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# Multiple co-infections (*Mycoplasma, Chlamydia*, human herpes virus-6) in blood of chronic fatigue syndrome patients: association with signs and symptoms

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Previously we and others found that a majority of chronic fatigue syndrome (CFS) patients showed evidence of systemic mycoplasmal infections, and their blood tested positive using a polymerase chain reaction assay for at least one of the four following *Mycoplasma* species: *M. fermentans*, *M. hominis*, *M. pneumoniae* or *M. penetrans*. Consistent with previous results, patients in the current study (n=200) showed a high prevalence (overall 52%) of mycoplasmal infections. Using forensic polymerase chain reaction we also examined whether these same patients showed evidence of infections with *Chlamydia pneumoniae* (overall 7.5% positive) and/or active human herpes virus-6 (HHV-6, overall 30.5% positive). Since the presence of one or more infections may predispose patients to other infections, we examined the prevalence of *C. pneumoniae* and HHV-6 active 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, and the converse was also found in active HHV-6-positive and -negative patients. 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. Severity and incidence of patients' signs and symptoms were compared within the above groups. Although there was a tendency for patients with multiple infections to have more severe signs and symptoms ($p<0.01$), the only significant differences found were in the incidence and severity of certain signs and symptoms in patients with multiple co-infections of any type compared to the other groups ($p<0.01$). There was no correlation between the type of co-infection and severity of signs and symptoms. The results indicate that a large subset of CFS patients show evidence of bacterial and/or viral infection(s), and these infections may contribute to the severity of signs and symptoms found in these patients.

Key words: Chronic fatigue syndrome; infection; *Mycoplasma*; human herpes virus-6; *Chlamydia*.

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Chronic fatigue syndrome or myalgic encephalomyelopathy (CFS/ME) is characterized by persistent or relapsing fatigue lasting 6 or more consecutive months, but it is also a heterogeneous illness in that patients can have somewhat different, overlapping signs and symptoms (1, 2). Although no single underlying cause has been established for all CFS patients, possibly due to the heterogeneity of the illness, patients' susceptibility and possible triggers, 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 ap-

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

Since chronic illnesses are usually complex, involving multiple, nonspecific, overlapping signs and symptoms, they are difficult to diagnose and even more difficult to treat (7–9). Chronic fatiguing illnesses for the most part do not have effective therapies, and therefore most CSF patients are not expected to completely recover from this chronic condition (9). Thus we consider it important to identify subsets of CFS patients who have treatable infections (10). 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 (11). Evidence for association with infectious agents or their possible role in the clinical conditions cited above comes from studies where the incidence or the degree of infection were slowly suppressed by appropriate antibiotics resulting in gradual recovery from most clinical signs and symptoms. Although the data are incomplete, the results from appropriate diagnosis and antibiotic therapy studies suggest that Mycoplasmas and perhaps other microorganisms (*Chlamydia*, *Brucella*, *Coxiella*, etc.), even if they are not the initial triggers for a variety of chronic illnesses, play a major role in disease progression and patient morbidity in important subsets of chronically patients. Identifying systemic infections, such as those produced by *Mycoplasma* species (3, 6, 8, 11–14), *Chlamydia pneumoniae* (10) or human herpes virus-6 (HHV-6) (15–17), is likely to be important in the treatment of 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 and to determine if these infections were related to severity of CFS signs and symptoms.

## METHODS

*Patients*

Patients diagnosed with CFS (ICD-10, 93.3) (n=200) reported their medical history, completed a sign/symptom illness survey and had routine laboratory tests. Clinical diagnoses were obtained from referring physicians according to the latest case definition. 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. The following criteria were used for patient classification: (a) unexplained relapsing or persistent fatigue of new or definite onset which is not caused by ongoing exertion, not relieved by rest and that results in a substantial reduction of activity compared to levels prior to onset; (b) four or more of the following signs and symptoms persist for at least 6 months: (1) impaired memory or concentration was enough to reduce levels of occupational, social, or personal activities; (2) sore throat; (3) tender cervical or axillary lymph nodes; (4) muscle pain; (5) multiple pain without swelling or redness; (6) new headache; (7) unfreshened sleep; (8) post-exertion malaise lasting more than 24 h; (c) 11/18 site-specific tender points and body pain above and below the waist. Patients were selected based on a routine physical examination, their case history, and clinical signs and symptoms. Patients with substance abuse were excluded from the study.

