

Prevalence of cytomegalovirus infection and its role in total immunoglobulin pattern in Iranian patients with different subtypes of multiple sclerosis

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SUMMARY

Multiple sclerosis (MS) is the most common autoimmune disease characterized by multifocal areas of inflammatory demyelination within the central nervous system. Cytomegalovirus (CMV) has a complex pathobiology and in most cases is simply asymptomatic. There is some recent controversy over the role of CMV in the pathology of MS. The aim of this study was to evaluate active CMV infection and its effect on the humoral immunity in patients with MS. Serum, plasma, peripheral blood mononuclear cells (PBMCs), saliva and urine collected from MS patients (n=78) and healthy subjects (n=123) were screened for the presence of anti-CMV antibodies and CMV-DNA by nephelometric and PCR methods. Concentrations of total antibodies in MS subtypes were measured using both nephelometric and enzyme linked fluorescent assay (ELFA) techniques. The results extend the observation of an increased frequency of CMV-DNA in patients, in contrast with controls ($p<0.001$). Furthermore, systemic CMV infections were found in 25.5% of patients and only 3.2% of controls ($p<0.001$). There was significant difference in the titers of anti-CMV IgG and total IgE in patient and controls ($P<0.001$). These results support the hypothesis that CMV may contribute to MS thought to establish systemic infection process and induce immune response.

KEY WORDS: Cytomegalovirus, Multiple sclerosis, Total antibodies, PCR detection

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INTRODUCTION

Multiple sclerosis (MS) is the most prevalent demyelinating disease of young adults, affecting many persons in the developed countries (Keegan & Noseworthy, 2002). Four major clinical subtypes

of MS based on disease pattern were introduced, relapsing-remitting (R.R), secondary progressive (S.P), primary progressive (P.P) and relapsing progressive (R.P) (Stevenson *et al.*, 1999). Therefore, a variety of types of evolution do exist for MS, not to mention the vast range of rates of accumulation of irreversible neurological disability during the disease from one patient to another. The risk of developing MS in the general population is approximately 0.2% (McMichael and Hall, 2001). Many studies have now been done on the efficacy of Interferon beta (IFN- β) and corticosteroids in both RRMS and SPMS (Sellebjerg *et al.*, 2005).

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Although the etiology of MS is unknown, it is generally believed that infection, genetic, immunologic and environmental factors are involved (Dyment *et al.*, 2004; Sadovnick *et al.*, 1999). The epidemiologic and pathologic characteristics of this disorder suggest that infectious agents may comprise the environmental component (Johnson, 1994). Although many viruses have been investigated as potential "triggers", to date no virus has been definitively associated with MS (Gilden, 2005). Many of the studies related to viral infection in MS are serological and involve the demonstration of increased antibody titers against particular viruses (Gilden, 2005). Human herpes virus (HHVs) has been associated with the pathogenesis of a wide range of severe diseases based on serologic, molecular and histopathology findings (Gaeta *et al.*, 2009; Richard, 2003). Cytomegalovirus (CMV) is a member of the *Herpesviridae* (beta herpes virus) with a double-stranded DNA (235kb) that infects more than 40-60% of the general population and up to 100% within some subpopulations and/or geographic areas (Astegiano *et al.*, 2010; Richard, 2005). CMV infection most commonly develops between ages 10-35 years and most people are exposed to CMV early in life and do not realize it because they have no symptoms. Laboratory signs of acute CMV infection and anti-CMV antibodies have been observed in autoimmune diseases (Barzilai *et al.*, 2007; Hjelmestaeth *et al.*, 2005; Kishore *et al.*, 2004; Mehraein *et al.*, 2004; Su *et al.*, 2007). Moreover, CMV has been implicated as a co-etiological agent in brain cancer and associated with a wide range of inflammatory diseases (Söderberg, 2008). CMV uses a variety of strategies that target host defenses, from the disruption of antigen-processing pathways to the modulation of cytokines (Tortorella *et al.*, 2000), all of which may contribute to the success of CMV in establishing coexistence. During active infection, CMV can be found in most tissues and organs, as well as in most bodily fluids especially urine and saliva (Long *et al.*, 1998). Considerable research has been directed towards finding abnormalities of the immunoglobulins (Igs) concentration in blood, which are characteristic of various neurologic disorders, especially infective diseases of the central nervous system (Pan-Hammarstrom & Hammarstrom, 2008; Freeman and Holland, 2008; Jacob *et al.*, 2008; Ochs, 2008). The present study attempted to determine the sero-

prevalence and distribution of CMV-DNA in different specimens of MS patients without any clinical evidence of CMV infection, and to determine the role of CMV infection in total immunoglobulin concentration and pathogenesis of MS.

