Clinical study

High frequency of systemic mycoplasmal infections in Gulf War veterans and civilians with Amyotrophic Lateral Sclerosis (ALS)

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Summary The presence of systemic mycoplasmal infections in the blood of Gulf War veterans (n = 8) and civilians (n = 28) with Amyotrophic Lateral Sclerosis (ALS) and age matched controls (n = 70) was investigated by detecting mycoplasma gene sequences with forensic Polymerase Chain Reaction (PCR) and back hybridization with a radiolabeled internal oligonucleotide probe. Almost all ALS patients (30/36 or ~83%) showed evidence of Mycoplasma species in blood samples, whereas <9% of controls had blood mycoplasmal infections (P < 0.001). Using PCR ALS patients with a positive test for any mycoplasmal infection were investigated for the presence of M. fermentans, M. pneumoniae, M. hominis and M. penetrans in their blood. All Gulf War veterans with ALS were positive for M. fermentans, except one that was positive for M. genitalium. In contrast, the 22/28 civilians with detectable mycoplasmal infections had M. fermentans (13/22, 59%) as well as other Mycoplasma species in their blood, and two of the civilian ALS patients had multiple mycoplasma species (M. fermentans plus M. hominis). Of the few control patients that were positive, only two patients (2/70, 2.8%) were positive for M. fermentans (P < 0.001). The results support the suggestion that infectious agents may play a role in the pathogenesis and/or progression of ALS, or alternatively ALS patients are extremely susceptible to systemic mycoplasmal infections.

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INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is an adult onset, idiopathic, progressive degenerative disease affecting both central and peripheral motor neurons. Patients with ALS show gradual progressive weakness and paralysis of muscles due to destruction of upper motor neurons in the motor cortex and lower motor neurons in the brain stem and spinal cord, ultimately resulting in death, usually by respiratory failure.1–4 The overall clinical picture of ALS can vary, depending on the location and progression of pathological changes found in nervous tissue.3,4

Although the cause of ALS remains unknown, there are several hypotheses on its pathogenesis: (a) accumulation of glutamate causing excitotoxicity; (b) autoimmune reactions against motor neurons; (c) deficiency of nerve growth factor; (d) dysfunction of superoxide dismutase due to mutations; and (e) chronic infection(s).5–12 Of these hypotheses, the role of chronic infections has attracted attention with the finding of enterovirus sequences in 15 of 17 spinal cord samples from ALS patients by Polymerase Chain Reaction (PCR).11 Although others had failed to detect enterovirus sequences in spinal cord samples from patients with or without ALS,15,16 some findings suggest that infectious agent(s), such as enterovirus, may play a role in the etiology of ALS.11 The possibility that one or more infectious agents could interact to cause ALS remains a distinct but unproven possibility.13

Here we report on detecting the presence of chronic, systemic bacterial (mycoplasmal) infections in the blood of ALS patients by PCR. Mycoplasmas are prokaryotes without cell walls of the class Mollicutes. They are small, free living, self-replicating organisms, some of which are pathogenic and have the capacity to invade various tissues, including the central nervous tissues.10–18 Although mycoplasmas are found commonly in the oral cavity and as symbiotic gut flora, some pathogenic species can cause acute and chronic illnesses when they penetrate into the blood vascular system and systemically colonize organs and tissues.17,18 For example, mycoplasmas, such as M. penetrans, M. fermentans, M. hominis and M. pneumoniae, can enter a variety of human tissues and cells and cause systemic signs and symptoms. Mycoplasmas have also been shown to have a complex relationship with the immune system.17,18 They are very effective at evading host immune responses, and synergism with other infectious agents has been seen.19 These properties make Mycoplasma species attractive as one of several possible infectious agents that could be involved in the pathogenesis or progression of ALS.

