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Bacterial and Viral Co-Infections in Chronic Fatigue Syndrome (CFS/ME) Patients

Garth L. Nicolson,[1] PhD, Marwan Y. Nasralla,[2] PhD, Kenny De Meirleir,[3] MD, PhD and Jeorg Haier,[4] MD, PhD

[1]The Institute for Molecular Medicine, Huntington Beach, California, USA,[2]International Molecular Diagnostics, Inc., Huntington Beach, California, USA, [3]Department of Internal Medicine, Free University of Brussels, Brussels, Belgium and [4]Department of Surgery, Wilhelm-University, Munster, Germany

Correspondence: Prof. Garth L. Nicolson, Office of the President, The Institute for Molecular Medicine, Huntington Beach, California 92647; Email: gnicolson@immed.org; Website: www.immed.org

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Abstract

A majority of Chronic Fatigue Syndrome (CFS) patients have systemic bacterial and viral infections. In our study of 200 CFS patients we found a high prevalence (52%) of mycoplasmal infections. Using forensic polymerase chain reaction we also examined whether these same CFS patients showed evidence of co-infections with various mycoplasmas, *Chlamydia pneumoniae* and/or Human Herpes Virus-6 (HHV-6). We found that 7.5% of the patients had *C. pneumoniae* and 30.5% had HHV-6 infections. Since the presence of one or more infections may predispose patients to other infections, we examined the prevalence of *C. pneumoniae* and HHV-6 infections in mycoplasma-positive and –negative patients. Unexpectedly, we found that the incidence of *C. pneumoniae* or HHV-6 was similar in mycoplasma-positive and -negative patients. Also, the incidence of *C. pneumoniae* in HHV-6-positive and –negative patients was similar. Control subjects had low rates of mycoplasmal (6%), HHV-6 (9%) or chlamydial (1%) infections, and there were no co-infections in control subjects. Differences in bacterial and/or viral infections in CFS patients compared to control subjects were significant ($P<0.001$). The results indicate that a very large subset of CFS patients show evidence of bacterial and viral co-infections.

Introduction

Chronic Fatigue Syndrome (CFS) is a syndrome with persistent or relapsing fatigue lasting six or more consecutive months plus additional signs and symptoms (1, 2). Although no single underlying cause has been established for all CFS patients, there is growing awareness that CFS can have an infectious nature that is either responsible (causative) for the illness, a

cofactor for the illness or appears as an opportunistic infection(s) responsible for aggravating patient morbidity (3). There are several reasons for this (4), including the nonrandom or clustered appearance of the illness, sometimes in immediate family members (5, 6), the presence of certain signs and symptoms associated with infection (1-4), the often cyclic course of the illness and its response to therapies based on treatment of infectious agents (7, 8).

Since chronic illnesses like CFS are usually complex, involving multiple, nonspecific, overlapping signs and symptoms, they are difficult to diagnose and even more difficult to treat (7-9). CFS for the most part does not have effective therapies, and therefore most CSF patients do not completely recover from their chronic condition (9). There is a growing international consensus to differentiate CFS into clinically relevant subcategories that may represent different disease states or co-morbid conditions or illnesses so they can be more effectively treated (11). Identifying systemic infections, such as those produced by *Mycoplasma* species (7,8,11-14), *Chlamydia pneumoniae* (10) and Human Herpes Virus-6 (HHV-6) (15-17), is likely to be important in determining the treatment strategies for many CFS patients. Here we examined CFS patients to see if a subset of patients had more than one type of chronic bacterial or viral infection.

Methods

Patients

All subjects (n=200) underwent a medical history, completed a sign/symptom illness survey and had routine laboratory tests. If necessary, medical records were also reviewed to determine if patients suffered from organic or psychiatric illnesses that could explain their symptoms. When positive results were found in any of the evaluations that met the Fukuda et al. (2) exclusionary criteria, the patients were not included in the study. Additionally, all subjects were questioned about medication use during the three months prior to the study, and they had to be free of antibiotic treatment for two months prior to blood collection. Controls (n=100) had to be free of disease for at least three months prior to data collection, and they had to be free of antibiotic treatment for three months prior to blood collection.