MATERIAL AND METHODS

Patients

The study, approved by the University of Zabol Multiple Institutional Review Board, was conducted with all clinical samples from MS patients who were treated at the Department of Neurology, Ali-ebn Abitaleb Hospital, Zahedan, Iran, and healthy blood donors who voluntarily entered in to the research at the central medical laboratory of Zahedan from December 2008 through July 2009. MS patients (in southeastern Iran) who had been diagnosed with magnetic resonance imaging (MRI) and McDonald criteria were collected (Polman *et al.*, 2005). We analyzed 201 different samples; 78 were from patients and 123 were from healthy controls. The patient group comprised 22 men (mean age, 28.8 years; age range, 17-48 years) and 56 women (mean age, 30.3 years; age range, 16-52 years). The control group of healthy blood donors comprised 34 men (mean age, 26.4 years; age range, 17-42 years) and 89 women (mean age, 26.0 years; age range, 17-50 years). Patients adjusted in definite RRMS ($n=46$; 10 men and 36 women), SPMS ($n=11$; 2 men and 9 women), PPMS ($n=10$; 5 man and 5 women), and RPMS ($n=11$; 5 man and 6 women) subtypes. Fifty-one patients were receiving only interferon- β_{1a} (Rebif) treatment, and twelve patients also received methylprednisolone (corticosteroids) prior to blood sampling.

Sample collection

Whole blood (10 mL) was collected in plasma separator tubes (contain EDTA, 0.5 M) and centrifuged for 15 min at 150 *g* at 20°C and then plasma used for serological assays. Serum was separated from 5 mL whole blood by the same centrifugation and stored for DNA detection. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque density gradient centrifugation according to the manufacturer's instructions. Between 10^6 and 10^7 cells (mean, 5×10^6) were obtained, resuspended in 200 ml of

phosphate-buffered saline. Whole saliva samples (1.0 ml) were collected by expectoration into sterile plastic tubes and directly used for DNA extraction. Urine samples (1.0 ml) were collected at the time of the visit in sterile containers and were centrifuged for 15 min at 180 *g* at 20°C to obtain cell-free supernatants. All specimens were stored at -20°C until the experiment was performed (Fischbach & Dunning, 2009). When multiple specimens were submitted for one patient, all of those were tested. When possible, clinical materials were tested more than once.

Molecular detection and serological studies

DNA extraction

Viral DNA from all clinical specimens was extracted using proteinase K-phenol-chloroform extraction procedure (Sambrook *et al.*, 1989). For example, 200 µL of serum samples was treated with lysis buffer (10 mM Tris-HCl pH 8.0, 0.2 M NaCl, 0.25% sodium dodecyl sulfate (SDS), 0.5 mg mL⁻¹ of proteinase K) for 2 h at 65°C. Pellet DNA was obtained by centrifugation at 13,000 rpm for 15 minutes. The pellet was then twice washed with 70% ethanol and air-dried. The pellet was dissolved in 50 µL of sterile distilled water and aliquot 5 µL into 25 µL of PCR reaction mixture.

Polymerase chain reaction (PCR)

In this study a pair of nucleotide primers (5'-CAT GCG CGA GTG TCA AGA C-3' and 5'-ACT TTG AGY(C or T) GCC ATC TGT TCC T-3') targeted to the *IE* gene, which is responsible for encoding of UL54-encoded DNA polymerase, was used in the PCR reaction. DNA was amplified by one rounds of PCR performed with 5 µL of DNA template in a 25 µL reaction mixture containing 1U of Smar Taq DNA polymerase (Takara, Japan), 0.5 µM of each primers, 240 µM of each dNTPs, 20 mM of Tris-HCl 3 mM MgCl₂, 50 mM KCl and 20 mM ammonium sulfate. Thermal cycling conditions were as follows: denaturation of 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. The amplification was followed by a final extension step at 72°C for 5 min. PCR products purified by precipitation with 2.5 M ammonium acetate and isopropyl alcohol and subsequently washed with 70% ethanol to remove the dNTPs and excess primers. The pellet was diluted in 20 µL of DNase-free sterile wa-

ter, and a 10 µL aliquot was analyzed by conventional (1.5% agarose) gel electrophoresis containing ethidium bromide (Sigma, Germany). The DNA concentration was estimated after visualization of bands and comparison with a standard DNA marker (100-bp ladder; Gibco-BRL).

DNA sequencing and analysis of sequences

To confirm the presence of CMV DNA detected in specimens, amplicons were sequenced (Gene Service Company, UK). Three samples that yielded single bands, with molecular weights corresponding to each of the putative genotypes, were selected for DNA sequencing. A WU-BLAST-2 search of the determined sequences against a nucleotide sequence database (EMBL, European Bioinformatics Institute) was performed.

Immune monitoring of anti-CMV and total antibodies

Concentration of plasma anti-CMV, IgG and IgM were measurement with MININEPHTM specific kits (Binding Site Ltd., Birmingham, UK) base on nephelometric method (Andersson, 1990). We also established an assay to detect total antibodies using a nephelometric and ELFA techniques. Concentration of plasma IgG, IgA and IgM were measurement with MININEPHTM specific kits (Binding Site Ltd., Birmingham, and UK.) and IgE was measurement with VIDAS kit (Biomérieux, France) in an automated instrument, according to the manufacturer's instructions (Bradwell, 1995). All serological assays are performed in Dr. Ahmadi medical laboratory, Zabol, Iran.

