with the occasional introduction of uridylate into the poly(C) strand, in order to produce supplexes free of adverse effects and containing specifically-configured regions which are not base paired (i.e. mismatched) at the position of the modifications [72] (Figure 2). This modified dsRNA is capable of activating TLR3-TRIF mediated pathway selectively, without activating other TLRs like TLR4 or RNA helicases like RIG-I or MDA-5, and without inducing proinflammatory responses and a TNFα/TNFR1 dependent toxicity, as seen with the non-selective TLR3 agonist poly (I):poly(C) in the phenomenon known as "cytokine storm" [19,56,77-79].

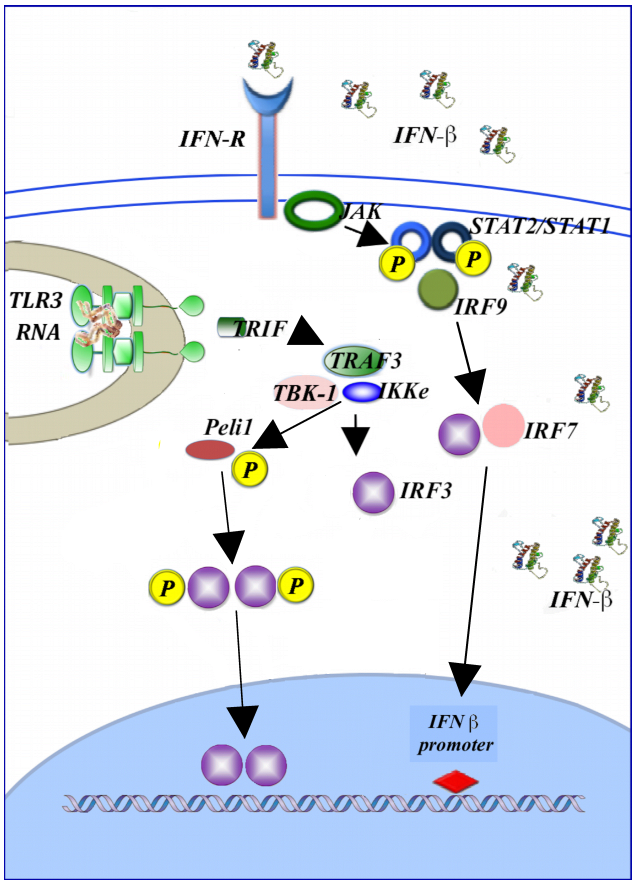

Figure 1: TLR3-TRIF mediated pathway. TLR3 is the only TLR that exclusively signals through the TRIF-dependent pathway. The association of TLR3/TRIF TIR domains activates TRAF3 and the downstream signaling events, represented by IKK/NF-κB pathway, that in turn is regulated by Peli 1. Peli 1, activated by TBK1 and IKKε, induces the IRF3 phosphorylation, dimerization and translocation to the nucleus, so leading to IFNβ gene transcription. IFNβ stimulates the JAK/STAT pathway, that induces the STAT1 and STAT2 phosphorylation, and the association of STAT1/2 with IRF9. This multimeric regulator stimulates IRF7, that in turn activates the IFNβ promoter either directly, or as IRF3/IRF7 heterodimer [67].

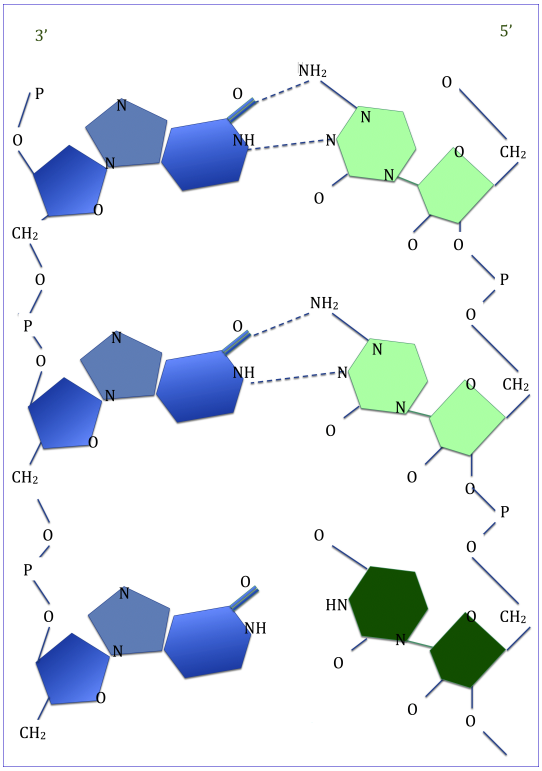

Figure 2: Partial view of poly(I):poly(C_{12}U) partially hybridized strands and the interaction of bases of individual poly(I) and poly(C_{12}U) strands [72].

