

Deregulation of the 2.5A synthetase RNase L antiviral pathway by *Mycoplasma spp.* in subsets of Chronic Fatigue Syndrome

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ABSTRACT

The deregulation of the 2.5A synthetase RNase L antiviral pathway and the prevalence of *Mycoplasma spp.* in subsets of Chronic Fatigue Syndrome [CFS] have been separately reported in the scientific literature. We hypothesised that a co-morbid pathophysiological mechanism involving infection by *Mycoplasma spp.* and the deregulation of the 2,5A synthetase / RNase L antiviral pathway may exist in CFS. Therefore, 186 consecutive CFS patients were enrolled. *Mycoplasma* detection was performed using forensic polymerase chain reaction. For RNase L determination, a radioactive probe was used to label 2,5A binding proteins in unfractionated peripheral blood mononuclear cell extracts. *Mycoplasma*-infected CFS patients presented with significantly elevated RNase L-ratio, compared to non-infected age- and sex-matched patients [$p = 0.016$]. These results suggest that *Mycoplasma* infections may cause deregulation of the 2,5A synthetase RNase L antiviral pathway in patients with CFS.

Keywords: Chronic Fatigue Syndrome, *Mycoplasma*, 2.5A synthetase RNase L antiviral pathway

INTRODUCTION

Chronic Fatigue Syndrome [CFS] is characterised by unexplained, persistent or relapsing

chronic fatigue that is of new or definite onset (1). Several reports suggested the importance of *Mycoplasma spp.* infections (2-7) and the activation / deregulation of the 2',5' oligoadenylate [2,5A] synthetase RNase L antiviral pathway (8-13) in subsets of CFS patients.

Mycoplasma are prokaryotes that lack a cell wall and certain cellular organelles. They contain circular DNA and some ribosomes. These microorganisms can cause opportunistic infections, as seen in the acquired immune deficiency syndrome and other chronic illnesses, and may produce some of the signs and symptoms seen in CFS (14, 15). Another feature of chronic illnesses such as CFS is the deregulation of the 2,5A synthetase RNase L antiviral pathway, which is characterised by the presence of a 37 kDa [low molecular weight] 2,5A binding protein, instead of the high molecular weight form [80 kDa] (10). Mark that this low molecular weight RNase L is not indicative of an activated 2,5A synthetase RNase L pathway, but instead suggests an improper functioning [deregulation].

We hypothesised that a co-morbid physiopathological mechanism involving *Mycoplasma spp.* infections and the deregulation of the 2,5A synthetase / RNase L antiviral pathway may exist in subsets of CFS. Indeed, *Mycoplasma spp.* are active in stimulating several components of the immune system. They can act as polyclonal T-cell and B-cell activators (15, 16), and they can produce components that activate macrophages in vitro (17). To bring about their phagocytic activity, monocytes produce the proteolytic enzyme elastase, which enables them to pass through connective tissues. Elastase is capable of cleaving 80 kDa RNase L (18), thus causing deregulation of the antiviral pathway. Therefore, we asked whether deregulation of the 2,5A synthetase RNase L antiviral pathway is associated with increased prevalence of *Mycoplasma* infections in CFS patients? To investigate this research question, we compared the deregulated antiviral pathway in *Mycoplasma*-infected and non-infected CFS patients defined by the criteria of Fukuda et al (1).

MATERIALS AND METHODS

Patients

recruitment

The study was conducted in Brussels, at a university-based tertiary referral outpatient clinic [Vrije Universiteit Brussel – V.U.B.]. We enrolled 186 consecutive patients seeking care for prolonged fatigue as major complaint between the first of January and the end of June 1999, who complied with the Fukuda et al (1) definition. All subjects signed written informed consent, authorising the laboratory to use their blood samples for research purposes. To fulfil the CDC criteria for CFS, clinically evaluated, unexplained, persistent or relapsing chronic fatigue that is of new or definite onset, should result in a substantial reduction in previous levels of occupational, educational, social, or personal activities (1). Additionally, at least four of the following symptoms must have persisted or recurred during 6 or more consecutive months and must have not predated the fatigue: impairment in short-term memory or concentration, tender cervical or axillary lymph nodes, muscle pain, multi-joint pain, headache, unrefreshing sleep and post-exertional malaise > 24 hours (1). Any active medical condition that may explain the presence of chronic fatigue prohibits the diagnosis of CFS. Therefore, all subjects underwent an extensive medical evaluation, consisting of a