Statistical considerations

Categorical variables were analyzed according to ² analysis and continuous variables by *t* tests and Mann-Whitney *U* tests for non-parametric variables. The nominal variable groups measured by three types of correlation: Pearson's, Kendall's tau and Spearman's rank correlation coefficient. The SPSS package (SPSS Inc., Chicago, IL, USA) were employed to statistical analyses. All P-values are Two-tailed and significant at *p*<0.05 or *p*<0.01 depending on the statistical method.

ETHICAL CONSIDERATIONS

The study conformed to the Helsinki Declaration

and was reviewed and approved by the local Research Committee and informed consent was obtained from all subjects.

RESULTS

Homology assay

Alignment of the sequence of the 180 bp PCR product (GenBank accession nos. **GU072731**,

GU072732 and **GU072733**) against sequences of the other isolates (GenBank accession nos. **GU072733**, **GU072732** and **BK000394**) showed 100%, 95% and 93% homology.

Prevalence of CMV infection in different specimens

The prevalence of CMV infection among MS patients and controls in southern Iran was quite high. Positivity for CMV-DNA (positive result or

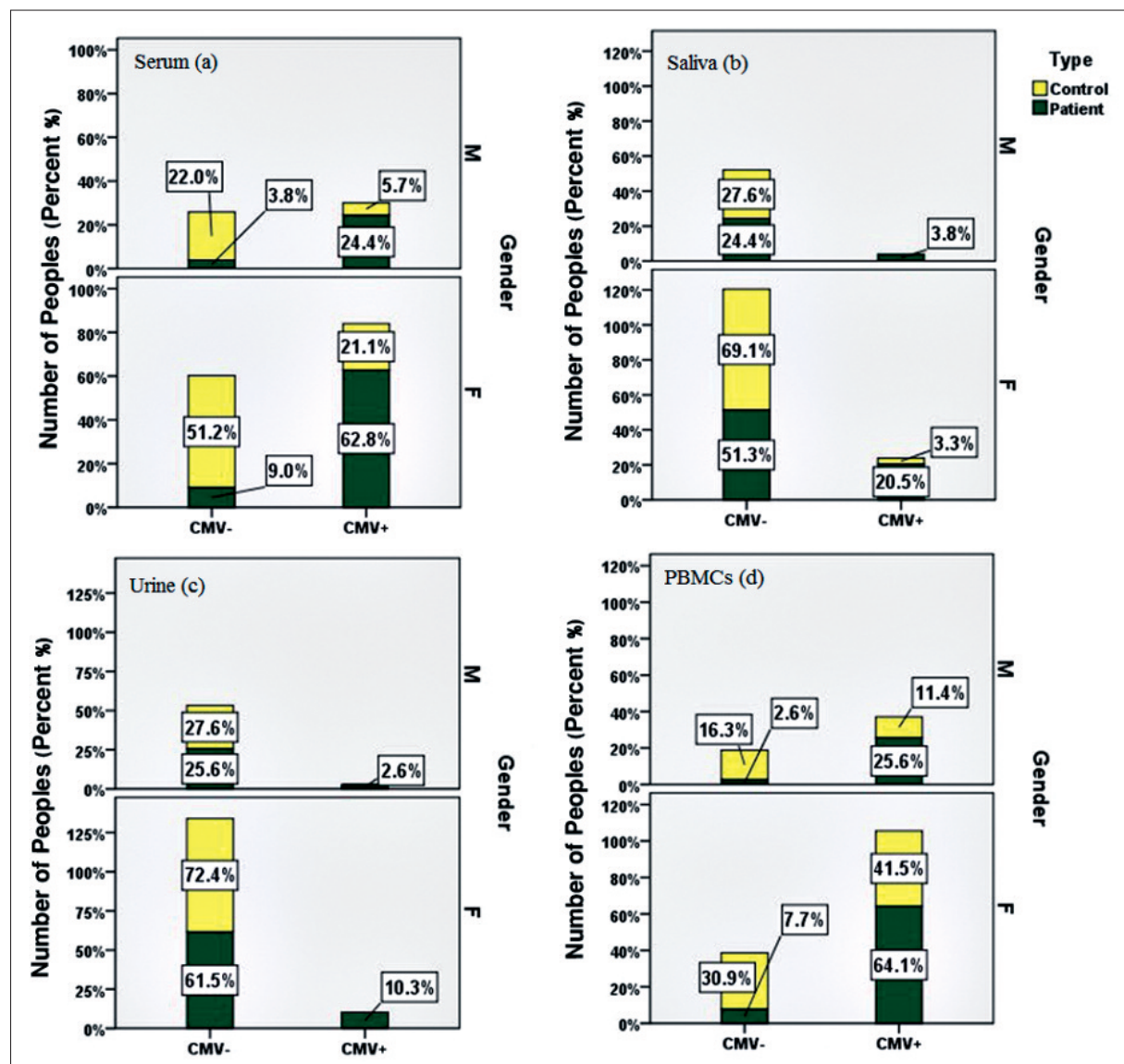


FIGURE 1 - Prevalence of CMV-DNA in different samples of controls and MS patients. The DNA from specimens (serum, saliva, urine and PBMCs) was purified and PCR for CMV-DNA was analyzed as described previously. Data are representative of three independent experiments.

