

Full Length Research Paper

Infection and genotype relationship in multiple sclerosis: Do *Chlamydomphila pneumoniae* and human herpes virus-6 infections together with APO E alleles have a role in the etiopathogenesis of multiple sclerosis?

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Despite numerous studies in many laboratories over several years, the etiology of Multiple Sclerosis (MS) is still unknown. It was suggested that some infectious agents play a role in the etiology of MS. This study included 39 patients with MS, 10 patients with other neurological disorders (OND) and a control group of 42 healthy people. There was no significant difference between MS, OND and HC groups for *Chlamydomphila pneumoniae* based on having past infection positivity ($p > 0.05$). Chronic *C. pneumoniae* infection was detected in 8 cases with MS and 3 cases in the healthy control group, and no chronic chlamydia infection was detected in patients with OND. No significant difference was found among the three groups. Antibody titres at 1/50 and higher IgG were detected in 34(87.2%), 8(80%) and 30(71.4%) of the patients with MS, OND and the healthy control group, respectively. There was no statistically significant difference among these groups. No *C. pneumoniae* and HHV-6 DNA was detected in CSF samples from the patients with MS and OND. There was no significant difference for the distribution of all APO E alleles for MS and healthy control groups. Moreover, no significant difference was found in the distribution of all APO E alleles for patients who had antibody titres for past infection with *C. pneumoniae* and HHV-6 between MS and control group. In conclusion, our data suggested that there was no contribution from the association of the *C. pneumoniae* and HHV-6 infections to the etiopathogenesis of the MS and our results are in concurrence with two important meta-analysis studies reported in 2006, but large scale, prospective new trials are needed to clarify this subject as proposed in meta-analysis considerations.

Key words: *Chlamydomphila pneumoniae*, human herpes virus-6, multiple sclerosis, APO E alleles.

INTRODUCTION

Chlamydomphila pneumoniae is one of the obligate bacterial intracellular pathogens of the respiratory tract. Like

all *Chlamydiae*, *C. pneumoniae* infects mucosal surfaces reportedly disseminating from its site of primary infection to other sites within the monocyte/macrophage and associated with chronic human diseases like atherosclerosis, cardiovascular diseases, and asthma besides respiratory tract diseases (Leinonen, 1993; Saikku et al., 1988; Kocazeybek, 2003). Human Herpes Virus 6 (HHV-6) infections are commonly seen in infants and boys.

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HHV-6 is neurotropic and persists or remains latent in numerous tissues including the central nervous system (CNS), and can be reactivated by stress or infections caused by other microbes (Swanborg et al., 2002; Challoner et al., 1995).

Multiple Sclerosis (MS) is a disease of the CNS effecting young or middle aged people. MS is the most commonly observed disease among the inflammatory demyelinating CNS diseases. The etiology of MS is unknown and it was suggested that some infectious agents can play a role in the etiology of MS. The widely accepted theory on the pathogenesis of the disease suggests that MS can be an autoimmune disease triggered by environmental factors; particularly microorganisms. Viruses like HTLV-1, HSV- 1, parainfluenzae virus 1, measles virus, coronal virus and CMV were thought to be the major triggering factors (Swanborg et al., 2002; Kurtzke, 1983). There is a growing interest for genetic markers in order to predict the disease level of MS. Apolipoprotein E (APOE) is an apoprotein found in the chylomicron and LDLs that binds to a specific receptor on liver cells and peripheral cells. It is essential for the normal catabolism of triglyceride-rich lipoprotein constituents. APOE, is an important factor in transport, uptake, and redistribution of cholesterol, which is significant to remodelling and repair of nerve tissue. Apo E is involved in neurodegenerative diseases and the most well known association is between Apo epsilon4 and Alzheimer's disease Pinholt et al., (2006). As the marker for membrane homeostasis, APO E, binds lipoprotein receptors which have less densities in CSF and play a role in cholesterol, triglycerid metabolism. APO E has been under investigation for the MS relationship with its alleles for some time (Masterman et al., 2002; Teunissen et al., 2005).

In this study, we aimed to investigate the role of *C. pneumoniae* and HHV-6 in the etiopathogenesis of MS and the effects of the relationship between these agents and APOE genotypes of patients for the etiopathogenesis of MS .

STUDY GROUPS

Patient and control groups

This study was designed as cross-sectional between May-October 2005 and three groups were included. This study was approved by the Cerrahpasa Medical Faculty, Ethical Committee, 6 May, 2005: Number:11001

MS group

Thirty nine patients of MS according to the The (PP) and relapsing-remitting (RR) MS) which were admitted to the neurology policlinics of Cerrahpasa Medical School were included in this study (McDonald et al., 2006). Thirty seven and two of the cases were detected primer pro-

gressive (PP) and relapsing-remitting (RR) MS, respectively.