The mismatched regions accelerate dsRNA hydrolysis, further reducing the toxicity [59,80], while retaining the ability of the nucleotide sequence to induce IFN synthesis, as well as its stability [81]. The hydrolysis is highly dependent on nucleic acid structure, as well as on the presence of nuclease and divalent cations, pH, and temperature. RNA is more susceptible to degradation than DNA, due to the ability of its 2'-OH groups to act as nucleophiles so facilitating hydrolysis [82]. Moreover, poly(I):poly(C_{12}U) was designed to be degraded more rapidly than other dsRNA in a nuclease-containing environment, such as blood and other tissue fluids. In contrast, it is stable in physiological salt buffers at room temperature, but overtime it is degraded in a time- and temperature-dependent pattern [72]. To improve the biological activity of Ampligen®, that resulted lower than expected, a new version of dsRNA with a superior biological and therapeutic profile, called "rugged" dsRNA, was recently obtained by purification (by HPLC or other cromatographic methods) from the Ampligen® mixture, by subjecting the partially hybridized strands of a population of dsRNAs to conditions that denature most dsRNAs, and then selecting dsRNA molecules that remained partially hybridized. Chemically, Ampligen® is a poly(I):poly(C30-35U), wherein C30-35U indicates of 1 U for 30-35 C. The minimal lenght of this rugged dsRNA is about 50 base pairs requiring about 4 to 5 helical turns. This "rugged" dsRNA is resistant to unfolding (i.e. denaturation) of its helical structure and shows a reduced tendency to form branched dsRNA molecules. In addition, it has an improved dsRNA activity as potent and highly selective TLR3 agonist. Immune cells may be susceptible to specific cytokine response patterns activated by rugged dsRNA. Ampligen® can be administered by intravenous infusion, intradermal, subcutaneous, or intramuscular injection, intranasal or intratracheal inhalation, oropharyngeal or sublingual application, or transocularly [72].

Clinical Pharmacology of Ampligen® and Mechanism of Action in CFS/ME

In 2009 Ampligen® was undergoing clinical trials to treat specific immune-mediated diseases, including CFS/ME, multidrug resistant HIV/AIDS, and hepatitis C. Most of preclinical data available so far were recently discussed in the Arthritis Advisory Commitee Meeting: Ampligen® - Treatment of chronic fatigue syndrome (2012). The safety profile and immunomodulatory effects of Ampligen®, were recently shown in the treatment of CFS/ME, a debilitating disorder characterized by disabling fatigue and a combination of flu-like symptoms [21,83-86], and considered by the Centers for Disease Control and Prevention (CDC) as an economically and emotionally devastating illness, whose functional impairment can be equivalent to MS [86]. Since the main hallmark of CFS/ME is represented by the fatigue, that appears not improved by bed rest and may be worsened by physical activity, cardiopulmonary exercise tolerance testing was used as an objective measurement of treatment efficacy and it was accepted as a regulatory standard for drugs ameliorating exertional fatigue [21]. Recent studies have shown that Ampligen® is able to induce objective improvement in exercise tolerance in CFS/ME patients receiving the drug for 40 weeks, related to concomitant medication usage as well as other secondary outcomes, including the drugs commonly used by patients in an attempt to alleviate the symptoms of the disease [21]. The mechanism of action of Ampligen®, that has reached Phase III clinical trials in CFS/ME [21], consists mainly of selectively modulating the activity of an important part of the innate immune defense, represented by RNase L enzymatic pathway that some studies suggest is disrupted in CFS/ME patients [87]. RNase L is an highly regulated latent endoribonuclease induced by INFs and activated by dsRNA. Regulated turnover and processing of ssRNA, such as RNA produced during the viral replication cycle, by RNase is essential for a complete IFN response. This pathway has recently been shown to contribute to innate immunity and appears to protect the CNS against viral-induced demyelination [88]. In the patent titled "Methods of treatment of chronic immune diseases", the inventors, who detailed their findings concerning the treatment of the two chronic immune diseases, represented by CFS/ME and MS, stated also that the CFS/ME diagnosis is accomplished by detecting the presence of RNase L fragments [89]. In addition to ability of Ampligen® to modulate RNase L enzymatic pathway, its agonistic activity towards TLR3, that is involved in the early recognition of pathogens, could also contribute to reduce the rates of opportunistic infections, that are increased in CFS/ME patients [90]. In CFS/ME patients, the beneficial effects of Ampligen® have shown to be related to its binding to the active sites of the TLR3 homodimer, represented by the N-terminals of each TLR3, with the subsequent IFN/RNase L enzyme pathway stimulation, IFN production, and oligoadenylate synthase-RNase L pathway activation [74,91]. RNase L plays a protective role in CNS demyelination viral-induced, since it is capable of both inhibiting the viral genome translation, and inducing apoptosis of infected cells, allowing then the propagation of IFNα/β pathways enhanced by RNA degradation products [92].