CMV⁺) was found in 73 (93.5%) patients and 67 (54.5%) healthy blood donors for all specimens (serum, saliva, urine and PBMCs) separately ($p<0.001$). In the serum samples, 87.2% of patients were CMV⁺ in contrast with 26.8% of the controls ($p<0.001$) (Figure 1a).

In the saliva samples, 24.3% of patients were CMV⁺ compared to 3.3% of the controls ($p<0.001$) (Figure 1b). In the urine samples, 12.9% of patients were CMV⁺ in contrast with none of the

controls ($p<0.001$) (Figure 1c). In the PBMC samples, 89.7% of patients were CMV⁺ in contrast with 52.9% of the controls ($p<0.001$) (Figure 1d). Moreover, in patient samples 9 (11.5%) individuals showed a positive result in all specimens together in contrast with none of the controls ($p<0.001$) (Figure 1).

On the other hand, five (6.4%) patients and 34 (27.6%) controls showed positive results only in PBMCs samples (negative in other samples)

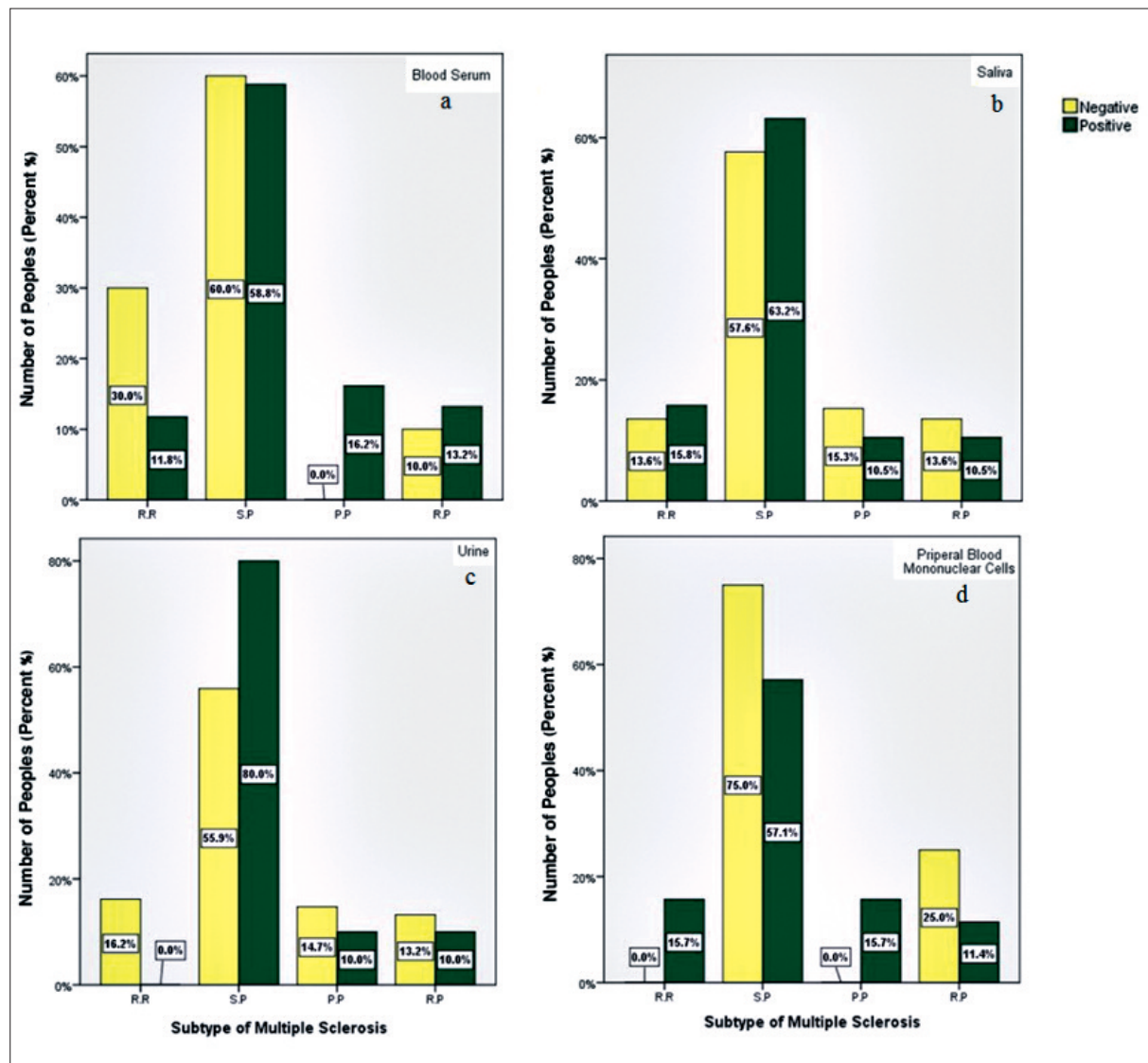


FIGURE 2 - Prevalence of CMV-DNA in patients with different MS subtypes. Patients adjusted in four definite subtypes (RR, SP, PP and RP) of M.S and DNA from specimens was purified. PCR for CMV-DNA was analyzed as described in material and methods. DNA negative = CMV- and DNA positive = CMV+. Data are representative of three independent experiments.