MATERIALS AND METHODS

Patients

Sporadic ALS patients had their diagnoses established clinically or pathologically according to established international criteria by a neurologist.9 Patients with ALS show gradual progressive weakness and paralysis of muscles due to destruction of upper motor neurons in the motor cortex and lower motor neurons in the brain stem and spinal cord.1–4 The diagnosis of ALS was obtained, if the following signs were found by clinical, electrophysiological or neuropathological examination: (a) lower motor neuron degeneration, (b) upper...
motor neuron degeneration, (c) progressive spread within a region or to other regions. Other disease processes that might explain the signs of upper or lower motor neuron degeneration were excluded by electrophysiological examination and/or neuroimaging (CT-scan, MRI). In a few patients muscle biopsies were obtained for further confirmation. None of the patients or control subjects had taken NSAP medication or antibiotics for at least 4 weeks before mycoplasma testing was performed. The patients were Gulf War veterans (all male) from the USA (n = 4), Great Britain (n = 3) and Australia (n = 1) and civilians (22 male, 5 female) from Great Britain (n = 16) and the USA (n = 11). The mean age of the Gulf War ALS patients was 34.7 years; whereas, the mean age of the civilian ALS patients was 44.6 years. Age- and sex-matched healthy control subjects (n = 70, average age = 42.6) were from the USA (n = 55), Great Britain (n = 10) and the Netherlands (n = 5) (Table 1).

**Blood samples**

Venous blood (5–10cc) was drawn from ALS patients and control subjects in plastic purple top tubes (containing EDTA), mixed, cooled and some were immediately frozen with dry ice (foreign shipments). The blood samples were immediately shipped with wet ice (overnight domestic shipments) or frozen with dry ice by air courier (foreign shipments) to the Institute for Molecular Medicine and International Molecular Diagnostics, Inc. of Huntington Beach, CA and processed for forensic PCR as described below.

**Purification of DNA**

Whole blood (50μl) was used for preparation of DNA using Chelex (Biorad, Hercules, USA) as follows. Blood cells were lysed with nanopure water (1.3ml) at room temperature for 30min. After centrifugation at 13000g for 2min, the supernatants were discarded. Chelex solution (200m) was added, and the samples were incubated at 56°C and at 100°C for 15min each. Aliquots from the centrifuged samples were used immediately for PCR or stored at −70°C until use. Multiple mycoplasma tests were performed on all patients.

**Amplification of gene sequences**

Amplification of the target gene sequences (Table 2) was performed by forensic PCR in a total volume of 50μl PCR buffer (10mM Tris–HCl, 50mM KCl, pH 9) containing 0.1% Triton X-100, 200μm each of dATP, dCTP, dGTP, dTTP, 100pmol of each primer (Table 2), and 0.5–1μg of chromosomal DNA. Purified mycoplasmal DNA (0.5–1ng of DNA) was used as a positive control for amplification. The amplification was carried out for 35–40 cycles with denaturing at 94°C and annealing at 60°C (genus-specific primers and M. fermentans) or 55°C (M. pneumoniae, M. hominis, M. penetrans). Extension temperature was 72°C in all cases. Finally, product extension was performed at 72°C for 10min. Negative and positive controls were present in each experimental run. ALS patient and control samples were blinded and processed together.

**Southern blot confirmation**

The amplified samples were run on a 1% agarose gel containing 5μl/100ml 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 the transfer, UV cross-linking was performed. The membranes were prehybridized with hybridization buffer consisting of 1× Denhardt’s solution and 1mg/ml salmon sperm as blocking reagent. Membranes were then hybridized with 32P-labeled internal probe (107cpm per bag). After hybridization and washing to remove unbound probe, the membranes were exposed to autoradiography film for 7 days at −70°C. The results were read by a technician who was blinded to the nature of the samples.

