Jundishapur J Microbiol. 2016 July; 9(7):e36582. doi: 10.5812/jjm.36582.

Published online 2016 July 3. **Research Article**

Prevalence of Cytomegalovirus in Patients With Multiple Sclerosis: A Case-Control Study in Northern Iran

Saeideh Najafi,[1,*] Masood Ghane,[1] Vahdat Poortahmasebi,[2] Seyed Mohammad Jazayeri,[2] and Shahrokh Yousefzadeh-Chabok[3]

[1]Department of Microbiology, Tonekabon Branch, Islamic Azad University, Tonekabon, Mazandaran, IR Iran
[2]Hepatitis B Molecular Laboratory, Department of Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, IR Iran
[3]Department of Neurosurgery, Guilan University of Medical Sciences, Rasht, Guilan, IR Iran

[*]*Corresponding author*: Saeideh Najafi, Department of Microbiology, Tonekabon Branch, Islamic Azad University, Tonekabon, Mazandaran, IR Iran. Tel/Fax: +98-1924272294, E-mail: saeedeh.najafi@yahoo.com

Received 2016 January 24; **Revised** 2016 May 25; **Accepted** 2016 June 18.

Abstract

Background: Multiple sclerosis (MS) is a chronic debilitating disease known as one of the most common neurological dysfunctions in young adults. Recent studies suggest that infections with herpesviruses play a critical role in the pathogenesis of MS.
Objectives: The present investigation aimed to detect the presence of cytomegalovirus (CMV) in patients with MS using polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) methods.
Patients and Methods: Plasma and peripheral blood mononuclear cells (PBMCs) were collected from MS patients (n = 82) and from blood donors as control group (n = 89). They were tested for the presence of CMV antibodies and DNA by ELISA and PCR, respectively.
Results: Anti-CMV was positive in 65 (79.3%) and 69 (77.5%) of the MS patients and healthy subjects, respectively (P= 0.853). Similarly, 23 (28%) and 2 (2.2%) patients were positive for CMV DNA among the MS and control groups, respectively. Statistical analysis showed that the frequency of CMV DNA in the MS patients was significantly higher than in the healthy controls ($P < 0.001$).
Conclusions: The results of this study showed a possible association between CMV infection and MS. Further experimental and epidemiological studies using case-control approaches are needed to confirm this association.

Keywords: Multiple Sclerosis, Cytomegalovirus, Autoimmune Disease

1. Background

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS) that affects approximately 400,000 individuals in the USA. Multiple sclerosis is characterized by the formation of lesions, inflammation, and the destruction of myelin sheaths of neurons (1, 2). The clinical courses of this disease are relapsing remitting MS (RRMS), secondary progressive MS (SPMS), primary progressive MS (PPMS), and progressive relapsing MS (PRMS). Relapsing remitting multiple sclerosis (approximately 85% of clinical cases) and PPMS (approximately 15% of clinical cases) are the two main types (3). Of the former, approximately 87% of patients experience acute attacks (relapses) followed by partial or full recovery (remission) (4). A growing body of literature has indicated that MS is an autoimmune and inflammatory disease; however, its underlying cause is still unclear. Many recent studies conducted on children and adolescents with MS suggest that viral infections play a critical role in its etiology (5, 6).

Cytomegalovirus (CMV) is a human pathogenic β-herpes viral agent in the most common congenital infections. Many genetically different strains of CMV circulate in the human population, but these antigenic differences are probably not important determinants in human disease (7). Approximately 40% - 60%, and up to 100%, of the general population is positive for anti-CMV, indicating primary infections during childhood or early adulthood (8). Reactivation of latent infections occurs in some individuals in the presence of humoral immunity. This virus has been isolated from the lung, liver, esophagus, colon, kidneys, monocytes, and T and B lymphocytes, and it can cause systemic infection. The primary disease consists of an infectious mononucleosis-like syndrome, although most CMV infections are subclinical. Similar to all herpesviruses, CMV establishes lifelong latent infections. The virus can be shed intermittently from the pharynx and in urine for months to years after the primary infection (7). Cytomegalovirus probably plays a causative role in the pathogenesis and onset of autoimmune disease. The prevalence of CMV crucially depends on factors such as age, ethnicity, socioeconomic status, and sexual history (9).