($p < 0.001$). Furthermore, five (6.4%) patients and 56 (45.5%) controls showed negative results in all samples ($p < 0.001$).

In all cases, female patients showed more positivity but it is not statistically different ($p = 0.4$) and systemic CMV infections (who showed CMV⁺ at last in tree specimens) were found in 25.5% of patients and only 3.2% of controls ($p < 0.001$) (Figure 1).

Prevalence of CMV infection in MS subtypes

Patients with SP-MS showed further positivity in all specimens in contrast with other subtypes ($p < 0.001$) and CMV-DNA was found in all serum samples of PP-MS subtypes (Figure 2 a-d). CMV-DNA was found in all PBMCs of RR-MS subtypes but we did not detect any CMV-DNA in their urine (Figure 2 c and d). Patients with SP-MS subtypes showed more individuals with CMV-DNA (seven) and also without CMV-DNA (six) for all cells free compartments (serum, saliva and urine) (Figure 2 a-c).

Four RR-MS and one PP-MS patients did not show CMV-DNA in any specimens. There were no significant differences in the comparison of CMV in separate specimens among the four MS subtypes.

Relationship between the anti-CMV immunoglobulin levels and total immunoglobulin pattern in different MS subtypes

Positivity for anti-CMV IgG and IgM antibodies was noted in 75 (96%) and 2 (2.5%) patients ($p < 0.001$) (Figure 3 a and 3 b) and 60 (49%) and 8 (6.5%) controls ($p = 0.3$) (Figure 4 a and 4 b). Superlative anti-CMV IgG was found in patients, especially in men (Figure 3 a), and higher than controls (Figure 4 a). There were no significant differences in the amount of total IgM in patients and controls (Figure 4 b). The detection of anti-CMV IgM from healthy volunteer donors and MS patients, irrespective of CMV-DNA detection in PBMCs, is indicative only of a new infection and found in 4.9% of the specimens (2 patients and 8 controls). Men with RRMS showed higher and women with RPMS showed lower concentrations of total IgG in patients (Figure 3 c) but there were no significant differences in the amount of total IgG in patients and controls (Figure 4 c). Women (9.71 ± 3.75) showed a wide ranging change in the amount of total IgG in contrast with men (9.64 ± 2.88) (Figure 3 c). On the other hand, total IgM in RRMS subtype was higher than other subtypes and men showed a wide range (Figure 3 d).

TABLE 1 - Correlation of CMV-DNA detection in separate specimens (CMV⁺) with CMV seroprevalence and concentration of total immunoglobulins in patients with MS.

		Concentration of IgG-CMV (UA/mL)	Concentration of IgM-CMV (UA/mL)	Concentration of total IgG (g/L)	Concentration of total IgM (g/L)	Concentration of total IgA (g/L)	Concentration of total IgE (IU/mL)
Kendall's tau_b	Serum	Correlation Coefficient	.582**	.216**	.173**	.046	.160**
		P-value	.000	.000	.003	.431	.006
	Saliva	Correlation Coefficient	.256	.079	.179**	.014	.123*
		P-value	.000	.194	.002	.807	.034
	Urine	Correlation Coefficient	.130*	.119	.109	-.007	.101
		P-value	.028	.052	.061	.898	.083
	PBMC	Correlation Coefficient	.536**	.154*	.384**	.347**	.373**
		P-value	.000	.012	.000	.000	.000
		Correlation Coefficient	.072	.242**	.094	.104	.094
		P-value	.215	.000	.104	.104	.094