Significant overlaps in MS and CFS/ME	
Symptomatic similarities	Disabling fatigue with chronic course, and relapsing-remitting nature
	Muscle symptoms: exercise intolerance, pain/myalgia, fasciculations
	Gastrointestinal dysfunction
	Brain and CNS symptoms: mental fatigue, cognitive dysfunction (problems with short-term memory, concentration and mainaining attention), disequilibrium, word finding disabilities, headaches and migraines
	Abnormalities in immune system function: sore throats, joint pains, intermittent flu-like feelings
	Problems related with control of the autonomic nervous system: palpitations, postural hypotension
	Disease exacerbated by stress and psychological stress
Similarities in immunoinflammatory, oxidative and nitrosative pathways	Oxidative and nitrosative stress (O+NS)
	Depressed levels of antioxidants and antioxidant enzymes (Vitamin E, ubiquinone, gluthatione peroxidase)
	Chronic activation of immunoinflammatory pathways
	Increased levels of proinflammatory cytokines (TNF-α, IL-1, IL-6)
	COX2 and NFkB upregulation
	T regulatory (T_{reg}) dysfunction.
	Coesistence of a T helper (Th)1 and Th2 response
Similarities in autoimmune responses	Oxidative and nitrosative stress (O+NS)
	Depressed levels of antioxidants and antioxidant enzymes (Vitamin E, ubiquinone, gluthatione peroxidase)
	Chronic activation of immunoinflammatory pathways
	Increased levels of proinflammatory cytokines (TNF-α, IL-1, IL-6).
	Detection of autoantibodies (anti-nuclear, anti-cardiolipin, anti-phospholipid, anti-neuronal, anti-muscle)
	IgM responses against palmitic and oleic acids
	Antibodies against byproducts of lipid peroxidation
	Response to B-cell depression treatment (e.g. Rituximab)

Table 1: Main similarities between MS and CFS/ME.

There are various local or systemic routes of administration available for Ampligen®, including enteral, topical, and parenteral. Consequently, permutations for administration, including acqueous solutions, powders, granules, containing different eccipients like binding agents, lubrificants, preservatives, and others are related to its use. In CFS/ME patients, Ampligen® is usually given intravenously twice weekly for an hour at the standard dose of 400 mg, even if the correct dosage is variable, often being too high and the appropriate dose being as low in some patients as 25 mg [93]. While 6 months may be enough to produce a significant response, 12-18 months are recommended depending on the patient's condition. Since the "rugged" dsRNA obtained from Ampligen® mixture represents a purified compound with rugged physico-chemical structure and highly specific biologic activity, its recommended dosage should be lower, being comprised from about 10 mg to about 40 mg, albeit the dose amount and/or frequency may be varied in response to the subject's symptoms, and with the age, conditions, gender, or health status. Recently, an oral form of Ampligen® (Oragen) is being evaluated [93]. No significant side effects are been reported, except for temporary and moderate flu-like symptoms, chills, vasodilatation and shortness of breath, that appeared increased in some patients, although these side effects appeared lower compared with other immunomodulating drugs [21]. Nevertheless, the high cost for the "infusion terapy", that ranges from $1-2,000/month; $15-25,000 a year, depending on dosage, could greatly reduce the probability of using this compound in day-to-day practice. At present, data from clinical trials are not yet considered sufficient by the US Food and Drug Administration (FDA), in order to obtain the approval of Ampligen in CFS/ME therapy. In particular, the FDA claims that CFS/ME does not have clear biomarkers such as blood tests, although cardiopulmonary exercise tolerance testing could be used to define which patients are most likely to respond to the drug [94]. However, studies performed in CFS/ME patients receiving Ampligen®, reported positive and significant effects on increasing exercise tolerance, and these data, together with the safety profile and high specific immunostimulatory properties of this molecule, may represent a strong motivation to assess its potential role in MS treatment [95]. The only question about Ampligen®, concerning the study showing that TLR3 can mediate West Nilus virus entry into the brain, causing lethal encephalitis [96], has been overcome in more recent studies that, in contrast, showed

that TLR3 can play a protective role also against West Nile virus, by partially restricting its replication in neurons [97]. Further studies concerning the manipulation of innate immune response as antiviral therapy showed that the use of Ampligen prevents Venezuelan encephalitis equine, despite it has not yet been evaluated in CNS infections [98]. In addition, independent researchers have demonstrated the antiviral activity of Ampligen against flaviviruses, including West Nile, Dengue fever virus and Japanese encephalitis virus as well as virus classes associated with bioterrorism [99].