Other neurological disorders (OND) control group

A total of ten patients, two patients with Guillian-Barreli, four patients with Neurobehcet and one patient with diabetic polyneuropathy, one patient with neurodegenerative disease, one patient with transvers myelitis and one patient with myelopathy were included in this study.

Healthy control (HC) group

Fourty two healthy blood donors who were in concordance with the gender and ages of MS group and did not have a respiratory system infection in the last 3 months and did not use antibiotic in the last month were included in this study as the healthy control group. The characteristics of MS and Control groups were shown in Table 1.

COLLECTION OF BLOOD SAMPLES AND LABORATORY METHODS

Two sets of 5 ml venous blood samples were collected into tubes, one set with EDTA for APOE genotyping and the other with no anti-coagulant for other serological tests from every MS patient in applying time and also 1 ml of CSF samples were taken from every MS patient. The second set of blood samples was collected for screening of seroconversion after 4 - 8 weeks. Five ml venous blood samples were collected into tubes, one set with EDTA for APOE genotyping and the other with no anti-coagulant from the healthy control group. Five ml venous blood and 1 ml CSF samples were also collected from the OND group (APOE was not investigated in this group).

All blood samples were centrifuged for the detection of *C. pneumoniae* and HHV-6 antibodies at 3000 rpm for 5 min, seperated serums were stored at -20°C . The CSF samples for *C. pneumoniae* and HHV-6 antibody detection were stored at - 80°C. Blood samples collected with EDTA for APOE genotyping were stored at +4°C.

Serological methods

IgG, IgM, IgA antibody titres against *C. pneumoniae* and IgG, IgM antibody titres against HHV-6 were detected by the immunofluorescence (MIF) method (Euroimmun Labordiagnostica, Germany). Elementary body in solid phase and Hep-2 cells infected with HHV-6 were used as antigens for *C. pneumoniae* and HHV, respectively. Phosphate-buffered Saline (PBS) -Tween mixture from the test kit was diluted with distilled water and used for futher dilution of sera. Dilutions for *C. pneumoniae* IgG were started at 1/32 and with doubled dilutions, final 1/512 dilution, was obtained. Dilutions by 1/16 and 1/40 were used for IgM and IgA, respectively. Dilutions were started from 1/50 and stopped at 1/1000 titers for HHV-6 IgG. Dilution by 1/10 was used for HHV-6 IgM antibody. The immunoabsorbtion method was used in order to avoid false IgM antibody before the study (100 l serum was mixed with 100 l Eurosorb Euroimmun, Germany and incubated for 20 min and centrifuged). Preparations were dyed with MIF method according to the manufacturer's protocol and preparations were evaluated in fluorescence microscope (Zeiss-Axiokop 40).

Table 1. The demographic characteristics of the study groups.

Study group	n	Gender		Mean age \pm SD	MS type		EDSS						PI		
		Female	male		RR	PP	0	1	1.5	2	2.5	3	4	<0.5	0.5
MS	39	27	12	31.03 \pm 8.22	37	2	2	20	5	6	2	2	2	9	19
OND	10	7	3	40.10 \pm 8.20	-	-				-					
Healthy Group	42	26	16	29.14 \pm 7.20	-	-				-					
Total	91	60	31												

MS: Multiple sclerosis, OND: Other Neurological Disorders, RR; Relapsing-Remitting, PP; Primer Progressive EDSS: Expanded Disability Status Scale P : Progression index

The evaluation of *C. pneumoniae* antibody titres

Seropositivity criterion for diagnosis of chronic *C. pneumoniae* infection were set to IgG 1/512 and IgA 1/40 and for the community population titres were set to IgG 1/32, based on the criterion established for previous infection. The highest result among the first or second blood samples obtained from both patients and the control groups were accepted as the final titers (Hannu et al., 1999; Kocazeybek, 2003).

The evaluation of HHV-6 antibody titres

IgG titres at 1/50 or higher were accepted as pre-infection criterion with HHV- 6 (Moore and Wolfson, 2002). Active infection criterion for HHV- 6 was accepted as 1/10 or higher IgM titers. The four-fold increase between 1 and 2 serum samples was accepted as active infection criterion (Pellett and Tipples, 2003).