Blood Collection

Blood was collected in EDTA-containing tubes and immediately brought to ice bath temperature as described previously (18-20). Samples were shipped with wet ice by air courier to the Institute for Molecular Medicine and International Molecular Diagnostics, Inc for analysis. All blood samples were blinded. Whole blood (50 μl) was used for preparation of DNA using Chelex (Biorad, Hercules, USA) as follows. Blood cells were lysed with nano-pure water (1.3 ml) at room temperature for 30 min. After centrifugation at 13 000 x g for 2 min, the supernatants were discarded. Chelex solution (200 μl) was added, and the samples were incubated at 56°C and at 100°C for 15 minutes each. Aliquots from the centrifuged samples were used immediately for PCR or flash frozen and stored at –70°C until use. Multiple aliquots were used for experiments on all patient samples.

Detection of Mycoplasma by Forensic PCR.

Amplification of the target gene sequences (Table 1, 20-23) was performed in a total volume of 50 μl PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 9) containing 0.1% Triton X-

100, 200 μm each of dATP, dTTP, dGTP, dCTP, 100 pmol of each primer, and 0.5-1 μg of chromosomal DNA. Purified mycoplasmal DNA (0.5-1 ng of DNA) was used as a positive control for amplification. The amplification was carried out for 40 cycles with denaturing at 94°C and annealing at 60°C (*M. penetrans*) or 55°C (*M. pneumoniae*, *M. hominis, M. fermentans*). Extension temperature was 72°C in all cases. Finally, product extension was performed at 72°C for 10 min. Negative and positive controls were present in each experiment (18-20). Different multiple primer sets were used to confirm the presence of specific mycoplasmas and not cross-reacting microorganisms. The amplified samples were run on a 1% agarose gel containing 5 μl/100 ml of ethidium bromide in TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA, pH 8.0). After denaturing and neutralization, Southern blotting was performed as follows. The PCR product was transferred to a Nytran membrane. After transfer, UV cross-linking is performed. Membranes were prehybridized with hybridization buffer consisting of 1x Denhardt's solution and 1 mg/ml salmon sperm as blocking reagent. Membranes were then hybridized with ^{32}P-labeled internal probe (10^7 cpm per bag). After hybridization and washing to remove unbounded probe, the membranes were exposed to autoradiography film for 7 days at -70°C (19, 20).

Chlaymdia pneumoniae Detection by Forensic PCR.

PCR detection of *Chlaymdia pneumoniae* was done as described above for various *Mycoplasma* species, except that the conditions and primers differ. PCR was carried out using the *C. pneumoniae*-specific primers:

5'-TGACAACGTTAGAAATACAGC-3' (upstream) and downstream 5'-CGCCTCTCTCTCCTATAAAT-3'. The DNA will be amplified for 30 cycles using standard cycle parameters, and the product evaluated by agarose-gel electrophoresis. The efficiency of the PCR process will be monitored by amplification of β-actin mRNA. The presence of amplifications inhibitors will be evaluated by spiking negative samples with 2 μl of DNA from stock. *C. pneumoniae*-specific oligonucleotides in the PCR product were identified by Southern Blot and dot-blot hybridization using a 21-mer internal probe:

(5'-CGTTGAGTCAACGACTTAAGG-3') 3' end-labelled with digoxigenin–UTP or ^{32}P-labeled probe.

HHV-6 Detection by Forensic PCR.