There has been recent controversy over the role of CMV in the pathogenesis of MS. Some investigations have suggested a positive association between CMV infection and

MS disease activity (10), while others have indicated that CMV infections are negatively associated with MS (11, 12).

2. Objectives

The present study aimed to determine the seroprevalence and distribution of CMV DNA in the peripheral blood mononuclear cells (PBMCs) of patients suffering from RRMS.

3. Patients and Methods

3.1. Patients

Eighty-two blood samples from MS patients and 89 from healthy blood donors (as the control group) in northern Iran were collected based on patient age and gender. MS was diagnosed with magnetic resonance imaging (MRI) and according to the McDonald criteria (13, 14). The patients had no clinical history of other autoimmune diseases, and all patients received treatment except for ten who were considered treatment-naive. The study protocol was reviewed and approved by the local ethics committee, and conformed to the ethical guidelines of Islamic Azad University, Tonekabon Branch, Iran.

3.2. Sample Preparation

Blood samples were collected from all of the MS patients and the healthy controls. The collected venous blood samples (~ 5 mL) were poured into plasma-separator tubes containing EDTA, then centrifuged. The serum samples were stored at -80°C until the enzyme-linked immunosorbent assay (ELISA) was performed. PBMCs were separated by Ficoll density gradient centrifugation according to the manufacturer's instructions (Sigma, Germany) and stored in liquid nitrogen until further testing.

3.3. DNA Extraction

DNA extraction from the PBMCs was performed using the commercial kit according to the manufacturer's instructions (Qiagen, Germany). The purity of the extracted DNA was confirmed based on its absorbance at 260 and 280 nm wavelengths by biophotometer (Eppendorf, Germany). The extracted DNA was eluted in 50 μL of elution buffer and stored at -80 °C until assayed.

3.4. CMV-Specific Antibody Detection

ELISA was used for identifying anti-CMV IgG in the plasma samples from the MS patients and the healthy controls. CMV serology was measured by ELISA using a commercial kit (EUROIMMUN Anti-cytomegalovirus IgG-ELISA, Germany).

3.5. Polymerase Chain Reaction

Specific primers were produced by TAG Copenhagen (Denmark) and used to amplify the CMV genes. A pair of nucleotide primers (5'-CATGCGCGAGTGTCAAGGC -3' and 5'-ACTTTGAGCGCCATCTGTTCCT-3') was targeted to the immediate early gene in the PCR reaction. Each reaction was performed in a total volume of 20 μL, which contained 10 μL of primer Taq premix (2X), 1 μL of 10 pmol of forward and reverse PCR primers, 3 μL of distilled water and 5 μL of DNA template. Thermal cycling (Biorad, Germany) conditions were as follows: denaturation at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 45 seconds, and extension at 72°C for 30 seconds. The amplification was followed by a final extension step at 72°C for 10 minutes. An aliquot of all PCR products was analyzed with conventional (1.5% agarose) gel electrophoresis. The DNA concentration was determined after visualization of the bands in comparison with a standard DNA marker (100-bp ladder; Fermentas-Russia).

Amplification of human beta-globin housekeeping gene was used in order to ensure the accuracy of the extracted DNA by using the following primers: beta-globin-F: 5'-TCCAACATCAACATCTTGG T-3' and beta-globin-R: 5'-TCCCCCAAATTCTAAGCAGA-3', produced by TAG Copenhagen (Denmark).

