



The MSMV hypothesis: Measles virus and multiple sclerosis, etiology and treatment

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Summary Multiple sclerosis (MS) is a progressive disease characterised by periods of quiescence and exacerbation. It is found more often in northern and southern climates, rather than those closer to the equator, where it is especially rare, and, therefore, cannot be considered as an autoimmune disease. We present the MSMV Hypothesis, involving novel ideas which encompass an understanding of the blood brain barrier (BBB) function, the lymphocyte population, together with the viral presence in the CNS of what we are calling the multiple sclerosis measles virus (MSMV) that is the immediate cause of MS, and which exhibits a similar immunologic response of the systemic virus. We assume that the geographical distribution of MS is related to MSMV's sensitivity to ultraviolet light and that it is feasible to assume a viral etiology for MS based on this. The methodology employed is eclectic and grounded on several differing approaches: involved are the meta-analyses of two comprehensive studies on the effects of azathioprine in the treatment of a large number of MS patients undertaken since the early 1990s, a pioneering pilot study that examined the effects of azathioprine treatment on a smaller set of patients in the late 1960s; and, finally, we also outline the results of several experiments in cell culture on two MV strains using a new drug lead that has been shown to effectively stave off the progression of MS by interfering with the normal replication process of the MSMV. In the latter case, strain Edmonston (MV-E) was employed, along with strain Halle (MV-H), which was obtained from a lymph node of a patient with subacute sclerosing panencephalitis (SSPE), which mimics various aspects of the pathology of neurological diseases, including demyelination. An analogue of a metabolite of azathioprine (ESP) was evaluated for antiviral activity against these two viral strains. The results proved positive for the MV-H infected cells as syncytia formation was reduced in a dose-dependent manner, and under protocols which avoided toxic effects, following ESP treatment ranging from 66% with 1 µg/ml and to 25% with 0.1 µg/ml. Since ESP is an analogue of the active metabolite of azathioprine, which exhibits positive outcomes when administered to MS patients, we submit that this metabolite is acting on MSMV, in a similar fashion to the action of ESP on MV-H.

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Introduction

Multiple sclerosis (MS) is a progressive disease characterised by periods of quiescence and exacerbation. It is found more often in northern and southern climates, rather than those closer to the equator, where it is especially rare. Although much

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of the scientific literature points to MS as an autoimmune disease, the clinical, laboratory and pathological findings support an infectious agent as the fundamental cause [1]. The discussion encompasses the measles virus (MV) in relation to the lymphocyte population and the antibody-antigen responses in the confined closed organ system of the CNS, which can only be entered through the blood brain barrier (BBB).

A simple search on the PubMed database reveals close to five hundred articles published between 1962 and 2008 on MS that refer in one way or another to MV. Many of these publications propose an autoimmune etiology. Significantly, Chaudhuri has argued convincingly against an autoimmune theory, and also puts forward the notion of a genetically determined disorder characterised by metabolically dependent neurodegeneration, indentifying as possible disease influences regulatory factors such as sunlight exposure and vitamin D metabolism which would be governed by seasonal fluctuations and put certain individuals who might carry the MS susceptibility gene or genes in the 'at-risk' category [2].

Here, however, we present the MSMV Hypothesis, involving novel ideas which encompass an understanding of the BBB function, the lymphocyte population, together with the viral presence in the CNS of what we are calling the multiple sclerosis measles virus (MSMV). We also outline the results of several experiments in cell culture on two MV strains using a new drug lead that will effectively stave off the progression of MS by interfering with the normal replication process of the MSMV.

This line of approach was first motivated by the results of a pilot study of seven patients undertaken in the late 1960s with confirmed diagnosis of MS, who were treated with azathioprine [3]. The results of that undertaking revealed the stabilization of MS symptoms, and no further exacerbations of the disease. In fact, with physical therapy, some patients showed consistent improvement over several years.