PCR detection of HHV-6A was done as described above, except that the conditions and primers differ. PCR were carried out using the following HHV-6A-specific primers:

5'-GCGTTTTCAGTGTGTAGTTCGGCAG-3' (upstream) and downstream 5'-TGGCCGCATTTCGTACAGATACGGAGG-3'. The nucleotides were amplified for 30 cycles using standard cycle parameters, and the product evaluated by agarose-gel electrophoresis. The efficiency of the PCR process was monitored by amplification of β-actin mRNA. The presence of amplifications inhibitors were evaluated by spiking negative samples with 2 μl of DNA from stock. HHV-6A-specific oligonucleotides in the PCR product were identified by Southern Blot and dot-blot hybridization using a 21-mer internal probe: (5'-ATCCGAAACAACTGTCTGACTGGCA-3') 3' end-labelled with digoxigenin–UTP or ^{32}P-labeled probe.

Southern Blot Confirmation

The amplified samples were run on a 1% agarose gel containing 5 ml/100 ml of ethidium bromide in TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA, pH 8.0). After denaturating and neutralization, Southern blotting was performed as follows. The PCR product was transferred to a Nytran membrane. After transfer, UV cross-linking was performed. Membranes were prehybridized with hybridisation buffer consisting of 1x Denhardt's solution and 1 mg/ml salmon sperm DNA as blocking reagent. Membranes were then hybridized with digoxigenin–UTP or ^{32}P-labeled internal probe (10^7 cpm per bag). After hybrization and washing to remove unbounded probe, the membranes were examined (digoxigenin-UTP-labeled probe) or exposed to autoradiography film (^{32}P-labeled probe) for 1- 2 days at –70°C.

Statistics

Subjects' demographic characteristics were assessed using descriptive statistics and students' t-tests (independent samples test, t-test for equality of means, 2-tailed). The 95% confidence interval was chosen for minimal significance.

Results

Chronic Infections in CFS Patients

Patients and control subjects were approximately similar in age characteristics (control subjects mean age = 34.6; CFS patients: mean age = 40.6). CFS patients differed significantly according to sex distribution ($P< 0.05$); 146 (73%) patients were female, while 54 (27%) patients were male. Similarly, 69 (69%) of control subjects were female, while 31 (31%) were male (Table 1). All patients fulfilled current international CDC case definition for Chronic Fatigue Syndrome (2).

Chronic infections were not found in 58 of 200 (29%) of CFS patients and 82 of 100 (82%) of control subjects (Table 3). When we examined CFS patients' blood for the presence of chronic infections, *Mycoplasma* species infections were found in 104 of 200 (52%) of CFS patients and 7 of 100 (7%) of control subjects. *C. pneumoniae* infections were found in 15 of 200 (7.5%) of CFS patients and in 1 of 100 (1%) of control subjects. HHV-6 infections were found in 61 of 200 (30.5%) of CFS patients and 9 of 100 (9%) of control subjects. The differences between chronic infections in CFS patients and control subjects were significant (*Mycoplasma* species, $P<0.001$; HHV-6, $P<0.01$; *C. pneumoniae*, $P<0.01$) (Table 3). We did not find any multiple co-infections in control subjects.

Co-Infections (Mycoplasmal) in CFS Patients

Using species-specific primers and forensic PCR the incidence of various *Mycoplasma* species was examined. Similar to previous results, *M. pneumoniae* infections were observed in 56 of 104 patients with CFS, *M. fermentans* infections occurred in 46 of 104 patients and *M. hominis* in 33 of 104 patients, whereas *M. penetrans* infections were found at lower (18 of 104) incidence (Table 4). We examined 100 control subjects who did not show clinical signs and symptoms and found that 7 were positive for a single species of mycoplasma (Table 3). Differences between CFS patients and control subjects were significant ($P<0.001$).