3.6. Statistical Analysis

Statistical program for social sciences (SPSS-16, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Continuous variables were expressed as the mean $\pm$ standard deviation (SD) or median, and were compared using the independent t-test. Categorical variables were expressed as percentages, and differences between groups were judged for significance using the chi-squared test or Fisher's exact test. P-Values of < 0.05 were considered significant.

4. Results

4.1. Demographic Features

A total of 171 different samples were analyzed; 82 were from MS patients and 89 were from healthy controls. The blood samples from the patient group were analyzed based on factors such as age, gender, geographical area, and history of viral infections. The patient group comprised 23 (28%) males (mean age 40.0 years, range 18 - 54 years) and 59 (72%) females (mean age 30.3 years, range 16 - 52 years) (Table 1). The control group of healthy blood donors was composed of 34 (38.2%) males (mean age 25.64 years, range 19 - 70 years) and 55 (61.8%) females (mean age 26.0 years, range 17 - 50 years) (Table 1). There were 15 (18.3%) MS patients with a familial history of MS.

Table 1. Demographic Characteristics and CMV Frequency in RRMS Patients and Healthy Controls[a]

Variable	RRMS (n = 82)	Healthy (n = 89)	P Value
Gender			
Male	23 (28)	34 (38.2)	0.194
Female	59 (72)	55 (61.8)	
Age (y), mean ± SD	36.9 ± 9.30	34.32±10.56	0.096
Disease-onset age (y), mean ± SD	27.46 ± 7.9	-	-
Definite diagnosis age (y), mean ± SD	30.04 ± 9.53	-	-
Family history		-	-
Yes	15 (18.3)		
No	65 (79.3)		
Unavailable	2 (2.4)		
CMV antibody			
Positive	65 (79.3)	69 (77.5)	0.853
Negative	17 (20.7)	20 (22.5)	
CMV DNA			
Positive	23 (28.0)	2 (2.2)	< 0.001
Negative	59 (72.0)	87 (97.8)	

[a]Values are expressed as No. (%) unless otherwise indicared.

4.2. Detection of CMV-Specific IgG Antibody

The prevalence of CMV-specific IgG antibody among the MS patients and healthy controls were 65 (79.3%) and 69 (77.5%), respectively (Table 1). The statistical analysis showed no significant relationship between the frequency of CMV antibodies and MS (P= 0.853). No significant associations were found when comparing the MS patients to the control group based on gender, drug-administration status, age, or type of drug treatment (P for all > 0.05) (Table 2).

Table 2. Frequency of CMV Antibodies in MS Patients

Variable	CMV Positive (n = 65)	CMV Negative (n = 17)	P Value
Gender			0.997
Male	18 (27.7)	5 (29.4)	
Female	47 (72.3)	12 (70.6)	
Age (y), mean ± SD	37.74 ± 9.42	33.76 ± 8.38	0.118
Family history, No. (%)			0.908
Yes	12 (18.5)	3 (17.6)	
No	51 (78.5)	14 (82.4)	
Unavailable	2 (3.1)	0 (0.0)	

4.3. PCR Results

Conventional standard PCR was used to identify CMV DNA in the PBMCs. The amplified fragments of human beta-globin gene and viral DNA were 180 bp and 267 bp, respectively. As indicated in Table 1, among the MS patients and healthy controls, 23 (28%) and 2 (2.2%) were positive for CMV DNA, respectively. The statistical analysis showed that the presence of CMV DNA was significantly different in both groups (P < 0.001). No significant differences were found between the MS patients and the controls based on gender, drug-administration status, age, and type of drug treatment in terms of CMV DNA positivity (P for all > 0.05) (Table 3).

