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Evidence for Bacterial (Mycoplasma, Chlamydia) and Viral (HHV-6) Co-Infections in Chronic Fatigue Syndrome Patients

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ABSTRACT. Using the blood of 100 CFS patients and forensic polymerase chain reaction we have found that a majority of Chronic Fatigue Syndrome (CFS) patients show evidence of multiple, systemic bacterial and viral infections (OR = 18.0, 95% CL 8.5-37.9, $P < 0.001$) that could play an important role in CFS morbidity. CFS patients had a high prevalence (51%) of one of four *Mycoplasma* species (OR = 13.8, 95% CL 5.8-32.9, $P < 0.001$) and often showed evidence of co-infections with different *Mycoplasma* species, *Chlamydia pneumoniae* (OR = 8.6, 95% CL 1.0-71.1, $P < 0.01$) and/or active Human Herpes Virus-6 (HHV-6) (OR = 4.5, 95% CL 2.0-10.2, $P < 0.001$). We found that 8% of the CFS patients showed evidence of *C. pneumoniae* and 31% of active HHV-6 infections. Since the presence of one or more chronic systemic infections may predispose patients to other infections, we examined the prevalence of *C. pneumoniae* and active HHV-6 infections in mycoplasma-positive and –negative patients. The incidence of *C. pneumoniae* or HHV-6 was similar in mycoplasma-positive and -negative patients, suggesting that such infections occur independently in CFS patients. Also, the incidence of *C. pneumoniae* in active HHV-6-positive and –negative patients was similar. Control subjects (N=100) had low rates of mycoplasmal (6%), active 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. The results indicate that a relatively large subset of CFS patients show evidence of bacterial and viral co-infections.

INTRODUCTION

Chronic illnesses like Chronic Fatigue Syndrome (CFS) are usually complex, heterogeneous and involve multiple, nonspecific, overlapping signs and symptoms (1, 2). Such illnesses are usually difficult to diagnose and treat (3-5). CFS for the most part does not have effective therapies, and therefore patients often do not completely recover from their illness, even with therapy (3). CFS patients can be subdivided into clinically relevant subcategories that may represent different disease states or co-morbid conditions or illnesses (6). Identifying systemic infections, such as those produced by *Mycoplasma* species (4-9), *Chlamydia pneumoniae* (10) and Human Herpes Virus-6 (HHV-6) (11-13), is likely to be important in determining the treatment strategies for many CFS patients.

Although no single underlying cause has been established for CFS, there is growing awareness that CFS can have an infectious nature that is either causative for the illness, a cofactor for the illness or appears as an opportunistic infection(s) that aggravate patient morbidity (14). There are several reasons for this (15), including the nonrandom or clustered appearance of CFS, sometimes in immediate family members (16, 17), the presence of certain signs and symptoms associated with infection, the often cyclic course of the illness and its response to anti-microbial therapies (4, 5, 14).

Here we examined CFS patients to see if a subset of patients had more than one type of chronic bacterial or viral infection. We were particularly interested in assessing whether patients with one type of infection were more likely to show evidence of additional infections.

MATERIALS AND METHODS

Patients

All patients were from North America (Canada and the United States, n=100) and 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. Control subjects (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 (18-20) 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. Additional primer sets were used to confirm the species specificity of the reaction. The amplification was carried out for 40 cycles with denaturing at 94°C and annealing at 60°C (genus-specific primers and *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. 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 described below (18-20).

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'-TGACAACAGGTATAATACAGC-3' (upstream) and downstream 5'-CGCCTCTCTCTCCTATAAAT-3'. Additional primer sets were used to confirm the species specificity of the reaction. The DNA was 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 b-actin mRNA. The presence of amplifications inhibitors will be evaluated by spiking negative samples with 2 ml 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.

Active HHV-6 Detection by Forensic PCR.