Single infections with one of the four mycoplasmas that were tested for were observed in 59 of the 104 (56.7%) mycoplasma-positive patients or 29.5% of the total (Table 4). In the seven control subjects that were positive for mycoplasmal infections we found two controls were positive for *M. fermentans*, three for *M. pneumoniae* and one for *M. hominis* and one for *M. penetrans*. Similar to a previous study (19), the most commonly observed infection was *M. pneumoniae* (56 of 104 patients, 28%), followed by *M. fermentans* in 46 patients (18.3%), *M. hominis* in 33 patients (16.5%) and *M. penetrans* in 18 patients (9%). Multiple mycoplasmal infections were detected in 45 of the 104 patients (43.3% or 22.5% of the total patients), whereas single infections were found in 59/104 (56.7% or 29.5% of the total). Similar to our previous results (19), we have not found patients positive for all four of the tested mycoplasma species. In previous studies on North American (19) and European (11) CFS patients with multiple mycoplasmal infections, all patients showed combinations of *M. pneumoniae* and/or *M. fermentans* (with or without other species). The combination of *M. hominis* and *M. penetrans* was not seen. Similar results were found here where the most commonly found combination of *Mycoplasma* species were *M. fermentans* plus *M. pneumoniae* (20 of 104 patients, 19.2%), *M. fermentans* plus *M. hominis* (14 of 104 patients, 13.5%) or *M. hominis* plus *M. pneumoniae* (5 of 104 or 4.8%). The most common triple infection found was *M. fermentans* plus *M. hominis* plus *M. pneumoniae* (5 of 104 patients, 4.8%) (Table 4).

Co-Infections (HHV-6) in CFS Patients

HHV-6 infections were found in 61 of 200 patients (30.5%) with CFS. This finding is similar but somewhat lower than previously reported for CFS patients (14-16). When we examined the incidence of HHV-6 infections in mycoplasma-positive and –negative patients, we found that there was no preference for HHV-6 infections in mycoplasma-infected patients (Table 4). In Mycoplasma-positive patients HHV-6 infections were found in 32 of 104 patients (30.7%), whereas in mycoplasma-negative patients HHV-6 infections were found in 29 of 96 patients (30.2%) (Table 4). There was also no preference for particular *Mycoplasma* species in HHV-6 co-infections (data not shown). In control subjects without evidence of signs or symptoms we found HHV-6 infections in 9 of 100 subjects. None of these HHV-6-positive control subjects had other infections (Table 3).

Co-Infections (C. pneumoniae) in CFS Patients

Chlamydia pneumoniae infections were found in 15 of 200 CFS patients (7.5%) (Table 4) and one control subject out of 100 that also did not have mycoplasmal or HHV-6 infections (Table 3). This finding is similar but somewhat lower than previously reported for CFS patients (10). When we examined the incidence of *C. pneumoniae* infections in mycoplasma-positive and –negative patients, we found that there was no preference for multiple infections, nor was there a preference for particular *Mycoplasma* species in *C. pneumoniae* mycoplasma co-infections. In Mycoplasma-positive patients *C. pneumoniae* infections were found in 8 of 104 patients (7.7%), whereas in mycoplasma-negative patients *C. pneumoniae* infections were found in 7 of 98 patients (7.3%). Similarly, in HHV-6-positive patients *C. pneumoniae* infections were

found in 5 of 61 patients (8.2%), whereas in HHV-6-negative patients *C. pneumoniae* infections were found in 10 of 139 patients (7.2%) (Table 4).

Discussion

In CFS patients we have found that chronic infections are a rather common feature of the illness. Previously we studied American and European CFS patients and found that most had mycoplasmal infections (11, 19, 20). Others studied CFS patients and also found evidence of widespread mycoplasmal infections (12-14). When we examined the incidence of particular mycoplasmal infections in CFS, we found that most patients had multiple infections (two or more species of mycoplasma), which were for the most part combinations of *M. fermentans* and other mycoplasma species (19). For example, in studying the prevalence of multiple mycoplasmal co-infections we found that double or triple infections occurred only when one of the species was *M. pneumoniae* and/or *M. fermentans* (11, 19). In a study on European CFS patients a slightly different picture was found (11). Examining 261 consecutive patients seen at a CFS clinic in Belgium 68.6% of patients were found to have one or more species of mycoplasma in their blood. In contrast to North American patients, however, the most common species found was *M. hominis*. This could indicate differences in demography and exposures between North American and Belgian CFS patients. We also found that more than 50% of North American patients with rheumatoid arthritis had mycoplasmal infections, and in the majority of these patients multiple infections with more than one species was found (18).