The illness survey questionnaire included demographic information, known environmental exposures, dates of illness onset, health status before onset of illness, during illness and current health status using scores on approximately 120 signs and symptoms (http//:www.immed.org/signsympt.htm). Additionally, all subjects were questioned about medication use during the 3 months prior to the study, and they had to be free of antibiotic treatment for 2 months prior to blood collection. Age- and gender-matched controls (n=100) had to be free of disease for at least 3 months prior to data collection, and they had to be free of antibiotic treatment and regular intake of NSAID for at least 3 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×*g* for 2 min, the supernatants were discarded. Alternatively, blood plasma was prepared and the Chelex isolation procedure was followed (for HHV-6 detection). Chelex solution (200 μl) was added, and the samples were incubated at

56°C and at 100°C for 15 min 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 (19–23) 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. Additional primer sets were used to confirm the species specificity of the reaction. Purified mycoplasmal DNA (0.5–1 ng of DNA) was used as a positive control for amplification in each experimental run. 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 are present in each experiment (18–20). The amplified samples are 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 (19, 20).

*C. pneumoniae detection by forensic PCR*

PCR detection of *C. pneumoniae* was done as described above, except that the conditions and primers differed. PCR was carried out using the *C. pneumoniae*-specific primers: 5′-TGACAACGTTAGAAATACAGC-3′ (upstream) and downstream 5′-CGCCTCTCTCTCCTATAAAT-3′. 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 β-actin mRNA. The presence of amplification inhibitors was 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-labeled with digoxigenin-UTP or $^{32}$P-labeled probe.

*HHV-6 detection by forensic PCR*

PCR detection of active HHV-6A was done as described above, except that the conditions and primers differ and plasma was used for polynucleotide isolation to detect active infections. PCR reactions 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 was evaluated by agarose-gel electrophoresis. The efficiency of the PCR process was monitored by amplification of β-actin mRNA. The presence of amplification inhibitors was 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-labeled 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 1× 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 unbound 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 (19, 20).

*Signs and symptoms incidence and severity score*

Illness survey forms (http//:www.immed.org/signsympt.htm) were given to each patient that analyzed the most common signs and symptoms of chronic illnesses at the time the blood sample was drawn, before and after onset of illness (19). Patients marked the intensity of 114 signs/symptoms prior to and after onset of illness on a 10-point self-rating rank scale (0: none; 10: extreme). The 114 questions were then grouped into 28 categories containing 3–9 questions each. An average score for each category was calculated as the average change of the intensity of all questions in the category (score=sum of differences between self-rating values prior to and after onset of illness divided by the number of questions in the category). The data from prior to onset of illness and after onset as well as within the last week before the blood was drawn were compared. A significant difference was obtained if the score after onset/within the last week was three or more points higher than prior to the illness (19). This was used to determine the incidence of change in signs/symptoms among groups of patients. Additionally, the average score change with illness and the duration of illness were correlated with the results of different infections found in blood. Surveys of patients with negative test results were used to compare score values (19).

*Statistics*

Subjects' demographic characteristics were assessed using descriptive statistics and students' *t*-tests

TABLE 1. *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 2. *Comparison of chronic infections between CSF patients and healthy subjects*

| | No infection (%) | HHV-6 infected (%) | Sign | *Mycoplasma* species infected (%) | Sign | *C. pneumoniae* infected (%) | Sign |
|---|---|---|---|---|---|---|---|
| CFS patients | 58 (29) | 61 (30.5) | | 104 (52) | | 15 (7.5) | |
| Control subjects | 88 (88) | 9 (9) | $p<0.01$ | 7 (7) | $p<0.001$ | 1 (1) | $p<0.01$ |

(independent samples test, *t*-test for equality of means, two-tailed). The 95% confidence interval was chosen for minimal significance. Illness survey data were statistically analyzed using Spearman rank correlation and Mann-Whitney tests.

## RESULTS

### *Patients and control subjects*

Demographic features of the patients are presented in Table 1. Patients and control subjects were approximately similar in age (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. All patients fulfilled the current international CDC case definition for chronic fatigue syndrome (2) None of the patients or control subjects had taken NSAID medication or antibiotics for at least 4 weeks before mycoplasma testing was performed.

Chronic infections were not found in 58 of 200 CFS patients (29%) and 88 of 100 control subjects (88%) (Table 2). When we examined CFS patients' blood for the presence of chronic infections, *Mycoplasma* species infections were found in 104 of 200 CFS patients (52%) and 7 of 100 control subjects (7%). *C. pneumoniae* infections were found in 15 of 200 CFS patients (7.5%) and in 1 of 100 control subjects (1%). HHV-6 infections were found in 61 of 200 CFS patients (30.5%) and 9 of 100 control subjects (9%). 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 2). We did not find any multiple co-infections in control subjects.