bromide in TAE buffer [0.04 M Tris-Acetate, 0.001 M EDTA, pH 8.0]. After denaturing and neutralization, Southern blotting was performed as follows. The PCR product was transferred to a Nytran membrane. After transfer, UV cross-linking was performed. Membranes were prehybridized with hybridisation buffer consisting of 1x Denhardt's solution and 1 mg/ml salmon sperm DNA as blocking reagent. Membranes were then hybridized with ³²P-labeled internal probe [107 cpm per bag]. After hybridization and washing to remove unbound probe, the membranes were exposed to autoradiography film for 1-2 days at -70°C.

RNase

L-ratio

determination

The assay is performed by 1/ preparation of a cytoplasmic extract of the patient's peripheral blood mononuclear cells, 2/ combination of this extract with a labelled probe that binds specifically to 2-5A binding proteins such as RNase L and the low molecular weight species, 3/ sodium dodecylsulfate polyacrylamide gel electrophoresis, and 4/ densitometry to determine the relative quantities of 2-5A binding proteins. A numerical value is calculated by densitometry as "the amount of low molecular weight protein present divided by the amount of native [high molecular weight] RNase L present" multiplied by a factor of 10. The next paragraph presents a more detailed description of the assay.

Within four hours of phlebotomy, peripheral blood mononuclear cells [PBMC] were separated from heparinized blood [30 ml] by Ficoll-Hypaque density gradient centrifugation. In addition, PBMC's were stored at -70°C until cytoplasmic extraction preparation. The latter was performed in the presence of protease inhibitors aprotinin, leupeptin, pepabloc-SC and EDTA [Roche Biochemicals, Mannheim, Germany]. Protease inhibitors are required for preventing proteolytic cleavage. Standard laboratory procedures were used to separate serum from coagulated blood, and to store it at -70°C until analysis. A modified Bradford assay method [Bio-Rad Laboratories, Hercules, CA] was used for quantification of total proteins in the patients' cell extracts and serum. The probe specifically attaches to 2'-5'A binding proteins like 80 kDa RNase L and 37 kDa RNase L. Briefly, 200µg of PBMC extract was incubated with a metaperiodate [10mM final concentration, pH 4.75] oxidized 2-5A trimer radiolabeled at the 3' end with ³²P-pCp as the receptor ligand, at 2-4°C for 15 minutes. In addition, it was covalently attached to the binding proteins by the addition of cyanoborohydride [20 mM in 100 mM phosphate buffer, pH 8.0]. This reduction reaction was allowed to progress for 20 minutes at 2-4°C. Sodium dodecylsulfate polyacrylamide gel electrophoresis [SDS-PAGE] buffer and a tracking dye were added to the samples, and incubated at 95°C for 5 minutes followed by separation using standard SDS-PAGE with a 4% stacking and a 10% separating gel. The gel was dried and autoradiography was used to detect the radioactivity of the marked probe [Bio-Rad Laboratories Molecular Imager® Fx, Hercules, CA]. Densitometric analysis of the autoradiographs was followed by quantification of any present 2'-5' A binding proteins [using specialized software: Quantity One® Software, Bio-Rad Laboratories, Hercules, CA]. RNase L-ratio was counted using following equation: RNase L-ratio = [low molecular weight RNase L] / [high molecular weight RNase L] x 10.