### Table 1 Clinical characteristics of patients with ALS or control subjects

<table>
<thead>
<tr>
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<th>ALS patients</th>
<th>Control subjects</th>
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<tbody>
<tr>
<td></td>
<td>Civilians</td>
<td>Gulf War veterans</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>28</td>
<td>8</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>44.6 ± 12.0 years (32–77 years)</td>
<td>34.7 ± 3.8 years (31–45 years)</td>
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<tr>
<td></td>
<td>(range)</td>
<td>(range)</td>
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<tr>
<td><strong>Duration of ALS symptoms</strong></td>
<td>12 ± 8 months</td>
<td>17 ± 16 months</td>
</tr>
<tr>
<td><strong>Number of patients with affected regions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulbar</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Cervical</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>Thoracic</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Lumbosacral</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td><strong>Number of patients with lower motor neuron signs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>weakness</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>atrophy</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>fasciculation</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td><strong>Upper motor neuron signs</strong></td>
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<td></td>
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<tr>
<td>pathological reflexes</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>clonus</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>cramps, spastic tone</td>
<td>21</td>
<td>6</td>
</tr>
</tbody>
</table>

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RESULTS

Clinical features of ALS patients

Analysis of ALS patients used in obtaining our preliminary data indicates that none had familial ALS (Table 1). In addition to ALS features, such as muscle weakness and wasting, fasciculation, speech and swallowing problems, cramping, among other signs (Table 1), several ALS patients had additional signs/symptoms. For the most part, these were rheumatic signs and symptoms or allergies. In addition to ALS features, patients had rheumatic signs/symptoms (26%), history of asthma, bronchitis or pneumonia (33%), allergies (26%), rashes (52%), night sweats (41%), diarrhea (29%), digestive problems (37%), sleep problems (44%), nausea (41%), overall fatigue (82%), dental problems (41%), and evidence of infections (data presented here). Duration of symptoms were not significantly different between civilians and Gulf War veterans diagnosed with ALS.

Mycoplasmal infections in ALS patients

We have studied the presence of systemic microbial infections in a preliminary number of ALS patients. We found that 8/8 Gulf War veterans diagnosed with ALS from three nations had systemic mycoplasmal infections. All but one patient had M. fermentans infections, and one patient had a systemic M. genitalium infection. In 22/28 nonmilitary ALS patients from the USA and Great Britain we have also found blood mycoplasmal infections. Of the mycoplasma-positive civilian patients who were further tested for M. penetrans, M. fermentans, M. hominis and M. pneumoniae, most were positive for M. fermentans (13/22, 59%), but we did find other Mycoplasma species, such as M. hominis (7/22, 31%) and M. pneumoniae infections (2/22, 9%). Two civilian ALS patients had multiple mycoplasmal infections (M. fermentans plus M. hominis, 9%). The difference in incidence of mycoplasmal infections between ALS patients and control subjects was highly significant (P < 0.001).

Using an internal probe mycoplasmal infections were confirmed with Southern back-hybridization of the PCR product. This technique is extremely sensitive and can specifically detect mycoplasma DNA down to 1–10 fg mycoplasma DNA in a clinical sample. Using internal probe mycoplasmal infections were confirmed with Southern back-hybridization of the PCR product. This technique is extremely sensitive and can specifically detect mycoplasma DNA down to 1–10 fg mycoplasma DNA in a clinical sample. For example, some of the back-hybridization results for M. hominis infections are presented in Fig. 1.

DISCUSSION

The involvement of persistent, chronic infectious agents in ALS was shown recently with the discovery of enterovirus RNA sequences in a high proportion (~88%) of formalde-hyde fixed spinal cord samples and at lower frequency in CSF patients but at only very low frequencies in other patients with neurological diagnoses or in control subjects. The detection of enterovirus sequences in patients with ALS supports a link between infectious agents and ALS, but the exact role of enteroviruses in the pathogenesis of ALS remains to be demonstrated. Indeed, recent re-examination of this finding has not confirmed the presence of enterovirus sequences in the brain or spinal cord of ALS patients. We have found a very high proportion of ALS patients have evidence of blood mycoplasmal infections, and this suggests that certain bacterial infections might be important in ALS morbidity.
M. fermentans has been found in the CNS of patients with lethal mycoplasmal infections.16,17 We found that all but one of the Gulf War veterans with ALS had M. fermentans infections. This finding is consistent with the finding of predominantly M. fermentans in the Gulf War Illness patients who are mycoplasma positive in blood tests.27-29