**Correlation is significant at the $P < 0.01$ level; *Correlation is significant at the $P < 0.05$ level.

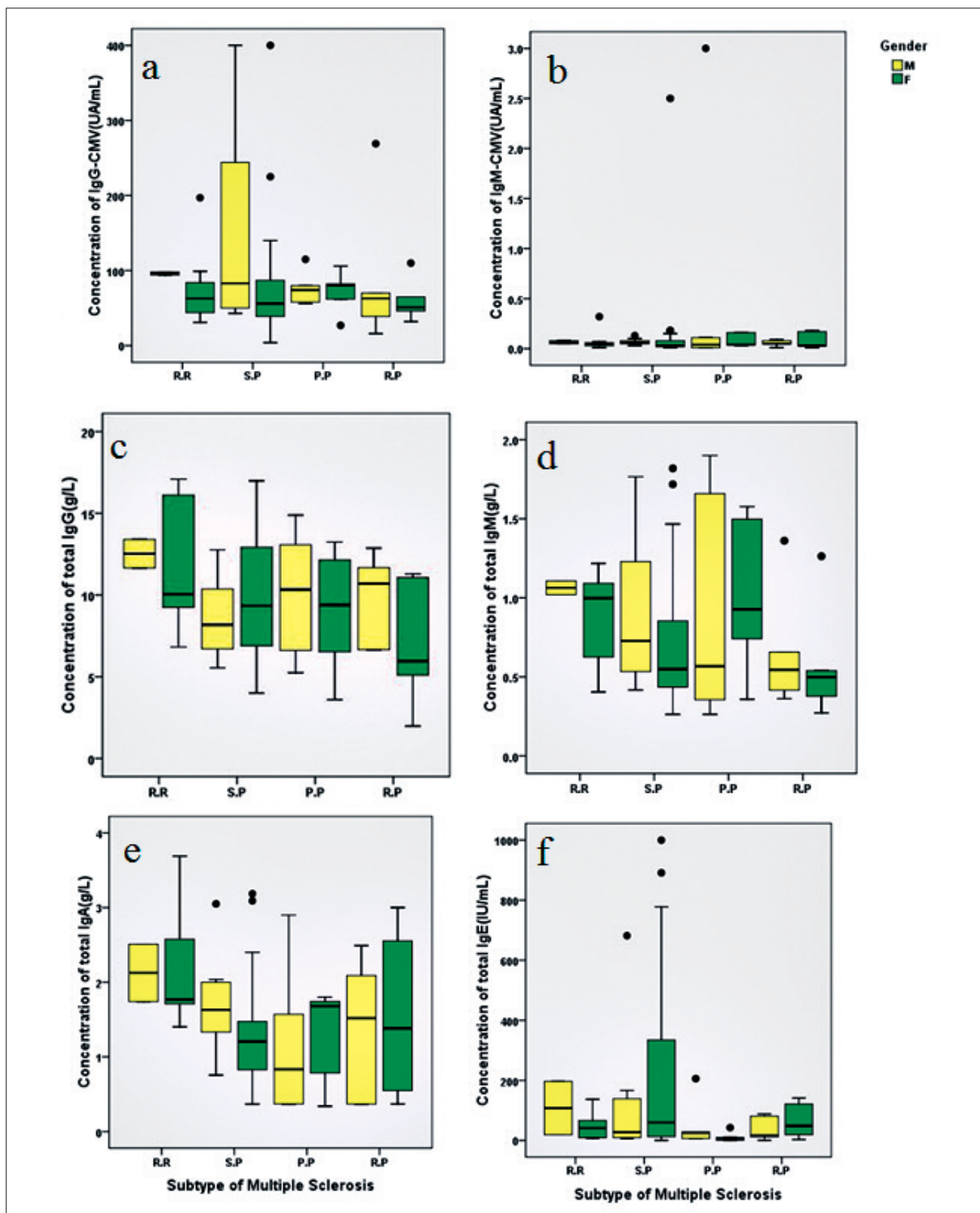


FIGURE 3 - Concentration of total and anti-CMV immunoglobulin levels in patients with different MS subtypes. Concentration of plasma anti-CMV, IgG (a) and IgM (b) and total IgG (c), IgM (d), IgA (e) and IgE (f) were measurement in an automated instrument, according to the manufacturer's instructions. Spots show samples with distance from normal distribution. Data are representative of three independent experiments.