5. Discussion

More than 80 types of disorders are known to result from the immune system attacking the body. The onset of autoimmunity depends on both genetic and environmental factors (e.g. viruses) (9, 15). MS is characterized by the infiltration of immune cells into the central nervous system (CNS) upon autoreactivity (16). Previous studies suggested that Epstein-Barr virus (EBV), a human γ-herpesvirus, was associated with the risk of progression to MS (17). Recently, some surveys indicated that CMV infection could be correlated with certain types of autoimmune disease. Sev-

Table 3. Frequency of CMV DNA in MS Patients

Variable	CMV DNA Positive (n = 23)	CMV DNA Negative (n = 59)	P Value
Gender, No. (%)			0.274
Male	4 (17.4)	19 (32.2)	
Female	19 (82.6)	40 (67.8)	
Age (y), mean ± SD	37.68 ± 8.96	36.60 ± 9.49	0.467
Family history, No. (%)			0.476
Yes	3 (13.0)	12 (20.3)	
No	19 (82.6)	46 (78.3)	
Unavailable	1 (4.3)	1 (1.7)	

eral investigations have demonstrated that CMV infection may trigger an immune attack against the patient's own cells by molecular mimicry. According to previous studies, a high prevalence of CMV IgG antibodies was found in patients with certain autoimmune disorders (9). CMV is probably capable of triggering some immune regulation-evasion mechanisms, which may decrease immune reactivity in MS patients (18). After an initial infection with CMV, the virus can remain in PBMCs for life, in the latent phase (19, 20).

The present study showed positivity for anti-CMV IgG antibodies in a remarkable 65 (79.3%) of the MS patients. However, there was no significant correlation with the presence of specific antibodies in MS patients compared to the controls (P = 0.853). These findings were similar to those of Sanadgol et al., indicating that the presence of anti-CMV antibodies alone may not be an important marker for MS development (10). Previous investigations showed that serum concentrations of the main immunoglobulin isotypes may be affected by systemic CMV infections (10). However, concentrations of immunoglobulin isotypes were not evaluated. On the other hand, a higher CMV DNA prevalence in MS patients compared with the healthy control group was found (P < 0.001). This finding was similar to the previous report by Sanadgol et al. (10). In the latter survey, CMV DNA was found at different frequencies in various samples of a majority of MS subtypes. In other reports, however, CMV has been found to be negatively correlated with MS (11, 21). In fact, Zivadinov et al. showed a protective effect of CMV against MS (18).

Taken together, the risk of developing MS may be increased via systemic CMV infection. Although this was a case-control study, we cannot conclusively confirm that CMV plays a critical role in MS disease. The present report and others from Iran have shown a remarkably high CMV prevalence in MS patients (10). However, further experimental and epidemiological studies are needed to confirm and prove our findings, using larger samples of MS patients.

Acknowledgments

This research was supported by Islamic Azad University, Tonekabon Branch. The authors are grateful to the staff of the microbiology department.

Footnotes

Authors' Contribution: All authors participated in the research and made substantial contributions to the conception and design of the study, according to the order of authors listed.

Financial Disclosure: None declared.

Funding/Support: None declared.

