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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 *Mycoplasama* 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, idopathic, 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).[4–12] 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,[14,15] 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]

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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.[16–18] 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.[3] 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 (5–10 cc) 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.[20–23] Previous studies have shown no differences in sample results between samples shipped overnight with ice or shipped with dry ice.[23]

Purification of DNA

Whole blood (50 µl) was used for preparation of DNA using Chelex (Biorad, Hercules, USA) as follows. Blood cells were lysed with nanopure water (1.3 ml) at room temperature for 30 min. After centrifugation at 13 000*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 min 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.[21,22]

Amplification of gene sequences

Amplification of the target gene sequences (Table 2) was performed by forensic PCR 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 (Table 2), 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 35–40 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 experimental run. ALS patient and control samples were blinded and processed together.[21–23]

Southern blot confirmation

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 the transfer, UV cross-linking was performed. The membranes were prehybridized with hybridization buffer consisting of 1× 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.[20–22] 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

	ALS patients		Control subjects
	Civilians	Gulf War veterans	
n	28	8	70
Age			
Mean ± SD	44.6 ± 12.0 years	34.7 ± 3.8 years	42.6 ± 4.9
(range)	(32–77 years)	(31–45 years)	(29–58)
Duration of ALS symptoms	12 ± 8 months	17 ± 16 months	—
Number of patients with affected regions			
Bulbar	13	5	
Cervical	20	5	
Thoracic	10	4	
Lumbosacral	18	3	
Number of patients with			
Lower motor neuron signs			
weakness	15	5	
atrophy	8	4	
fasciculation	17	7	
Upper motor neuron signs			
pathological reflexes	5	4	
clonus	5	3	
cramps, spastic tone	21	6	

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Table 2 Sequences, target genes and size of amplified products from mycoplasmal DNA used for mycoplasma genus-specific and species-specific PCR

Sequence name	Sequence	Target	Size [bp]	Source
GPO1 primer	ACT CCT ACG GGA GGC AGC AGT A	16S mRNA	717	Van Kuppeveld et al.[24]
MGSO primer	TGC ACC ATC TGT CAC TCT GTT AAC CTC	genus		
UNI- probe	TAA TCC TGT TTG CTC CCC AC			
SB 1 primer	CAG TAT TAT CAA AGA AGG GTC TT	*tuf* gene	850	Berg et al.[25]
SB 2 primer	TCT TTG GTT ACG TAA ATT GCT	*M. fermentans*		
SB 3 probe	TTT TTC AGT TTC GTA TTC GAT G			
MP5-1 primer	GAA GCT TAT GGT ACA GGT TGG	unknown gene	144	Bernet et al.[26]
MP5-2 primer	ATT ACC ATC CTT GTT GTA AGG	*M. pneumoniae*		
MP5-4 probe	CGT AAG CTA TCA GCT ACA TGG AGG			
Mhom1 primer	TGA AAG GCG CTG TAA GGC GC	16S mRNA	281	Van Kuppeveld et al.[24]
Mhom2 primer	GTC TGC AAT CAT TTC CTA TTG CAA A	*M. hominis*		
GPO1 probe	ACT CCT ACG GGA GGC AGC AGT A			
IMM-7 primer	GGA AAC GGG AAT GGT GGA ACA GAT	P35 gene (lipoprotein)	704	Haier et al.[21]
IMM-5 primer	TTC TGC TAA TGT TAC AGC AGC AGG	*M. penetrans*		
IMM-3 probe	AGG GAA TCT GTG ATC TTA TTC			

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.[20–23] For example, some of the back-hybridization results for *M. hominis* infections are presented in Fig. 1.

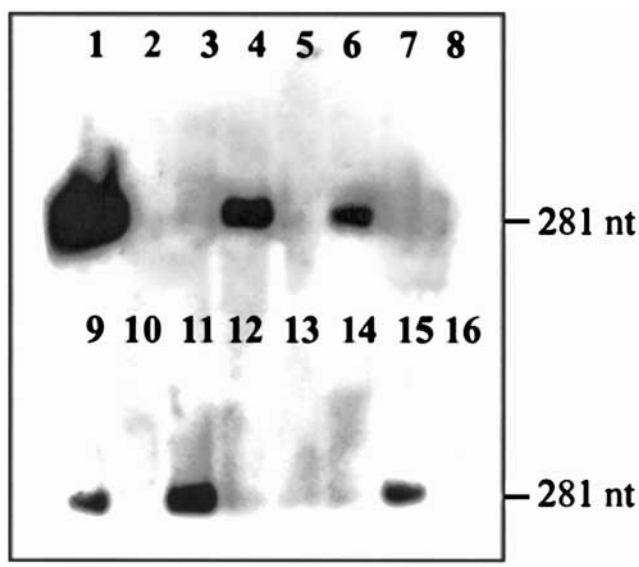

Fig. 1 Detection of mycoplasmal DNA by PCR and back-hybridization of several control and ALS patient blood samples. PCR and back-hybridization autoradiography using specific ^{32}P-labeled internal probe was performed as described in Materials and Methods. Mycoplasma DNA was detected using the primers shown in Table 2. The example shown in this figure is detection of *M. hominis*. Lane 1, positive control, 100 fg mycoplasma DNA; lane 2, negative control; lanes 3–8, patient samples; lane 9, positive control, 10 fg mycoplasma DNA; lane 10, negative control; lanes 11–16, patient samples. Note that duplicate samples are in lanes 4 and 11. The size marker is 281 nucleotides.

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 formaldehyde 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.[7,11] 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.[15] 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.

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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 enteroviruses[7,11] 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 myocarditis[30] 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,[11,29,32] 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 patients[5] could also be related to virus-[7] 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 ccu/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 *Mycoplasmae*. 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, cardiac infections, oral infections, peridontal 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.[32]

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