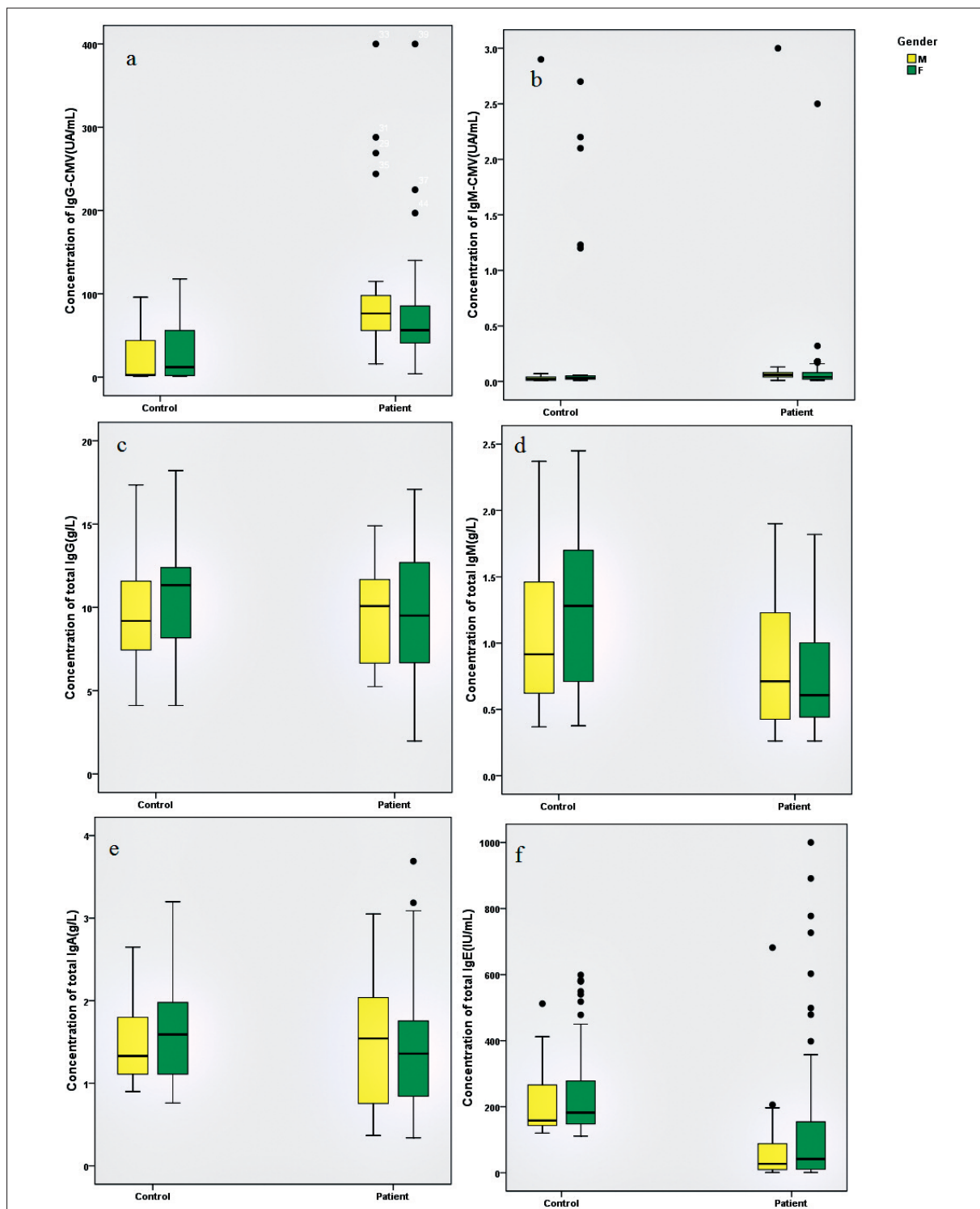


FIGURE 4 - Concentration of total and anti-CMV immunoglobulin levels in patients and controls. Concentration of plasma anti-CMV, IgG (a) and IgM (b) and total IgG (c), IgM (d), IgA (e) and IgE (f) were measurement in an automated instrument, according to the manufacturer's instructions. Spots show samples with distance from normal distribution. Data are representative of three independent experiments.

Women patients had a lower concentration of total IgM in contrast with controls (Figure 4 d). Men (0.865 ± 0.497) showed wide ranges in the amount of total IgM in contrast with women (0.745 ± 0.397) (Figure 3 d). Moreover, a significant change in total IgA concentration was observed in RRMS subtypes (Figure 3 e) but there were no significant differences between patients and controls (Figure 4 e). A higher concentration of total IgE was detected in male patients with RRMS subtype in contrast with other subtypes ($p=0.004$) (Figure 3 f) but it was lower than controls ($p=0.2$). An increase in seropositivity with rising total IgE concentration was also observed ($p=0.035$) (Table 2) and the difference between the patients and controls was statistically significant (Figure 4 f). Patients who had a higher titer of anti-CMV IgG, showed more positivity for CMV-DNA in serum and saliva samples in contrast with other samples (Table 1). Moreover, detection of CMV-DNA in urine was directly related to the increase in total IgE in patients ($P<0.001$) (Table 1). Indeed, increase in total IgG

and IgM was directly related to the increase in anti-CMV immunoglobulins (IgG and IgM) ($p<0.001$) (Table 2). Furthermore, the change in the amount of total IgA was significantly dependent on the variation in amount of total IgM and IgG (Table 2).

DISCUSSION

It has been suggested that viral infection may initiate or exacerbate organ-specific autoimmune diseases (Olson *et al.*, 2001). A viral trigger involved in multiple sclerosis was suggested more than a 100 years ago (Larner, 1986; Kurtzke, 1993), and an extensive list of candidate viruses has emerged since then. Several clinical studies have suggested that MS in general as well as episodes of disease exacerbation are associated with concomitant viral or microbial infections (Buljevac *et al.*, 2002; Gilden, 2005). Viruses may play an important role in relapsing episodes, and MS relapses are often associated with common

TABLE 2 - Correlation of CMV seroprevalence and concentration of total immunoglobulins together in patients with MS.