PCR detection of active HHV-6A was done as described above, except that blood plasma was used instead of whole blood and the conditions and primers differed. PCR reactions were carried out using the following HHV-6A-specific primers:

5'-GCGTTTTCAGTGTAGGTTCGGCAG-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. Additional primer sets were used to confirm the specificity of the reaction. The efficiency of the PCR process was monitored by amplification of b-actin mRNA. The presence of amplification inhibitors was evaluated by spiking negative samples with 2 ml 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 hybridization 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

Patients and Control Subjects

Patients and control subjects were approximately similar in age characteristics (control subjects mean age = 34.6; CFS patients: mean age = 39.7). CFS patients differed significantly according to sex distribution ($P < 0.05$); 72% of the patients were female, while 28% of the patients were male. Similarly, 69% of control subjects were female, while 31% were male (Table 1). All CFS patients fulfilled current international CDC case definition for Chronic Fatigue Syndrome (2).

Chronic Infections in CFS Patients

Chronic infections were not found in 29% of CFS patients and 88% of control subjects (Table 2). When we examined CFS patients' blood for the presence of chronic infections using forensic PCR, evidence for *Mycoplasma* species infections were found in 51% of CFS patients and 7% of control subjects (Odds Ratio = 13.8, 95% CL = 5.8-32.9, $P<0.001$). Evidence for *C. pneumoniae* infections were found in 8% of CFS patients and in 1% of control subjects (Odds Ratio = 8.6, 95% CL = 1.0-71.1, $P < 0.01$), and evidence for active HHV-6 infections were found in 31% of CFS patients and 9% of control subjects (Odds Ratio = 4.5, 95% CL = 2.0-10.2, $P < 0.001$). We did not find any multiple co-infections in control subjects. The differences between chronic infections in CFS patients and control subjects were significant (Odds Ratio = 18.0, 8.5-37.9, $P < 0.001$) (Table 2).

Using species-specific primers and PCR the incidence of various *Mycoplasma* species in the blood of CFS patients was examined. *M. pneumoniae* infections were observed in 29 of 51 mycoplasma-positive CFS patients (Odds Ratio = 13.2, 95% CL = 3.8-45.4, $P < 0.001$), *M. fermentans* infections occurred in 22 patients (Odds Ratio = 13.8, 95% CL = 3.1-61.1, $P < 0.001$) and *M. hominis* in 16 patients (Odds Ratio = 18.8, 95% CL = 2.4-147.0, $P < 0.001$), whereas

M. penetrans infections were found at lower (8 of 51 mycoplasma-positive patients) incidence (Odds Ratio = 8.6, 95% CL = 1.0-71.1, $P < 0.01$) (Table 2). 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 2). Differences between CFS patients and control subjects were highly significant (Odds Ratio = 18.0, 95% CL = 8.5-37.9, $P < 0.001$).

Multiple Mycoplasmal Co-Infections in CFS Patients

Single infections with one of the four mycoplasmas that were tested were observed in 29 of the 51 (56.9%) mycoplasma-positive patients (Table 2). In the seven control subjects that were positive for mycoplasmal infections we found two controls that were positive for *M. fermentans*, three for *M. pneumoniae* and one for *M. hominis* and one for *M. penetrans* but these were found only as single infections. Similar to a previous study (19), the most commonly observed infection was *M. pneumoniae* (29 of 51 mycoplasma-positive patients), followed by *M. fermentans* in 22 patients, *M. hominis* in 16 patients and *M. penetrans* in 8 patients. Multiple mycoplasmal infections were detected in 22 of the 51 mycoplasma-positive patients (43.1% of the mycoplasma-positive patients), whereas single infections were found in 29/51 (56.9% of the mycoplasma-positive patients). The few control or healthy subjects that showed evidence for mycoplasmal infections only had single species infections ($Chi^2 = 24.7$, $P < 0.001$). Similar to our previous results (25), we have not found patients positive for all four of the tested mycoplasma species. In previous studies on North American (19) and European (6) 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*, *M. fermentans* plus *M. hominis* or *M. hominis* plus *M. pneumoniae*. The most common triple infection found was *M. fermentans* plus *M. hominis* plus *M. pneumoniae* found in two patients (Table 2).