A second motivation presented itself early in 2008 when we commissioned a preliminary cell culture study in which a line of human cervical carcinoma cells (HeLa) were used in conjunction with two strains of MV: (i) strain Edmonston (MV-E) was obtained from the serum of a patient with MV; and (ii) strain Halle (MV-H) was obtained from a lymph node of a patient with subacute sclerosing panencephalitis (SSPE), which mimics various aspects of the pathology of CNS diseases, including demyelination [4]. An analogue of a key metabolite of azathioprine (we call it ESP) was evaluated for antiviral activity against these two viral strains. The results proved positive for the MV-H infected cells as syncy-

tia formation was reduced in a dose-dependent manner, and under protocols which avoided toxic effects, following ESP treatment ranging from 66% with 1 µg/ml to 25% with 0.1 µg/ml.

Medical hypothesis and principal assumptions

We propose the following: there exists a specific multiple sclerosis measles virus (MSMV) that is the immediate cause of MS, and which exhibits a similar immunologic response of the systemic virus – the MSMV Hypothesis. As a corollary, any new drug developed to treat MS needs to have the capacity to target the MSMV directly.

In the context of this hypothesis we base our analysis in the present paper on four principal assumptions:

- (i) A singular approach to the identification of MS.
- (ii) The crucial role of the BBB as blocker to entry or exit into or out of the CSF.
- (iii) Favourable evidence for MV as causative agent of MS.
- (iv) The geographical distribution of MS as being related to MV's sensitivity to ultraviolet light.

Identification of MS

We define the disease entity of MS as being characterised by acute exacerbations of neurological dysfunction with inter-current periods of improvement and quiescence. The lesions underlying this sequence of events are multi-focal plaques of demyelination disseminated irregularly throughout the cerebral spinal axis, and occur in successive crops over a period of years. Demyelination begins at the peripheral myelin sheath, and with recurring episodes spreads inwardly towards the axis cylinder. In fresh lesions lymphocytic infiltration is associated with acute myelin edema; whereas in older foci a secondary gliosis develops.

Here, we propose that the neurologic diseases that do not follow this characterization cannot be classified as MS, such as the one time visual problems lasting a few days or the sudden onset of acute neurological disease with rapid deterioration to the end point of death. In this context our position differs from the *status quo*, as reflected in the criteria put forward by Poser or MacDonald for PPMS (primary progressive MS), although we would accept the criteria that distinguish the RRMS category (relapsing remitting MS) [5–6].

Blood brain barrier

The CNS is a confined closed organ system totally separated from the rest of the body with controlled entry through the BBB. Lymphocytes are not normal in the CSF. When there is an inflammatory disease in the CNS damage occurs to the endothelium of the capillaries and through this damaged area the lymphocytes enter into the CSF. This would be one probable route through the BBB for cellular bodies such as lymphocytes. Albrecht et al. who have examined MV intra-BBB antibody production in MS patients have reported on a variety of viruses including vaccinia, rubella, varicella, and herpes virus simplex, state that: measles virus was the only agent studied against which there was a significantly higher incidence of CNS antibody production in MS patients than in controls [7]. Furthermore, as regards azathioprine treatment, although generally considered as immunosuppressive, our position is that this drug is antiviral in the present context, and that its principal metabolite passes through the BBB.

Favourable evidence for MV as causative agent of MS

Close to two hundred papers, revealed by a PubMed search, include both MS and MV in their titles. The majority of these interpret MS as an autoimmune disease, but many also accept the fact that MS is in part an inflammatory response to environmental agents such as viruses, in these cases specifically MV. Several studies in particular have identified MV as being in the fore front of the etiologic picture. The evidence for this position comes from several considerations in MS patients: (i) an association between MV and demyelination, or oligodendrocyte damage [8–9]; (ii) positive CSF titers for MV, accompanied by associated lymphocytes in the CSF [10–12]; (iii) the presence of significant numbers of antibodies to MV (primarily IgG) in the CSF as compared to blood serum [7]; (iv) certain laboratory tests that reveal a connection between MV and MS [13]; (v) results from researchers who have found an association between MS and the

age of onset of the initial MV infection: in these cases while the mean age of measles in MS was seven, in controls infection occurred at age four [14–15]; (vi) the fact that lymphocytes of MS patients adhere to measles-infected epithelial cells in substantially greater numbers than lymphocytes from healthy volunteers or patients with other neurologic diseases, and that lymphocyte adherence values increase during clinical exacerbations [16]; and (vii) various reports on electron microscopic examination of inclusion bodies that revealed microtubular structures of MV nucleocapsids, and other reports that MV antigens, especially N, can be identified by immunocytochemistry [17].