		Concentration of IgG-CMV (UA/mL)	Concentration of IgM-CMV (UA/mL)	Concentration of total IgG (g/L)	Concentration of total IgM (g/L)	Concentration of total IgA (g/L)	Concentration of total IgE (IU/mL)
Concentration of IgG-CMV (UA/mL)	Pearson correlation		-.125	.189**	.024	.120	-.148*
	P-value		.077	.007	.733	.088	.035
Concentration of IgM-CMV (UA/mL)	Pearson correlation	-.125		.382**	.402**	.267**	.349**
	P-value	.077		.000	.000	.000	.000
Concentration of total IgG (g/L)	Pearson correlation	-.189**	.382**		.668**	.590**	.320**
	P-value	.077	.000		.000	.000	.000
Concentration of total IgM (g/L)	Pearson correlation	.024	.402**	.668**		.574**	.388**
	P-value	.733	.000	.000		.000	.000
Concentration of total IgA (g/L)	Pearson correlation	.120	.267**	.590**	.574**		.322**
	P-value	.088	.000	.000	.000		.000
Concentration of total IgE (IU/mL)	Pearson correlation	.148*	.349**	.320**	.388**	.322**	
	P-value	.035	.000	.000	.000	.000	

**Correlation is significant at the $P<0.01$ level; *Correlation is significant at the $P<0.05$ level.

viral infections (Panitch, 1994). Moreover, increased titers of viral antibodies, particularly to the measles virus, have been found in the serum and CSF of MS patients (Cook *et al.*, 1995). By molecular methods, there is evidence that human herpesviruse-6 (Solden *et al.*, 1997), Epstein-Barr virus (Wandinger *et al.*, 2000) and Chlamydia pneumonia (Wandinger *et al.*, 2000) may be linked with MS.

In recent years, there has been an improved understanding of the epidemiology, pathogenesis, and long-term disabilities associated with systemic CMV infection (Michaux *et al.*, 2010). CMV is probably capable of triggering some immunomodulating/immune evasion mechanisms which may decrease immune reactivity in MS patients.

Furthermore, the CMV infection probably triggers an immune reaction against components of peripheral nerve myelin (Yuki *et al.*, 1998). Although CMV initially triggers the accumulation of many IFN-stimulated mRNAs, it is eventually able to disarm this antiviral response, at least in part (Evers *et al.*, 2004). However, experimental evidence suggested that CMV encodes at least one additional gene product, synthesized at immediate-early (IE) times after infection, which suppresses the up-regulation of IFN-dependent gene expression (Browne *et al.*, 2001).

After initial infection, CMV can remain in the PBMCs in a latent state (Sinclair & Sissons, 1996; Taylor *et al.*, 1991), with reactivation being a particular problem after MS.

Reactivation of latent CMV in a subset of patients with MS may result in the release of virus in cell-free compartments, such as saliva or urine (Griffiths, 2004). Consequently, CMV may caused myelin-specific lymphocytes shed to CSF and induce demyelinating lesions (Erin and Robyn, 2007). Using a large, geographically diverse population of MS patients (southeast of Iran), we found an approximate 98% seropositivity rate for anti-CMV antibodies in contrast with 52% of controls ($p < 0.001$).

Although the presence of anti-CMV antibodies alone may not be highly predictive of MS risk, our observation that 93.5% of patient samples were positive for CMV-DNA confirms our results. The study results extend the observation of an increased frequency of CMV-DNA in cell-free compartments of patients with MS. Of note, the

prevalence rate of CMV-DNA was found to be higher in women than men in both groups. Evaluation of the utility of the assay for risk stratification in the context of ongoing clinical studies will help elucidate if the risk may be significantly lower in a seronegative group compared with that of a seropositive group.

Humoral autoimmune phenomena may also be generated by nonspecific B-cell hyperactivation caused by CMV. For evaluation of immunomodulatory role of CMV we also monitored changing in total immunoglobulins during CMV infection. According to our results, median IgA and IgM values were higher in both males and females with RRMS, whereas median IgE values were higher only in females with RRMS. Sex differences in immunoglobulin concentrations, specifically high IgG levels in males with RRMS, maybe attributed to hormonal effects on B lymphocytes. But, when the concentration of total immunoglobulins in patients was compared with controls we observed a dramatic difference between CMV infected groups and non infected groups. Significant changes in serum IgM and IgE were observed with systemic CMV infection. A novel result in the current study was the down-regulation of total IgE expression in MS patients with systemic CMV infection compared with other individuals. The possible role of changing IgE concentrations in the development of MS should be investigated further. Serum IgA and IgG levels tended to decrease with progression of MS. Moreover, serum IgM and IgE concentrations tend to be lower in MS patients.

In summary, the present study shows that serum concentrations of the main immunoglobulin isotypes may be affected by systemic CMV infection. In conclusion, the immunomodulatory role of CMV remains to be finally proven. However, based on recent clinical findings we feel that the evidence is stronger.

The results obviously revealed that systemic CMV infection may induce humoral immunity toward down-regulation of total IgE and IgM and provide higher concentration of IgG-CMV but not total IgG and IgA in MS patients. Finally, we believe that the risk of developing MS may increase via systemic CMV infection but although this study is prospective in design, we cannot definitively prove that CMV plays a causative role in MS. How CMV manipulates the immune re-

sponse to induce autoimmune phenomena is not fully understood. Much remains to be learned about the pathogenic role of CMV in autoimmune diseases.

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