Nucleic acid and APO E studies and the discrimination of *C. pneumoniae* and HHV-6 DNA

We have used polymerase chain reaction (PCR) based assays to detect the *C. pneumoniae*, HHV-6 and ApoE alleles. *C. pneumoniae* and HHV-6 DNA were isolated from the CSF samples using the Qiagen DNA isolation kit according to a manufacturer's protocol. The presence of *C. pneumoniae* was tested using CP-F:5'-gtgttcacatgaaggcctact-3' and CP-R:5'-tgcataacctacggtgtgtt-3' primers (Campbell et al., 1992). Nested PCR was used to monitor HHV-6 using the following primers; HHV-6F1:5'-agtcatcagcatcgcgctgctatc-3', HHV6-R1:5'-tatctagcgcaatcgctatgctg-3', HHV-6-F2:5'-tcgactctcaccctactgaacgag-3', and HHV-6R2:5'-tgactagagagcgacaaattggag-3' (Kurukuyu et al., 2005) The successful amplification of 437 bp and 163 bp amplicon confirms the presence of *C. pneumoniae* and HHV-6, respectively.

As a negative control for amplification, reaction mixtures containing reagents only, and HHV-6 and *C. pneumoniae* negative sample were amplified (in the case of the nested PCR, in both rounds). As a positive control for extraction and amplification, DNA of a sample containing the HHV-6 or *C.pneumoniae* in a low copy number was extracted and amplified. PCR assays were analysed in duplicate.

The ApoE gene was genotyped by the PCR-RFLP method. The 227 bp region of the ApoE gene was amplified with the ApoE-F:5'-tccaaggagctgcaggcggcgca-3' and ApoE-R:5'-acagaattcgccccggcctggtactgcccac-3' primers (Wenham, 1991). The amplified product was digested with HhaI restriction enzyme. Each of the isoforms was distinguished by a Nigie combination of HhaI fragment sizes that enabled unambiguous typing of all homozygotic and heterozygotic combinations. 3/ 3 (91, 48 bp), 3/ 2 (91, 81, 48 bp) and 3/ 4 (91, 72, 48 bp) alleles were genotyped according to restriction fragments.

All PCR assays were performed according to quality standards for nucleic acid amplification techniques published by the German Society for Hygiene and Microbiology (Roth, 2001) included physical separation of working areas for the preparation of amplification reaction mixtures, specimen preparation, preparation of positive controls and the amplification and detection of nucleic acids.

Statistical methods

Data given as mean \pm standard deviation, frequencies and percentages: The data were statistically analyzed with Chi-square test, unpaired t test, One-Way ANOVA and post hoc tests used were Bonferroni (multiple comparisons) and Dunnett-t (comparisons against a control group). All statistical tests were two-sided and p value lower than 0.05 was considered statistically significant. Statistical analyses were performed with the SPSS[®] 11.5 for Windows[™] (SPSS Inc., Chicago, IL).

RESULTS

The demographic characteristics of the study groups were shown in Table 1. IgG, IgM and A antibody seropositivities against *C. pneumoniae* and IgG, IgM antibody seropositivities against HHV-6 and distribution of different APO E genotypes in MS, OND and the healthy control group were shown in Table 2. There was no significant difference between MS, OND and HC groups for *C. pneumoniae* based on having past infection seropositivity as 1/32 ($p > 0.05$). No *C. pneumoniae* IgM positivity was detected in MS, OND and HC groups. There was also not a significant difference between MS, OND and HC groups for HHV-6 seropositivity on having past infection seropositivity for IgG and IgM as 1/50 and 1/10, respectively ($p > 0.05$). Titre of IgM antibody response against HHV-6 equal or higher than 1/10 was only seen in one patient in the MS group type RR. No HHV- 6 DNA positivity was detected in the CSF samples of 39 MS, 10 OND and 49 HC cases.

Our results revealed that APOE 3/ 3 is the most common allele type in both MS patients and the HC, and all alleles were distributed with similar percentages in both groups. A significant difference was not found between MS patients and the healthy control group for APOE alleles distribution (Table 2). Moreover, there was no significant difference between MS and HC groups for APOE alleles distribution based on having past *C. pneu-*

Table 2. The distribution of the antibodies for *C. pneumoniae* and HHV-6, and APOE alleles in all groups.