Mycoplasmas are found commonly in the oral cavity, urogenital tract and as symbiotic gut flora, but some species can cause acute and chronic illnesses when they penetrate into the blood vascular system and systemically colonize organs and tissues (3, 24-26). For example, *M. penetrans*, *M. fermentans, M. hominis* and *M. pirum* can enter a variety of tissues and cells and cause systemic signs and symptoms. Mycoplasmas have also been shown to have a complex relationship with the immune system. They are very effective at evading host immune responses, and synergism with other infectious agents has been seen (24). In addition to CFS, mycoplasmas are thought to contribute to patients' morbidity in rheumatoid arthritis (18, 27), systemic lupus erythematosis (28), demyelinating and axonal neuropathies (29), HIV-AIDS (24, 26, 30) and chronic respiratory conditions (31-33). Mycoplasmal infections have been reported as co-infections with other microorganisms (34, 35).

Certain types of non-mycoplasmal infections are also commonly found in CFS patients. The most common viral infection found is HHV-6 (15-17). Although HHV-6 infections are commonly found in children, in adults such infections are considered latent but can be reactivated in certain illness states. In CFS patients HHV-6 is frequently reactivated and appears in blood leukocytes, in contrast to control subjects (15-17). In at least one of these studies peripheral blood mononuclear cells were cultured and tested for two HHV-6 glycoproteins using specific monoclonal antibodies and HHV-6 genes were probed using nested PCR (17). In 23 out of 35 patients (65.7%) peripheral leukocytes showed active infection by PCR.

The use of PCR techniques for detection of microorganism infections in patients has been questioned in studies where different methods were used in different laboratories without

validation. The PCR tests that we used to identify bacterial and viral infections are very sensitive and highly specific. These tests are a dramatic improvement on the relatively insensitive serum antibody tests that are routinely used to assay for systemic infections (36). For example, in the determination of mycoplasmal infections we used primer sets for various genes found in specific species (19-23). Since the universal primers GPO-1 and UNI that have been used to detect all *Mycoplasma* species are capable of possible cross-reactions with mycoplasma-related organisms (21), we used only unique primers and conditions that detect only specific species (Table 1). Similar to a previous study (20), we examined the reliability of the methods by performing multiple assays (repeated 3-6 times), and the results were completely reproducible. The sensitivity of mycoplasma detection by the described method was assessed by the detection of control mycoplasma DNA and by internal Southern blot hybridization using mycoplasma-specific probes. Using serial dilutions of mycoplasma DNA the method was able to detect as low as a few fg of DNA (20). In other experiments, mycoplasma was added to control blood samples at various concentrations. We were able to detect specific products down to 10 ccu/ml blood. Thus with the use of specific Southern hybridization the procedure can result in specific test results of high sensitivity, down to the presence of a few microorganisms in a clinical sample (19, 20). In our experience, conventional PCR yields similar results to forensic PCR with extracellular microorganisms, but not with clinical samples that contain intracellular microorganisms. Although the reason for this is not known, it could be due to inhibitors present in the clinical samples or to loss of mycoplasma DNA in the conventional extraction procedures due to protein complexing or degradation by cellular nucleases (20).

The multiple co-infections in CFS probably play an important role in determining the severity of systemic signs and symptoms found in CFS patients (19, 25). Since most CFS patients that previously tested positive for mycoplasmal infections have benefited from therapies directed at their chronic infections (25, 37), we consider it important that such infections be carefully considered in any treatment program for CFS patients (37-39).