### *Mycoplasma species infections in CFS patients*

Using species-specific primers and forensic PCR the incidence of various *Mycoplasma* spe-

TABLE 3. *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*[a] | 56 | 28 |
| *M. fermentans*[a] | 46 | 23 |
| *M. hominis*[a] | 33 | 16.5 |
| *M. penetrans*[a] | 18 | 9 |
| Single mycoplasmal infection | 59 | 29.5 |
| Multiple mycoplasmal infections | 45 | 22 |
| *M. fermentans*+*M. hominis* | 14 | 7 |
| *M. fermentans*+*M. pneumoniae* | 20 | 10 |
| *M. pneumoniae*+*M. hominis* | 6 | 3 |
| *M. fermentans*+*M. hominis*+*M. pneumoniae* | 5 | 2.5 |
| 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 |

[a] Alone or in combination with another species of *Mycoplasma*.

cies 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 3). 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 significant ($p<0.001$).

*Multiple mycoplasmal infections in CFS patients*

Single infections with one of the four mycoplasmas tested were observed in 59 of the 104 (56.7%) *Mycoplasma*-positive patients or 29.5% of the total (Table 3). In the seven control subjects that were positive for mycoplasmal infections we found two controls positive for *M. fermentans*, three for *M. pneumoniae*, one for *M. hominis*, and one for *M. penetrans.* Similar to a previous study (19), the most commonly observed infection in CFS patients 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 mycoplasma-positive patients (43.3% or 22.5% of all patients), whereas single infections were found in 59/104 (56.7% or 29.5% of all patients). Similar to our previous results (19), we have not found patients positive for all four of the *Mycoplasma* species tested. In previous studies on North American (19) and European (11) CFS patients with multiple mycoplasmal infections, all of these 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 was *M. fermentans* plus *M. pneumoniae* (20 of 104 *Mycoplasma*-positive 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 3).

*Multiple co-infections with HHV-6 in CFS patients*

Active HHV-6 infections were found in the blood plasma of 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 active HHV-6 infections detected in plasma in *Mycoplasma*-positive and -negative patients, we found that there was no preference for HHV-6 infections in *Mycoplasma*-infected patients (Table 3). In 104 *Mycoplasma*-positive patients HHV-6 infections were found in 32 patients (30.7%), whereas in *Mycoplasma*-negative patients HHV-6 infections were found in 29 of 96 patients (30.2%) (Table 3). 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 2).

*Multiple co-infections with* C. pneumoniae *in CFS patients*

*C. pneumoniae* infections were found in 15 of 200 CFS patients (7.5%) (Table 3) and one control subject out of 100 that also did not have mycoplasmal or HHV-6 infections (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 104 *Mycoplasma*-positive patients *C. pneumoniae* infections were found in 8 patients (7.7%), whereas in 98 *Mycoplasma*-negative patients *C. pneumoniae* infections were found in 7 patients (7.3%). Similarly, in 61 HHV-6-positive patients *C. pneumoniae* infections were found in 5 patients (8.2%), whereas in 139 HHV-6-negative patients *C. pneumoniae* infections were found in 10 patients (7.2%) (Table 3).

*Signs and symptoms incidence and severity scores*

Similar to a previous study (19), we found that the severity of signs/symptoms in CFS patients was independent of the time of onset of

TABLE 4. *Changes in average severity of signs and symptoms scores in CFS patients with single (*Mycoplasma *species, HHV-6 or* C. pneumoniae*) or multiple (combinations of* Mycoplasma *species, HHV-6 or* C. pneumoniae*) infections in their blood after onset of illness*