Antibody titres	MS (n:39)	OND (n:10)	Healthy control group (n:42)	p
<i>C. pneumoniae</i>				
IgG /32	35	10	32	NS
IgG 1/512	4	--	--	NS
IgA 1/40	13	2	4	NS
IgM 1/16	--	--	--	NS
IgG 1/512 and IgA 1/40	8	--	3	
HHV-6				
IgG 1/50	34	8	30	NS
IgM 1/10	1	--	--	NS
APO E distribution				
2 / 3	8	not done	4	NS
3 / 3	26	not done	21	NS
3 / 4	2	not done	4	NS
2 / 4	--	not done	1	

NS: Not significant

Table 3. Comparing the patient and the control groups with regard to seropositivity with the titre of 1/32 for *C.pneumoniae* 1/50 for HHV-6 for APO E alleles.

	APOE alleles					
	2 3		3 3		3 4	
	n	%	n	%	n	%
<i>C. pneumoniae</i> antibody 1/32						
MS Group (n:32)	7	21	23	71	2	6.3
Healthy Control Group (n:23)	4	17.4	16	69.6	3	13.0
Total	11		39		5	
p	NS		NS		NS	
HHV-6 antibody 1/50						
MS Group (n:31)	7	22.6	22	71.	2	6.5
Healthy Control Group (n:23)	4	17.4	16	69.6	3	13
Total	11		38		5	
p	NS		NS		NS	

NS: Not significant

moniae infection seropositivity as 1/32. Similar results were detected for HHV-6 based on having past infection seropositivity as 1/50 ($p > 0.05$) (Table 3).

DISCUSSION

Etiology of MS is unknown and current research efforts are focused on understanding the etiology of MS. Lesions caused by MS include inflammatory, primarily composed of T and B lymphocytes, plasma cells with mononuclear cells like macrophages are in concordance with active infection in perivascular region takes attention

in active plaques that were playing a role in MS pathology. Also, it was thought that both proinflammatory (TH1 originated) and antiinflammatory cytokines play a role in MS pathogenesis. Since the beginning of 2000, *C. pneumoniae* and HHV-6 have been added to the list of possible etiologic agents (Swanborg et al., 2002; Sarchielli et al., 1993; Kurtzke, 1983; Gilden, 2005).

Antibody titres at 1/32 IgG or higher (past infection criterion) for *C. pneumoniae* were detected in 35 (97%), 10 (100%) and 32 (76.2%) of MS, OND and HC groups, respectively. There was no significant difference between MS, OND and HC groups for *C. pneumoniae* based on having past infection seropositivity ($p > 0.05$).

C. pneumoniae IgM positivity was not observed in any of these three groups. No *C. pneumoniae* DNA was detected in 10 patients with MS and 49 patients with OND.

The association of CNS with *C. pneumoniae* and MS was initially suggested by the Helsinki Study Group, after a serologic study of patients with encephalitis and CNS infection. Two years later, detection of *C. pneumoniae* in patient's CSF with high EDSS score was reported in a study conducted at Vanderbilt University. Neurological involvement closely paralleled antibiotic treatment. (Swanborg et al., 2002; Campbell, 2002) Higher *C. pneumoniae* antibodies were detected in the MS group against the control group and *C. pneumoniae* culture and *C. pneumoniae* DNA positivity were detected in 24 and 36 of 37 CSF samples of MS patients, respectively in a study including 37 MS patients and OND group that was performed by the same study group. (Sotgiu et al., 2001). In a similar study, Gieffers et al., (2001) reported that *C. pneumoniae* was present in significantly higher levels in the MS patients than the control group in a study including 58 MS patients and 47 OND control group. Conversely, Krametter et al., (2001) have shown that there was no significant difference between MS patients and control groups with inflammatory and non-inflammatory neurological diseases. They concluded that there was no relationship between *C. pneumoniae* seropositivity and type, duration, clinical presentation, progression index and EDSS of MS similar to our serological results. A study by Dong-Si et al. (2004) indicated that *C. pneumoniae* was not a specific microorganism for MS in a study including 84 MS and 89 OND control groups for DNA positivity. Bagos et al. (2006) reported that their findings are insufficient to establish an etiologic relation between *C. pneumoniae* infection and MS in a meta-analysis consideration. The other studies indicated that *C. pneumoniae* may play a role in MS pathogenesis (Sriram et al., 1999; Fainardi et al., 2004) and association of this organism with MS remains in doubt as reported by other investigators (Swanborg et al., 2003; Campbell, 2002; Gilden, 2005).