References

1. Calabresi PA. Diagnosis and management of multiple sclerosis. *Am Fam Physician.* 2004;**70**(10):1935–44. [PubMed: 15571060].
2. Goldberg LD, Edwards NC, Fincher C, Doan QV, Al-Sabbagh A, Meletiche DM. Comparing the cost-effectiveness of disease-modifying drugs for the first-line treatment of relapsing-remitting multiple sclerosis. *J Manag Care Pharm.* 2009;**15**(7):543–55. doi: 10.18553/jmcp.2009.15.7.543. [PubMed: 19739877].
3. Riccio P, Rossano R. Nutrition facts in multiple sclerosis. *ASN Neuro.* 2015;**7**(1) doi: 10.1177/1759091414568185. [PubMed: 25694551].
4. Goldenberg MM. Multiple sclerosis review. *P T.* 2012;**37**(3):175–84. [PubMed: 22605909].
5. Pender MP, Greer JM. Immunology of multiple sclerosis. *Curr Allergy Asthma Rep.* 2007;**7**(4):285–92. [PubMed: 17547851].
6. Pawate S, Sriram S. The role of infections in the pathogenesis and course of multiple sclerosis. *Ann Indian Acad Neurol.* 2010;**13**(2):80–6. doi: 10.4103/0972-2327.64622. [PubMed: 20814489].
7. Brooks GF, Carroll KC, Butel JS, Morse SA. Jawetz, Melnick, and Adelberg's medical microbiolog. Medical pub division: McGraw-Hill; 2013.
8. Griffiths PD, Panjwani DD, Stirk PR, Ball MG, Ganczakowski M, Blacklock HA, et al. Rapid diagnosis of cytomegalovirus infection in immunocompromised patients by detection of early antigen fluorescent foci. *Lancet.* 1984;**2**(8414):1242–5. [PubMed: 6150279].
9. Halenius A, Hengel H. Human cytomegalovirus and autoimmune disease. *Biomed Res Int.* 2014;**2014**:472978. doi: 10.1155/2014/472978. [PubMed: 24967373].
10. Sanadgol N, Ramroodi N, Ahmadi GA, Komijani M, Moghtaderi A, Bouzari M, et al. Prevalence of cytomegalovirus infection and its role in total immunoglobulin pattern in Iranian patients with different subtypes of multiple sclerosis. *New Microbiol.* 2011;**34**(3):263–74. [PubMed: 21811746].
11. Sundqvist E, Bergstrom T, Daialhosein H, Nystrom M, Sundstrom P, Hillert J, et al. Cytomegalovirus seropositivity is negatively associated with multiple sclerosis. *Mult Scler.* 2014;**20**(2):165–73. doi: 10.1177/1352458513494489. [PubMed: 23999606].

12. Waubant E, Mowry EM, Krupp L, Chitnis T, Yeh EA, Kuntz N, et al. Common viruses associated with lower pediatric multiple sclerosis risk. *Neurology.* 2011;**76**(23):1989–95. doi: 10.1212/WNL.0b013e31821e552a. [PubMed: 21646624].
13. McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann Neurol.* 2001;**50**(1):121–7. [PubMed: 11456302].
14. Polman CH, Reingold SC, Edan G, Filippi M, Hartung HP, Kappos L, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Ann Neurol.* 2005;**58**(6):840–6. doi: 10.1002/ana.20703. [PubMed: 16283615].
15. Selgrade MK, Cooper GS, Germolec DR, Heindel JJ. Linking environmental agents and autoimmune disease: an agenda for future research. *Environ Health Perspect.* 1999;**107 Suppl 5**:811–3. [PubMed: 10502548].
16. Steinman L. Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. *Cell.* 1996;**85**(3):299–302. [PubMed: 8616884].
17. Ascherio A, Munger KL, Lennette ET, Spiegelman D, Hernan MA, Olek MJ, et al. Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study. *JAMA.* 2001;**286**(24):3083–8. [PubMed: 11754673].
18. Zivadinov R, Nasuelli D, Tommasi MA, Serafin M, Bratina A, Ukmar M, et al. Positivity of cytomegalovirus antibodies predicts a better clinical and radiological outcome in multiple sclerosis patients. *Neurol Res.* 2006;**28**(3):262–9. doi: 10.1179/016164106X98134. [PubMed: 16687051].
19. Sinclair J, Sissons P. Latent and persistent infections of monocytes and macrophages. *Intervirology.* 1996;**39**(5-6):293–301. [PubMed: 9130040].
20. Taylor-Wiedeman J, Sissons JG, Borysiewicz LK, Sinclair JH. Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *J Gen Virol.* 1991;**72 (Pt 9)**:2059–64. doi: 10.1099/0022-1317-72-9-2059. [PubMed: 1654370].
21. Pirko I, Cardin R, Chen Y, Lohrey AK, Lindquist DM, Dunn RS, et al. CMV infection attenuates the disease course in a murine model of multiple sclerosis. *PLoS One.* 2012;**7**(2):eee32767. doi: 10.1371/journal.pone.0032767. [PubMed: 22393447].