Co-Infections with HHV-6 in CFS Patients

Similar to others, we found evidence of (active) HHV-6 infections in the plasma in approximately 31% of patients with CFS (Odds Ratio = 4.5, 95% CL = 2.0-10.2, $P < 0.001$). This finding is similar but somewhat lower than previously reported for CFS patients in other studies (11-13). When we examined the incidence of HHV-6 infections in mycoplasma-positive and –negative patients, we found that there was no preference for active HHV-6 infections in mycoplasma-infected patients (Table 2). Evidence for active HHV-6 infections was found by examination of blood plasma, and we found that 31.4% of mycoplasma-positive CFS patients also had HHV-6. Similarly, in mycoplasma-negative patients evidence of active HHV-6 infections was found in 29.4% of patients (Table 2). 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 evidence for active HHV-6 infections in 9 of 100 subjects. None of these HHV-6-positive control subjects showed other infections (Table 2).

Co-Infections with C. pneumoniae in CFS Patients

Chlamydia pneumoniae infections were found in 8% of CFS patients and one control subject out of 100 that also did not have mycoplasmal or HHV-6 infections (Odds Ratio = 8.6, 95% CL = 1.0-71.1, $P < 0.01$) (Table 2). 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 or –negative patients *C. pneumoniae* infections were found in 4 patients in each group. Similarly, in HHV-6-positive patients *C. pneumoniae* infections were found in 3 of 31 patients (9.7%), whereas in HHV-6-negative patients *C. pneumoniae* infections were found in 5 of 69 patients (7.3%) (Table 2). Thus there appeared to be no preference for particular combinations of co-infections in CFS patients.

DISCUSSION

Chronic infections appear to be a rather common feature of CFS. Many patients report that their CFS signs and symptoms slowly evolved after acute infections, chemical exposures, multiple vaccinations, severe trauma or other conditions that are associated with immune suppression and opportunistic infections. Previously we studied North American and European CFS patients and found that most showed evidence of mycoplasmal infections (6, 19, 20). Others who studied CFS patients also found evidence of widespread mycoplasmal infections (7-9). When we examined the incidence of particular mycoplasmal infections in North American CFS patients, 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). In our study on the prevalence of multiple mycoplasmal co-infections in CFS patients we found that double or triple infections occurred only when one of the species was *M. pneumoniae* and/or *M. fermentans* (19). In a study on European CFS patients a slightly different picture was found (6). Over two-thirds (68.6%) of 261 consecutive patients seen at a CFS clinic in Belgium were found to show evidence of mycoplasmas in their blood. In contrast to North American patients, however, the most common species found was *M. hominis,* and there was a lower overall rate of multiple mycoplasmal co-infections in the European CFS patients. 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 mycoplasmal co-infections infections were found (18).

Infections of the class *Mollicutes* can invade a variety of tissues but they can also present as superficial infections (21, 22). 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 (4, 5, 22-23). For example, *M. penetrans*, *M. fermentans, M. pneumoniae, 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 mycoplasmas can cause changes in cytokine production (24, 25). In addition to CFS, mycoplasmas are thought to contribute to patients' morbidity in rheumatoid arthritis (22, 26), systemic lupus erythematosis

(27), demyelinating and axonal neuropathies (28), HIV-AIDS (29, 30) and chronic respiratory conditions (31-33).

In CFS patients we found that multiple co-infections involving mycoplasmas were common (19 and the results here), and such mycoplasmal co-infections also occurred with *C. pneumoniae* and HHV-6. Mycoplasmal infections have been reported as co-infections with other microorganisms (29, 30). In some cases synergism with other infectious agents has been seen (34).