| Category[a] | Average score value changes[b] | | Average score value changes | | | |
|---|---|---|---|---|---|---|
| | Infection present | | No. of infections identified | | | |
| | None | Positive | One | Two | Three | Four |
| Fatigue/sleep problems | 2.99 | 3.49 | 3.31 | 3.47 | 3.66 | 3.52 |
| Depression | 3.43 | 4.16 | 3.67 | 4.00 | 4.70 | 4.56 |
| Memory problems | 2.75 | 3.29 | 3.09 | 3.11 | 3.57 | 3.53 |
| Balance problems | 2.54 | 3.76 | 3.46 | 3.65 | 4.00 | 3.95 |
| Muscle pain/ache | 3.21 | 4.17 | 3.61 | 3.88 | 4.75 | 4.71 |
| Joint pain/ache | 2.21 | 2.64 | 2.41 | 2.92 | 3.18 | 3.16 |
| Infections | 2.39 | 3.39 | 3.34 | 3.41 | 3.53 | 3.48 |
| Skin disorders | 1.86 | 2.33 | 2.22 | 2.41 | 2.53 | 2.51 |
| Hair/scalp disorders | 1.25 | 1.79 | 1.68 | 1.82 | 1.84 | 1.99 |
| Skin rash/sensitivity | 1.49 | 2.31 | 2.26 | 2.39 | 2.55 | 2.59 |
| Genital disorders | 1.33 | 2.19 | 1.91 | 2.22 | 2.45 | 2.52 |
| Alimentation | 0.90 | 1.37 | 1.27 | 1.38 | 1.67 | 1.69 |
| Sense disorders | 2.33 | 2.51 | 2.39 | 2.58 | 2.88 | 2.72 |
| Head/neck ache | 2.00 | 2.39 | 2.30 | 2.44 | 2.56 | 2.40 |
| Swelling (tissue) | 2.60 | 2.82 | 2.76 | 2.86 | 2.81 | 2.97 |
| Night sweats/fever | 2.20 | 2.48 | 2.43 | 2.59 | 2.73 | 2.74 |
| Gastrointestinal problems | 1.38 | 2.67 | 2.49 | 2.85 | 2.99 | 2.98 |
| Urinary problems | 2.10 | 2.48 | 2.41 | 2.59 | 2.69 | 2.66 |
| Bleeding | 2.22 | 2.31 | 2.29 | 2.50 | 2.63 | 2.61 |
| Mouth cavity problems | 1.20 | 1.69 | 1.63 | 1.67 | 2.00 | 1.99 |
| Visual disorders | 1.31 | 1.57 | 1.51 | 1.61 | 1.73 | 1.71 |
| Audial disorders | 2.00 | 2.60 | 2.17 | 2.83 | 2.71 | 2.75 |
| Eye problems | 2.28 | 1.96 | 2.69 | 2.31 | 1.63 | 1.61 |
| Taste/smell problems | 2.26 | 2.46 | 3.00 | 2.44 | 2.81 | 1.76 |
| Nasopharyngeal problems | 1.72 | 2.18 | 2.54 | 2.18 | 2.19 | 1.95 |
| Breathing problems | 1.93 | 2.64 | 2.38 | 2.10 | 2.94 | 2.95 |
| Heart problems | 1.97 | 2.26 | 2.18 | 1.86 | 2.64 | 2.24 |
| Chemical sensitivity/allergy | 2.65 | 2.29 | 2.58 | 1.97 | 2.52 | 2.17 |

[a] Severity of signs and symptoms was assessed using a Patient Illness Survey Form that included 114 signs and symptoms.

[b] The intensity of signs and symptoms was marked by patients on a 10-point scale (0: none; 10: extreme) prior to and after onset of illness. Scores were determined in each category (3–9 questions) as the sum of differences between values prior to and after onset of illness/number of questions in the category. Differences between scores of patients without evidence of infection compared to patients with evidence of infection were significant ($p<0.01$), and differences in scores between patients with multiple infections compared to patients with one infection were significant ($p<0.01$).

illness, and the average of severity score was similar during the week the blood was drawn compared with the values after the onset of the disease. All categories for signs and symptoms showed an increase in score values (3 or more points) after onset of illness in a majority of the patients. Similar to previous findings (19), significant differences in the duration of patients' illnesses between the different infections were not found (data not shown).

To evaluate the possible influence of specific bacterial or viral infections or multiple co-infections on the severity of illness we compared the average increase of the various scores prior to onset of illness with the values after onset of illness and also during the week the blood was drawn. Significant differences in scores were not found between the onset of illness and at the time of drawing of blood. As found previously (19), the highest increases in score values of CFS patients were found in fatigue/sleep problems, depression, memory loss, balance disturbances, muscle and joint pain or problems, head/neck aches, and night sweats/fevers. Significant