Ampligen® in MS Treatment

MS is considered a form of encephalomyelitis disseminata showing remarkable levels of similarity with CFS/ME. Indeed, both disorders, classified as diseases of the CNS by the World Health Organization, show remarkable phenomenological and neuroimmune overlaps [100] (Table 1). Other remarkable levels of similarity concern "the findings produced by neuroimaging techniques that appear quite similar in both illnesses and show decreased cerebral blood flow, atrophy, gray matter reduction, whit matter hyperintensities, increased cerebral lactate and choline signaling, and lowered acetyl-aspartate levels" [101]. The neuroimmune similarities between MS and CFS/ME, are mainly based on shared immunoinflammatory oxidative and nitrosative stress, autoimmune and mitochondrial pathways. However, the two diseases show also remarkable differences, since MS is considered an immunologically mediated disease resulting in the demyelination of brain and spinal cord white matter disease, characterized by multifocal lesions, the MS plaques, which consist of a well-demarcated hypocellular area characterized by the loss of myelin, the formation of astrocytic scars, and the mononuclear cell infiltrates concentrated in perivascular spaces, where activated mononuclear cells, including lymphocytes, microglia, and macrophages destroy myelin [101]. Biochemical analysis of the spinal fluid provides evidence of the inflammatory response in the CNS. The two well-established spinal fluid markers used for the diagnosis of MS are represented by "oligoclonal bands and IgG index" something that is not found in CFS. Altogether, both MS and CFS/ME remain unsolved disorders with multiple symptoms and no single causative factor, with a potential to be described as inflammatory diseases of the CNS disorder yet to be identified [102,103].

It has been recently reported that innate immune phaenomena concur in activation of autoimmune and inflammatory responses, as also shown by the increased expression of TLRs observed whitin the CNS during MS [13,104,105].

TLRs have shown to play a critical role in modulating cytokine and chemochine secretion in MS and its animal models and, more specifically, whereas MyD88-dependent pathways have shown to contribute to MS and EAE pathology [106], MyD88-independent pathways appear to mitigate disease severity. In particular, TLR3 agonists might favour the inhibition of signaling responsible for autoimmune and inflammatory responses in MS and EAE [107]. In addition, TLR3 stimulation leads to endogenous induction of IFNβ, that has shown to prevent inflammation and demyelination and, unlike the exogenous IFN used in MS therapy, does not induce antibodies anti-IFN [13,105]. IFNβ has also shown to increase the expression of CD73 on endothelial cells. CD73 ectoenzyme on CNS produces adenosine from AMP and adenosine possess both anti-inflammatory and neuroprotective activity [108].