Certain types of non-mycoplasmal infections are commonly found in CFS patients. One of the most common viral infections found is HHV-6 (11-13). HHV-6 appears to play a role in several chronic illnesses (12, 13). Although several studies have associated HHV-6 with CFS (13, 35-38), there are also reports that could not find an association with CFS (39, 40). 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 and blood plasma. In contrast, in control subjects HHV-6 may be present as a latent infection, and although some active HHV-6 may be found in controls, it is generally found at lower levels compared to CFS patients (11-13). After peripheral blood mononuclear cells from CFS patients were cultured, specific HHV-6 glycoprotiens could be found in the culture medium using monoclonal antibodies. Also, HHV-6 genes could be found using nested PCR (17). The peripheral leukocytes from a majority of CFS patients showed active HHV-6 infections. Most of these patients test positive using antibodies reactive with the HHV-6A variant (11-13).

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 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. For example, in the determination of mycoplasmal infections we used primer sets for various genes found in specific species (18-20). Since some primers that have been used to detect *Mycoplasma* species are capable of possible cross-reactions with mycoplasma-related organisms (41), we used multiple unique primer set and conditions that detect only specific *Mycoplasma* species. As in a previous study (20), we examined the reliability of the methods by performing multiple assays, and the results were completely reproducible. The sensitivity of mycoplasma detection by the described method was assessed by the detection of control mycoplasma 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 a few ccu/ml blood. We have used a specific DNA isolation procedure to avoid inhibitors, contaminating nucleases and protein complexing. With the use of specific Southern hybridization the procedure can result in specific test results of high sensitivity and can detect a few microorganisms in a clinical sample (19, 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, 23, 42). Since CFS patients that previously tested positive for mycoplasmal infections have benefited from therapies directed at their chronic infections (5, 22, 23), we consider it important that such infections be carefully considered in the clinical management of CFS.

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Table 1. Patient demographic data.

	N	**Mean age (SD)**	**Range**	**Males (%)**	**Females (%)**
Patients	100	39.7 (8.9)	18-66	28 (28)	72 (72)
Controls	100	34.6 (9.1)	21-58	31 (31)	69 (69)
Female patients	72	39.8 (9.8)	18-66	0 (0.0)	72 (100.0)
Male patients	28	39.2 (10.3)	20-60	28 (100.0)	0 (0.0)

TABLE 2. Prevalence and Odds Ratio Analysis of Chronic Infections Between 100 CSF Patients and 100 Healthy Control Subjects.

Type of infection	**CFS Patients N = 100**	**Control Subjects N = 100**	**Odds Ratio, 95% CL, *P* or Chi^2**
Number Infected	71	12	**18.0**, 8.5-37.9, $P < 0.001$
HHV-6	31	9	**4.5**, 2.0-10.2, $P < 0.001$
C. Pneumoniae	8	1	**8.6**, 1.0-71.1, $P < 0.01$
Mycoplasma spp.	51	7	**13.8**, 5.8-32.9, $P < 0.001$
M. pneumoniae	29	3	**13.2**, 3.8-45.4, $P < 0.001$
M. fermentans	22	2	**13.8**, 3.1-61.1, $P < 0.001$
M. honinis	16	1	**18.8**, 2.4-147.0, $P < 0.001$
M. penetrans	8	1	**8.6**, 1.0-71.1, $P < 0.01$
Single mycoplasmal infection	29	7	**13.8**, 5.8-32.9, $P < 0.001$
Multiple mycoplasmal infections	22	0	Chi$^2 = 24.7$, $P < 0.001$
M. fermentans +M. pneumoniae	10	0	Chi$^2 = 10.5$, $P < 0.001$
M. fermentans +M. hominis	7	0	Chi$^2 = 7.3$, $P < 0.007$
M. pneumoniae +M. hominis	3	0	Chi$^2 = 3.1$, $P < 0.08$
M. fermentans +M. hominis + M. pneumoniae	2	0	Chi$^2 = 2.0$, $P = 0.16$
Mycoplasma + HHV-6	16	0	Chi$^2 = 17.4$, $P < 0.001$
Mycoplasma + *C. pneumoniae*	4	0	Chi$^2 = 4.1$, $P < 0.04$
C. pneumoniae + HHV-6	3	0	Chi$^2 = 3.1$, $P < 0.08$