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Table 1. Sequences, target genes and size of amplified products from mycoplasmal DNA used for mycoplasma species-specific PCR

Sequence name	**Sequence**	**Target**	**Size [bp]**	**Source**
GPO1 primer MGSO primer UNI- probe	**ACT CCT ACG GGA GGC AGC AGT A** **TGC ACC ATC TGT CAC TCT GTT** **A A C C T C** **TAA TCC TGT TTG CTC CCC AC**	16S mRNA genus	717	Van Kuppeveld et al. 1992 (20)
SB 1 primer SB 2 primer SB 3 probe	**CAG TAT TAT CAA AGA AGG GTC TT** **TCT TTG GTT ACG TAA ATT GCT TTT TTC AGT TTC GTA TTC GAT G**	*tuf* gene *M. fermentans*	850	Berg et al. 1995 (21)
MP5-1 primer MP5-2 primer MP5-4 probe	**GAA GCT TAT GGT ACA GGT TGG ATT ACC ATC CTT GTT GTA AGG CGT AAG CTA TCA GCT ACA TGG AGG**	unknown gene *M. pneumoniae*	144	Bernet et al. 1989 (22)
Mhom1 primer Mhom2 primer GPO1 probe	**TGA AAG GCG CTG TAA GGC GC** **GTC TGC AAT CAT TTC CTA TTG** **C A A A** **ACT CCT ACG GGA GGC AGC AGT A**	16S mRNA *M. hominis*	281	Van Kuppeveld 1992 (20)
IMM-7 primer IMM-5 primer IMM-3 probe	**GGA AAC GGG AAT GGT GGA ACA GAT** **TTC TGC TAA TGT TAC AGC AGC AGG** **AGG GAA TCT GTG ATC TTA TTC**	P35 gene (lipoprotein) *M. penetrans*	704	Nasralla et al. 1999 (18)

Table 2. Patient demographic data.

	n	**Mean age (SD)**	**Range**	**Males (%)**	**Females (%)**
Patients	200	40.6 (8.8)	18-68	54 (27)	146 (73)
Controls	100	34.6 (9.1)	21-58	31 (31)	69 (69)
Female patients	146	40.8 (9.8)	18-60	0 (0.0)	146 (100.0)
Male patients	54	39.3 (10.3)	18-60	54 (100.0)	0 (0.0)

Table 3. Comparison of chronic infections between CSF patients and healthy subjects.

	n	**No infection (%)**	**HHV-6 infected (%)**	***Sign***	***Mycoplasma* species infected (%)**	*Sign*	***C. pneumoniae* infected (%)**	*Sign*
CFS patients	200	58 (29)	61 (30.5)	-	104 (52)	-	15 (7.5)	-
Control subjects	100	82 (82)	9 (9)	$P<0.01$	7 (7)	$P<0.001$	1 (1)	$P<0.01$

Table 4. Prevalence of *Mycoplasma* species, *C. pneumoniae* and HHV-6 infections in 200 CFS patients.

Type of infection	**n**	**% of total**
None	96	48
*M. pneumoniae**	56	28
*M. fermentans**	46	23
*M. honinis**	33	16.5
*M. penetrans**	18	9
Single mycoplasmal infection	59	29.5
Multiple mycoplasmal infections	45	22.
M. fermentans +M. hominis	14	13.5
M. fermentans +M. pneumoniae	20	19.2
M. pneumoniae +M. hominis	6	5.8
M. fermentans +M. hominis + M. pneumoniae	5	4.8
HHV-6	61	30.5
HHV-6 – Mycoplasma	29	14.5
HHV-6 + Mycoplasma	32	16
C. pneumoniae	15	7.5
C. pneumoniae – Mycoplasma	7	3.5
C. pneumoniae + Mycoplasma	8	4
C. pneumoniae – HHV-6	10	5
C. pneumoniae + HHV-6	5	2.5

* alone or in combination with another species of Mycoplasma