At light of this, Ampligen® could offer promising results for this autoimmune disease that at present has no cure. Despite the pharmacological armamentarium for MS has been significantly expanded in the last years and new effective therapies have been proposed to modify the disease course, the most common treatments usually focus on strategies to treat MS attaks, manage symptoms and reduce the progress of the disease. As inflammation is the main factor contributing to axonal pathology, aggressive anti-inflammatory treatment is used to contribute to reducing the lesions and preventing axonal injury. A preliminary trial of poly ICLC in chronic progressive MS was conducted in 1985 [109,110]. In these studies, performed before the discovery of TLR-mediated mechanisms, MS patients were treated in an open preliminary trial of poly ICLC to induce endogenous type I IFN; such studies were limited by poly-IC toxicity. Another more recent study, that evidenced the beneficial role of endogenous production of IFNβ TLR3-mediated, showed that myeloid heme oxygenase-1 was required for the regulation of IFNβ production after TLR3 stimulation [111]. This study confirmed the previous observations of Touil et al. in SJL/J mice with relapsing EAE and C57BL/6 mice with chronic EAE [16]. IFNβ1b (Betaseron) was the first disease-modifying therapy approved by US FDA and also recognized as Betaferon by European Medicines Agency (EMA), in 1995. To date, a number of approved disease-modifying treatments have been shown to slow the progression of MS. These drugs may be included into two main categories: treatments that allow to improve symptom management, and treatments capable of slowing the progression of the disease. Currently, there are at least 8 different products approved by the FDA as disease modifying treatments for MS [112]. These include IFNβ1a compounds, such as Avonex® and Rebif®, IFNβ1b, such as Betaferon®/Extavia, the mimetic compound glatiramer acetate (Copaxone®), containing a group of aminoacids that look like myelin, the chemotherapic agent mitoxantrone (Novantrone®), acting by suppressing the activity of T and B cells, and the humanized monoclonal antiboby natalizumab (Tysabri), able to bind to specific receptors on immune cells that allow it to enter the brain and the spinal cord. Nevertheless, none of these compounds have shown to be effective against this autoimmune disease. In addition, the most commonly used first-line agent, represented by IFNβ, in a range of treated people comprised between 2 and 45%, can induce neutralizing antibodies that significantly reduce its biological activity, so leading to loss of clinical effects [113]. Many different innovative compounds are been approved for the safety and efficacy profiles in MS therapy, including specific immune-targeting humanized monoclonal antibodies such as alemtuzumab, immunomodulators, such as dimethyl fumarate, immunosoppressive compounds such as teriflunomide, but we are still far from finding a cure for MS. In addition, more drugs require long-term and regular administration via parenteral or subcutaneous, and this results unconfortable and inconvenient for the patients affected by this chronic disorder. Therefore, the most important factor in the future development of MS drugs is represented by the achievement of effective medications, possibly orally administrable, together with additional therapies for halting neurodegeneration, promoting remyelination and neuronal repair [114]. At light of this, Ampligen®, and more in particular, the new version of "rugged" molecule, can show promise as a relatively safe and efficacious drug in the treatment of MS, both as monotherapy or as an add-on agent to first-line disease-modifying agents (DMAs). In addition, the possibility of oral administration makes it an attractive option in addition to the available therapeutic armamentarium to manage relapsing MS. Among the adverse drug events that could affect MS patients taking

Ampligen®, already reported in some clinical trials, in addition to flu-like syndrome, chills, vasodilatation and dyspnea, that altogether are side effects not particularly severe, a systemic inflammatory response dose-dependent TLR3-induced could contribute to the disease progression. However, despite toxicological analysis demonstrated the occurrence of systemic dose-dependent inflammatory cytokine responses in rats, more recent studies showed that primates are resilient to inflammatory cytokine toxicity induced by non-MyD88-dependent, TRIF-mediated activation of TLR3 [115,116], and these data appear consistent with differential TLR3 nuclear transcriptional activities, with the NF-κB pathway in inflammatory cytokine production in primates playing a relatively minor role compared with the IRF-3/IRF-7 nuclear IFN inducers [115].

Conclusions and Future Directions

Although there are many effective ways of managing MS, at present there is no cure for this disease. The modest effects in stabilizing disease, due to induction of anti-drug neutralizing antibodies against the first-line immunotherapeutic compound, represented by IFNβ, have reduced the efficacy of this important drug, similarly to many other biopharmaceuticals used in other chronic diseases (e.g. insulin, factor VIII). Nevertheless, the efficacy of IFNβ in order to counteract inflammatory processes, always reduced by host response, should encourage implementation of alternative approaches capable of triggering endogenous IFNβ production. In this case, Ampligen® could be effectiveness if used as an add-on agent to DMAs, being potentially able to induce similar or greater effects, when compared to exogenous IFNβ administration, togheter with the added convenience of oral administration. More specifically, Ampligen® might be considered for use in MS patients with suboptimal responses to IFNβ and who are reluctant or unable to use other approved DMAs. Future results from ongoing large-scale phase III clinical trials performed on CFS/ME patients will provide additional information on its effectiveness and tolerability, although its high cost could reduce the probability that this treatment can be incorporated into daily practice. Overall, the future appears bright for MS patients, since the modulation of innate immune response with this small molecule TLR3-targeting might represent a promising approach. The future will likely see other specific immunomodulators being utilized, perhaps along with other drugs able to prevent axonal degeneration and stimulate repair of damaged axon. Another winning point of TLR-targeting drugs is that they have fewer side effects and lower or no toxicity, compared with drugs commonly used in MS treatment, and this represents an important feature, since MS is a chronic disease that requires long-term treatments. Ongoing studies of innate immune pathways involved in autoimmunity and neurodegeneration could reveal new biological insights. In view of the likely overall complexity of TLR functions, additional advances will arise through the use of genomic, proteomic and other systems biology approaches. These approaches will allow to identify processes and pathways amenable to therapeutic manipulation, in order to ehnance innate immune responses and counteract autoimmune and neuroinflammatory processes, improving overall clinical outcomes in MS patients.

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