Similar to the possible role of enteroviruses in the pathogenesis of ALS, the exact role that mycoplasmal infections play in the pathogenesis or progression of ALS is not known. They could be cofactors in the pathogenesis of ALS, or they could simply be opportunistic infections that cause morbidity in ALS patients, such as the respiratory, rheumatic symptoms and other problems often found in ALS patients. They could also be involved in the progression of ALS rather than in its inception. The mean age of ALS patients, especially the Gulf War cohort, was generally lower than in other studies, suggesting that the ALS group studied here may not be indicative of ALS patients as a whole. For example, the military ALS cases were generally younger than civilian ALS patients, and generally younger than ALS patients as a whole, but this may be related to the average age of veterans of the Gulf War, most of which were under the age of 25.

Mycoplasmas like M. fermentans are particularly interesting because they have the capacity, like enteroviruses7-11 to penetrate the CNS, and they possess the potential to cause persistent neurological signs and symptoms.16-18 Our results on mycoplasmal infections in ALS patients suggest that coinfections with certain persistent viruses and bacteria might be important in ALS. We also found that ALS patients have some of the signs and symptoms seen in a variety of chronic illness patients, consistent with their having mycoplasmal infections that are also found in these patients.18,20,28,29

Similar to chronic mycoplasmal infections,21-23,27-29 enteroviruses have also been found in patients with chronic myocardiitis30 and chronic fatigue.31 It is interesting that both enteroviruses and mycoplasmas have the capacity to cause slow, persistent infections that can eventually result in cellular dysfunction and eventually cell death,12,20,32 and mycoplasmal infections have been implicated in infectious neurological diseases.17,28 Mycoplasmas have also been implicated in autoimmune diseases,17,18,20,28,29,32 and one hypothesis on the pathogenesis of ALS suggests autoimmune involvement.33 The deficits in glutamine uptake by the brain and spinal cord seen in ALS patients3 could also be related to virus2 and/or mycoplasma induced changes in membrane transport.19 Mycoplasmal infections can also affect gene expression.34 Although the exact role of mycoplasmal infections in ALS could not be determined in this study, the results suggest that these intracellular infections could promote the condition or enhance its progression. It is extremely unlikely that such infections on their own cause ALS without additional cofactors or coordinate genetic causes.

We were able to detect mycoplasmal genetic sequences in the blood of a high percentage of ALS patients using a very sensitive and specific assay. The sensitivity of mycoplasmal detection by the forensic PCR method we used was assessed by the detection of control mycoplasma DNA and by internal Southern hybridization using mycoplasma species specific probes. Using serial dilutions of mycoplasma DNA, the method was able to detect as low as a few fg of mycoplasma DNA.20-23 In other experiments, M. fermentans was added to control blood samples at various concentrations. We were able to detect specific products down to 10 ecu/ml blood. Thus with the use of highly specific Southern hybridization this procedure can result in specific test results of high sensitivity and specificity, down to the presence of a few microorganisms in a clinical sample.20-23

More than 100 bacteria belong to the genus of Mycoplasma. They are widely distributed in nature, and they have been found attached to the external surfaces of host cells or residing and replicating inside host cells.17,18 It has been only recently that some mycoplasmas have been identified as important pathogens in humans, animals, plants and insects.17,18,28,32 In humans mycoplasmas have been found to be associated with certain chronic and acute diseases where they can function as causative agents, cofactors or opportunistic infections that cause patient morbidity.16-18,28,32 For example, mycoplasmas have been found at high incidence as systemic infections in patients with respiratory illnesses, urogenital infections, Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, Fibromyalgia Syndrome, Rheumatoid Arthritis, autoimmune diseases, complications affecting the CNS, cardiovascular infections, oral infections, periarticular diseases, sexually transmitted diseases and systemic infections in leukemias or immunosuppression diseases, such as HIV-AIDS.16,18,28,32 Although the incidence of mycoplasmal infections in these illnesses was high (~40-60%), it was much lower than found here in ALS patients. Future efforts will be directed at finding whether mycoplasmal infections are opportunistic or play a role in causing neuropathology in ALS patients and whether treatment of mycoplasmal infections in these patients is of any value in the clinical management of ALS.35

REFERENCES