Many of these studies, however, also point out that MV may be only one of a collage of possible viral candidates implicated in the etiology of MS. Although there may be some examples of a single viral causative agent for MS posited on a hypothetical level [18], no one researcher or group ever take a conclusive stand on a specific virus as principal causal factor in the etiology of MS. However, our position is more aggressive, and we propose that the etiology of MS is directly related to the action of what we are calling MSMV in the CSF, leading to classical RRMS symptoms. Furthermore, we propose that the early diagnosis and treatment of MS can be accomplished with the first suggestion of clinical symptoms by doing a lumbar puncture and the measles viral titer on the CSF.

The geographical distribution of MS is related to MV's sensitivity to UV light

It is feasible to assume a viral etiology for MS based on the geographical distribution of this disease in relation to the intensity of ultra violet light around the globe.

First, Ohara has reported that the geographical distribution of MS is very specific, and that for every 100,000 people, the cases of MS surface along the lines shown in Table 1.

Significantly, in South Africa, the prevalence rate of MS among immigrants who were born in Northern Europe was found to be 49 per 100,000 in comparison with the prevalence of 11 per

Table 1 The geographical distribution of MS as of the end of the 20th century

Geographical region			MS cases per 100,000
Northern United States	Southern Canada	Northern Europe	30–80
Southern United States		Southern Europe	6–20
Asia	Central Africa	Caribbean Coasts	1–4
	South Africa		11

100,000 among native-born English-speaking white South Africans noted in Table 1. Ohara goes on to point out that the risk of developing MS was reduced to less than one third of that expected risk among those who immigrated under the age of 15 or 16, suggesting that exposure before puberty to some environmental factor such as an infectious agent could play an important role in the pathogenesis of MS [11].

Second, it has been shown that exposure to sunlight has a protective effect in patients with MS [19]. There is also some evidence to suggest that UV light diminishes the spread of the measles disease amongst young children in schools [20], as well as stimulating the proliferation of lymphocytes [21].

We therefore, posit that this is why the incidence of MS is much lower in regions closer to the equator, where MSMV would be exposed to a greater intensity of ultraviolet light; and why we witness a greater incidence of MS in northern and southern regions of the globe where the UV intensity is not as great, and, therefore, would allow for a more liberal spread of MSMV.

Finally, the epidemiological data suggest an infectious agent in the pathogenesis of MS and this interpretation outweighs by far arguments in favour of the autoimmune theory of MS. As Chaudhuri has persuasively pointed out: the age effect on migration and risk of acquiring MS and the geographic variations in disease prevalence cannot be explained by the autoimmune hypothesis [2].

Methods and results

We base the first part of this section on the meta-analyses of two comprehensive reports on the effects of azathioprine in the treatment of MS patients: the first was undertaken in the early 1990s; the results of the second were published the latter part of 2007 [22–23]. We will then elaborate on the pioneering pilot study mentioned previously, and conclude with the cell culture study undertaken in the early part of 2008 employing a new drug lead (ESP) on MV.

Effects of azathioprine: two meta-analyses (1991, 2007)

First, Yudkin et al. took a close look at the efficacy of azathioprine in the treatment of multiple sclerosis in 793 patients who participated in randomised, controlled trials (5 double-blind, and two single). Although they found that the Kurtzke disability sta-

tus scores did not differ much for the treated vs. untreated groups, the chance of relapse in the first, second, or third year of treatment was significantly lower in the azathioprine-treated groups vs. the controls. In general their analysis identified positive clinical outcomes as regards reduction of MS relapses, but they also characterised certain azathioprine induced side-effects, which included leucopenia, anorexia, diarrhoea and vomiting, abnormal liver function, skin rashes, as well as abdominal pain and other gastrointestinal disturbances. Moreover, the risk of cancer loomed large in several of the studies analysed by Yudkin et al., however, they pointed out that there is little risk of cancer during the first five years of treatment with azathioprine.