IgG antibody titres at 1/50 and higher were detected in 34 (87.2%), 8 (80%) and 30 (71.4%) of the patients with MS, OND and the healthy control group, respectively. There was no statistically significant difference among these groups ($p > 0.05$). HHV-6 DNA was found in neither 39 patients with MS nor 10 patients with OND. Wilborn et al., (1994) reported that HHV-6 DNA was detected in 3 out of 21 patients in the MS group, but not present in 26 patients with OND; the HHV-6 serum antibody titers were significantly higher in MS patients compared with 26 patients with OND. Soldon et al. (1997) reported that the IgM responses in patients with MS (RR type) detected significantly higher compared to the control group. Presence of HHV-DNA in 30% of the patients was in concordance with the reactivation of the infection in MS patients. In a larger study by Challoner et al., (1995), HHV-6 DNA was found in 78% of the MS patients and 74% of the patients with OND, led to a conclusion that

there was no relationship between HHV-6 and MS. While Enbom et al., (1999) reported presence of HHV-6 IgM only in 1 out of 55 patients, while there was a IgG response in 15 patients, and concluded that there was no etiological relationship between HHV-6 and MS. Liedtke et al., (1995) have shown HHV-6 IgM was found 1 out of 36 MS patients; however, no IgM positivity was found in 24 patients with OND (control group). These results were in agreement with those of Enbom et al's and Liedtke et al's. No *C. pneumoniae* and HHV-6 DNA was detected in CSF samples from the patients with MS and OND.

In recent years, APO E, which has (32, 33, 34, 35) 2, 3 and 4 alleles, has been used in monitoring the prognosis for MS patients and as a marker for axonal damage. While the most common of APOE allele is 3, there were cases reported indicating that Alzheimer patients carrying the APO E $\epsilon 4$ allele have the worst and fastest disease progression (8, 36), (Masterman et al., 2002; Pinholt et al., 2006; Schmidt S et al., 2002; Burwick et al., 2006; Savettieri et al., 2003; Fazekas et al., 2001). It was reported that there was no statistically significant difference of the prevalence of 4 allele between the MS patients and the patients who have ONDs or controls (Chapman et al., 2000; Fazekas et al., 2001; Balerini et al., 2000; Savettieri et al., 2003), but Gerard et al. (1999) and Chapman et al. (2000) reported that signs of the MS disease were observed earlier and progressed faster in patients with 4 allele, and this association was not reported in other studies (Gerard et al., 1999). Burwick et al. (2006) reported that their findings do not support a role for APO E in MS pathogenesis but they emphasized the importance of using large sample sizes to detect any relation in a meta-analysis consideration. Our results revealed that APOE 3 is the most common allele type in both MS patients and the healthy control group, and all alleles were distributed evenly in both groups. In our study, in 26 (72.2%) out of 36 MS patients, and in 21 (70%) out of 30 healthy control group, 3 was the most common allele type, and 2 patients in the MS group and 5 subjects in the healthy control group had heterozygote 3 4 allele ($p > 0.05$). In our study, 1 (patient no 26) out of 2 patients in the MS group who had 3 4 allele had EDSS 4, which was in concordance with literature and had a faster disease progress (in 3 months); these were not exhibited in the other subject. The synergistic roles of the APO E allele genotypes of patients and the *C. pneumoniae* and HHV-6 infections in the etiopathogenesis and especially-prognosis of the MS have been debated in recent years. Further studies are needed to confirm if there is any effect of *C. pneumoniae* and genotype relationship on the etiopathogenesis and the prognosis of the MS (Fainardi et al., 2004). Gerard et al. reported a relationship between the APOE 4 allele and *C. pneumoniae* pathobiology, which was about the arthritis unrelated with MS, the reason of this relationship was unknown Gerard et al., (1999). We could not manage to reach a

case or a comprehensive serial study related with the effects of APO E alleles together with *C. pneumoniae* and APO E alleles together with HHV-6 to the MS pathogenesis until 2007. In our study, which is the first study with its comprehensive content, we have demonstrated that there was no significant difference of APO E allele incidence between the MS patients who had *C. pneumoniae* (1/32 or higher) and HHV-6 (1/50 or higher) infections and who had not these titres (< 1/32 for *C. pneumoniae* and < 1/50 for HHV-6) ($p > 0.05$). Moreover, we also shown that there was no significant difference of APO E allele incidence between the MS subjects with IgG titres 1/32 for *C. pneumoniae* and 1/50 for HHV-6 and the healthy control subjects with the same titres ($p > 0.05$). In conclusion, our data suggested that there was no contribution from the association of the *C. pneumoniae* and HHV-6 infections to the etiopathogenesis of the MS and our results are in coincidence with two important meta-analysis study reported in 2006, but large scale, prospective new trials are needed to clarify this subject as proposed in meta-analysis considerations.

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