differences were not found in the scores of signs and symptoms between patients with different types of infections (*Mycoplasma* species, HHV-6 or *C. pneumoniae*) or with different *Mycoplasma* species (data not shown). However, there were differences between the patients with multiple infections of any type and those with single infections and also between those patients with any evidence of infection compared to those without evidence of infection (Table 4). Greater increases in the severity of signs and symptoms were found in patients with infections compared to the other patients ($p<0.01$), and there was a tendency for greater scores in patients with multiple infections of any kind (Table 4). This became significant ($p<0.01$) when scores of patients with multiple infections were compared to patients with only one infection of any type (Table 4); however, we did not find significant differences between the scores of patients with particular combinations of multiple infections. In addition, when the incidence rates of increase in particular signs/symptoms were compared between patients with evidence of multiple infections and no evidence of infection, there were some differences. As expected, patients with infections scored higher (more severity of signs/symptoms) in categories associated with infections (night sweats, fever, gastrointestinal problems, urinary problems, dental problems, muscle, joint pain). Patients with evidence of chronic infections had higher incidence of signs/symptoms in most categories (Table 4).

## DISCUSSION

Chronic infections are a feature of chronic fatiguing illnesses such as CFS in a rather large subset of patients. Previously we studied American and European CFS patients and found that most had mycoplasmal infections (11, 19, 20). Our results are similar to data published by others who studied CFS patients and also found widespread evidence of mycoplasmal infections (12–14). 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 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). We also found that CFS patients infected with multiple *Mycoplasma* species generally had a longer history of illness, suggesting that patients may have contracted additional mycoplasmal infections with time (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 revealed that 68.6% of patients had one or more species of *Mycoplasma* in their blood, and most patients had only single species infections. In contrast to North American patients, the most common species found was *M. hominis*. This could indicate differences in demography and exposures between North American and European 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 *Mycoplasma* species could be detected (18).

Mycoplasmas are small, free-living, self-replicating prokaryotes without cell walls of the class *Molecutes* (24–26). Although mycoplasmas are commonly found in the oral cavity, urogenital tract and as symbiotic gut flora, 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, Mycoplasmas, such as *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 patient morbidity in rheumatoid arthritis (18, 27), systemic lupus erythematosus (28), demyelinating and axonal neuropathies (29), HIV-AIDS (3, 24, 26, 30) and chronic respiratory conditions (31–33). Mycoplasmal infections have also been reported as co-infections with other microorganisms (34, 35).

Certain types of viral infections are commonly found in CFS patients, one of the most

common being HHV-6 (15–17). Antibodies against HHV-6 are routinely found in CFS patients, and most of these patients have the HHV-6A variant (15–17). HHV-6 appears to play a role as a major contributing factor in several chronic illnesses (15, 16). Although several studies have associated HHV-6 with CFS (17, 36–40), there are also reports that could not find an association with CFS (41, 42).

An unexpected finding in our study was that *Mycoplasma* species, *C. pneumoniae* and HHV-6 infections were not clustered together in CFS patients. When we examined the incidence of *C. pneumoniae* or HHV-6 in *Mycoplasma*-positive or -negative CFS patients, we found no differences, indicating that clustering of these infections is not more likely in certain CFS patients. Although patients with multiple infections had on average more severe signs and symptoms, we did not find differences between the types of infections and signs and symptoms. The signs and symptoms of CFS are relatively non-specific; therefore, the possibility of unique patient subsets with different patterns of signs/symptoms based on the types of infections present is probably unlikely and consistent with the results that we obtained.

We used PCR to detect the presence of specific microbial genes in blood or blood plasma. In contrast to the detection of immunological responses against infectious agents using serological techniques the detection of specific gene sequences has to be considered as direct evidence for the presence of living microorganisms in the tested sample. Therefore, PCR has been established as a highly sensitive routine procedure for various infections diseases, such as hepatitis and tuberculosis, that allows identification of infections prior to serological changes and even in immunocompromised patients. Use of PCR techniques for detection of microorganism infections 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. For example, in the determination of mycoplasmal infections we used primer sets for various genes found in specific species (19–23) and for conformation supplemented these primers with additional primer sets as new sequence information became available. This was done to eliminate possible cross-reactions with *Mycoplasma*-related organisms (21). Similar to a previous study (20), we examined the reliability of the methods by performing multiple assays (repeated up to six 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, mycoplasmas were added to control blood samples at various concentrations. We were able to detect specific products down to a few 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 finding of multiple infections in CFS patients has implications in the treatment of CFS. Although treatment of particular types of chronic infections has shown some benefit in certain CFS patients, this result is far from universal (7, 8). Identifying and treating the possible multiple, specific chronic infections in individual CFS patients may be useful in the clinical management of this illness.

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