Second, the meta-analysis of Casetta et al. was rigorous and comprehensive. They studied the results of clinical studies totaling 698 patients in all. They undertook a sophisticated meta-analysis of randomised controlled trials lasting at least one year and comparing azathioprine and placebo groups for patients with MS. Five major studies were analyzed, both double-blind and single blind. Adverse events were also scrutinised from non-randomised and observational studies.

The conclusions of this second meta-analysis are more positive than the Yudkin analysis. Taking into account the disability progression and the number of relapses, the authors found evidence that azathioprine reduced the number of patients who had relapses during the first year of treatment, and at two and three years' follow-up as well. Azathioprine treatment also reduced the number of patients who progressed during the first two to three years of therapy.

Adverse effects such as gastrointestinal disturbances, bone marrow suppression and hepatic toxicity occurred frequently, but they were known and anticipated, thus quite easily managed; withdrawals due to adverse events were few, and mainly due to gastrointestinal intolerance. On the question of cancer, this study group concluded that treatment with azathioprine would have to endure for over ten years with cumulative doses above 600 grams before a possible risk of cancer would manifest itself.

Azathioprine: the MS pilot study (1969)

We emphasize that the pilot study undertaken in the late 1960s of seven patients with confirmed diagnosis of MS was the first of its kind in relation to the use of azathioprine in the treatment of MS. Results of that time, although based on a small

number of patients – six of these were reported on [3] and one was monitored closely over the years – seem to parallel the results of the more elaborate studies undertaken over the past few decades, and reviewed in detail by the Yudkin and Casetta groups.

To summarize, the pilot study included patients who had a minimum of two years of cyclic exacerbations and were in the quiescent phase of disease. Each patient had an increased gamma globulin and an elevated measles titer in the CSF. The measles titer in the serum was normal to absent with no correlation to the CSF titer. All seven patients were treated with azathioprine. Treatment consisted of a tablet (1.4–2 mg/kg/d) taken orally in a divided dose twice a day for six months. These dosage levels were close to one-half of those employed in the studies analyzed by Yudkin and Casetta, which were typically 2.2–3 mg/kg/d, but sometimes as high as 4.4 mg/kg/d, administered on a daily basis.

Finally, the pilot study results were positive. Patients exhibited minor improvement in visual, motor, and sensory responses at four weeks. The maximum improvement occurred by the eighth week. The visual showed the greatest improvement; the motor and sensory showed some improvement. Moreover, patients exhibited visual, motor, and sensory improvement after treatment was started in the quiescent phase of disease. Some additional motor improvement occurred in the months following with physical therapy. This endeavor had revealed no exacerbations of disease and some patients were out to several years with no appreciable signs of the reappearance of symptoms.

Cell culture experiments (2008)

The following protocols were employed in the cell culture experiments:

1. *Drug Lead (ESP) Preparation*: stock solutions of the drug lead compound were prepared in dimethylsulfoxide at 10 mg/ml followed by sonication for 20 min at room temperature (RT) to ensure complete solubilization. Working concentrations were subsequently prepared in DMSO from the stock solution at concentrations that ensured a final vehicle concentration of 0.1% (v/v) and stored in 50 μ l aliquots at -20°C until used.
2. *Cells and cell culture*: HeLa cells (human cervical carcinoma) were used for these studies. Cells were maintained in DMEM medium supplemented with 10% fetal calf serum (FCS) and antibiotics in a humidified, 5% CO_2 envi-

ronment. Cells were passaged as required by trypsinization.

3. *Viruses*: two strains of MV were evaluated in this study: (i) strain Edmonston (MV-E), the prototypical lab-adapted strain originally derived from the blood of a human patient in the acute phase of a typical infection, and (ii) strain Halle (MV-H), a neurovirulent strain derived from a lymph node biopsy of a human patient with subacute sclerosing panencephalitis (SSPE).
4. *Infection conditions*: cells (4×10^4 cells/well) were seeded in 24-well culture plates and pre-treated with ESP for 24 h prior to infection. Cells were incubated with 100 μ l of MV at the indicated MOI for 1 h at 37°C before conditioned media containing the test compound and 5% FCS was replaced for the assay duration. MV replication was evaluated daily by microscopic analysis until cells were stained with 0.1% cresyl violet dye and scored for cytopathic effects.
5. *Toxicity screening*: ESP was evaluated using MV-E and MV-H under a protocol modified in an attempt to avoid the effects of toxicity. The viruses were investigated at three MOIs (1.0, 0.1, 0.01) using ESP at 5, 2.5, 1, 0.5, 0.25, 0.1 and 0 $\mu\text{g/ml}$.
6. The results of these experiments revealed that ESP did not significantly decrease syncytia formation in cells infected with MV-E compared to untreated controls. In contrast, syncytia formation in MV-H infected cells was reduced in a dose-dependent manner following ESP treatment ranging from 25% with 0.1 $\mu\text{g/ml}$ to a 66% reduction with 1 $\mu\text{g/ml}$, as shown in Fig. 1.

Discussion

Measles virus is a negative stranded RNA, large at 120–250 nm in diameter and consists of six major polypeptides [24]. Thus, it differs significantly from German measles (Rubella) which is a single strand RNA of positive polarity structured of membrane glycoproteins and nucleocapsid protein. Rubella encephalopathy is not associated with demyelination and, therefore, is not a consideration for the etiology of MS [25].

In determining the etiology of MS it is necessary to consider that under normal circumstances the BBB blocks entry of MV, as well as lymphocytes, into the CSF. Furthermore, MV and lymphocytes cannot leave the CSF through the BBB. However,

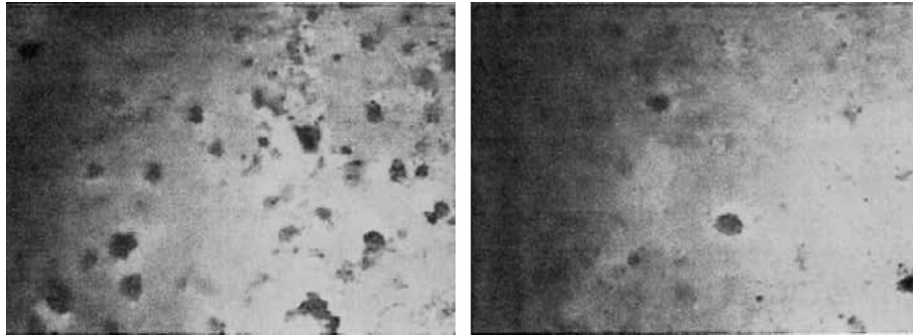


Figure 1 Representative images of HeLa cells infected with MV in the presence or absence of ESP at 10× magnification. MV-H infected cells are shown on the left with MOI = 0.1. Inhibition results due to the action of ESP on syncytia formation appear on the right as plaques at the same magnification and MOI, revealing a 66% reduction with an ESP dose level of 1 µg/ml.

in MS, we propose that a small quantity of the MSMV enters the CSF through the olfactory nerve pathway at the time of onset of the measles disease. Indeed, many of the infectious bacteria and viruses cannot pass through the BBB but obtain entry into the CSF through the olfactory nerve route. The olfactory nerve is derived from ectoderm, and it is non-myelinated. It is the only nerve which is constantly regenerating from its own stem cells. Each branch has a sheath of dura mater and pia arachnoid that passes through the cribriform plate [26], giving direct access to bacteria and viruses to enter the CSF.

With the initial entry of the virus into the CSF, a very minimal viral myelin attack occurs, which is not evidenced clinically. This minor viral inflammatory attack injures the endothelium of the capillaries, which allows the release of lymphocytes into the CSF, and then the production of antibodies (gamma globulins) commences. This immune response quiets the small lesions in the nerves, and in turn the endothelium heals. Since the lymphocytes cannot exit through the BBB they enter a quiescent phase and the lymphocytes maintain this state until the life span of the lymphocyte runs out [27]. Let us call this process or mechanism the 'BBB effect'. With the lymphocytes now absent in the CSF, the latent measles virus starts the next exacerbation. As the patient ages, and more and more attacks occur, the clinical neurologic symptoms become evident.

In the quiescent phase of MS, the lymphocytes in the CSF are concentrated in the G_0 phase of the cell cycle – this is the early G_1 phase or sometimes termed the G_0 or resting phase [28]. In the active phase of the disease the concentration is in the S phase, indicating continuous ongoing immune activity.

With the initial systemic measles infection, the T lymphocyte moves from its G_0 to the S phase,

and starts the process of immunological response. As the immunologic commander, it directs the B lymphocytes to produce immunoglobulin to control the systemic infection. When the infection subsides, the lymphocytes return to G_0 . Now with the exacerbation of the MS disease, the G_0 phase T lymphocytes enter the CSF through the damaged capillary which is infected and inflamed. The T lymphocytes responding to the infection move to the S phase. With the recognition of the measles virus in memory, the T lymphocytes respond to the infection and recruit B lymphocytes that have arrived through the same port of entry to start the production of IgG and IgA, which now produces the quiescent phase of disease. In one study it was found that lymphocytes from the CSF of an MS patient synthesised IgG and IgA but not IgM. The kappa and lambda determinants of the newly formed IgG were the same relative amounts as the IgG in the CSF. Blood lymphocytes of the same patient synthesised in vitro a completely different IgG electrophoretic pattern. The oligoclonal IgG of MS patients CSF cells synthesize intrathecally and are antigenically stimulated within the CNS in vivo. The synthesis is depressed during steroid therapy but the oligoclonal bands are not eradicated [29–30].

The electrophoretically separated CSF of normal subjects shows a homogenous appearance but in MS and other neurological diseases with known infectious etiology the IgG has a restricted heterogeneity – the oligoclonal bands of antibody are diffuse and suggest the presence of immuno dominant antigens. The result of antibody and antigen reaction in soluble immune complex formation is the result of continued antigen stimulation. The elevated IgG concentration in the CSF of MS patients is consistent with an ongoing infectious disease process [29]. Abnormal gamma globulins are found in the CSF of MS patients [31]. Increased

immunoglobulin IgG and intrathecally produced oligoclonal bands are characteristic of inflammatory CNS disease and are directed at the cause of the disease. Lymphocytes in the CSF and the less abundant plasma cells are the sources of oligoclonal bands directed against MV [32].

MV attaches to and penetrates a target cell, the virus genome is then discharged into the cytoplasm, crossing through the cytoplasm to enter the nucleus where the measles virus replication takes place. MV returns to the cytoplasm and attaches to the inner cell wall and budding takes place, followed by separation from the cell [24]. In the CSF the virus would be free, encapsulated in cytoplasm, with a cell wall which the lymphocyte recognizes as self and, therefore, without an inflammatory component will not react to it. However, when inflammation ensues, the lymphocyte will attack the cell infected with the virus as a normal response to the inflammation.

During viral replication in the host cell, a large amount of glycoprotein is inserted into the cell membrane which causes it to develop the capacity to haemadsorb, promoting fusion with adjacent cells. Multinucleated giant cells are formed, which is pathognomic for regular MV infection. The fusion kills the cells more rapidly than the virus and, if prevented, the cells survive longer and produce an increased yield of viruses. The antibody titers to MV increase in MS patients the longer the disease has been present and with exacerbations.

Conclusion

In summary, we suggest that the description of the immunologic response to the systemic virus reviewed above is analogous to the response in the CNS to MSMV, and this together with the notion of MSMV as the immediate cause of MS make up the principal components of the MSMV Hypothesis.

Moreover, the cell culture experiments have shown that the action of our drug lead, ESP, on measles virus strain MV-H thwarts proliferation of the virus, and that this action is both viral titer and concentration dependent, and manifested under a protocol which avoids the effects of toxicity. Since ESP is an analogue of the active metabolite of azathioprine, which exhibits positive outcomes when administered to MS patients, we propose that this metabolite is acting on MSMV, in a similar fashion to the action of ESP on MV-H.

In closing, we submit that any new drug developed to treat MS needs to have the capacity to target the MSMV directly, and the litmus test of this will be ESP human trials, which we aim to undertake in the near future.

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