Statistics

Subjects' age characteristics were analysed using descriptive statistics and students' t-test [independent samples t-test, 2-tailed]. Sex differences were assessed using Fisher's exact test [2-tailed]. RNase L ratios were transformed logarithmically to obtain a normal distribution and stabilize the variances. Independent samples student's t-test was used to test the difference of mean log RNase L ratios between infected and non-infected patients. Equality of variances was examined using Levene's test. Mean log RNase L ratios were compared among different subsets of CFS [subgrouping in accordance to the different *Mycoplasma spp.* detected] using exactly the same statistical procedure. To investigate possible interactions between different species of *Mycoplasma*, a full factorial ANOVA [analysis of variances] was performed. Three factors were used for ANOVA [*M. pneumoniae* / *M. fermentans* / *M. hominis*] with each factor having two levels [absence or presence]. Pearson correlation coefficients were computed to search for possible associations between log RNase L and age in different subsets [*Mycoplasma* + or -]. The significance level of the different tests was at 0.05. The data were processed using SPSS 7.5 © for Windows [SPSS Inc. Headquarters, 233 S. Wacker Drive, 11th floor Chicago, Illinois 60606, USA].

RESULTS

Demographic features of the data sample are shown in Table 1. These patients presented with a mean age of 35.8 years, and the age ranged from 18 to 64. One hundred and sixty-three CFS-patients [87.6 %] were female. In fifty-five out of 186 CFS patients [29.6 %], *Mycoplasma spp.* infections could not be detected. One hundred and thirty-one patients [70.4 %] were infected by at least one *Mycoplasma spp.* Among *Mycoplasma*-infected patients, *M. hominis* was observed most frequently [n = 45; 24 % of overall sample]. *M. penetrans* was not detected in this patient group. Multiple infections were detected in 31 CFS patients [16.7 %], with *M. fermentans* – *M. pneumoniae* being the most frequently observed combination. With the absence of *M. penetrans* in mind, all possible combinations were present [Table 2].

Thirty-one out of 186 patients [16.7 %] presented with a RNase L ratio of less than 0.5, while all others had values of at least 0.5 [155 = 83.3 %]. In 98 CFS patients [52.7 %] the RNase L ratio exceeded the threshold value of 2.0. Mean logarithmic transformed RNase L ratios [log RNase L ratio] were compared among *Mycoplasma*-infected and non-infected patients [Table 3 and Figure 1]. The two groups were matched according to age [mean age of *Mycoplasma* + patients = 35.7; mean age of non-infected patients = 36.2 years; p = 0.746] and sex [113 [90 %] versus 50 [86 %] women; p = 0.470] characteristics. Mean log RNase L ratio was significantly elevated [p = 0.016] in *Mycoplasma*-infected CFS patients compared to non-infected subjects, suggesting an association between *Mycoplasma spp.* and deregulation of the 2,5A synthetase RNase L antiviral pathway in patients with CFS. Additionally, mean log RNase L ratios were compared among different subsets of CFS [subgrouping in accordance to the different *Mycoplasma spp.* detected] [Table 4 and Figure 2]. Eight patients who presented with a combination of *M. pneumoniae* and *M. hominis*, showed significantly elevated log RNase L ratios [p = 0.048], compared to age-matched [p = 0.604] non-infected CFS patients. No other significant differences were observed among subgroups, probably due to the small sample size of some groups. Indeed, interactions plots suggested higher numbers might lead to statistically significant differences [interactions

plots not shown]. These results were validated using Mann-Whitney U-test [data not shown]. A full factorial ANOVA revealed no synergic effects of different *Mycoplasma spp.* on log RNase L [data not shown].

Correlation analysis [Pearson's R, 2-tailed] revealed no statistically significant correlation between log RNase L and age in *Mycoplasma*-infected CFS patients [$p = 0.070$], nor in non-infected CFS patients [$p = 0.298$].

DISCUSSION

These results suggest interactions between *Mycoplasma* infections and deregulation of the 2.5A synthetase RNase L antiviral pathway. Indeed, mean logarithmically transformed RNase L ratios were significantly elevated in *Mycoplasma*-infected CFS patients, compared to patients with no *Mycoplasma spp.* detected. These observations suggest a co-morbid physiopathological mechanism between *Mycoplasma spp.* and the deregulation of the 2,5A synthetase / RNase L antiviral pathway in CFS patients. On the other hand, this study does not address the exact nature of these interactions.

Mycoplasma infections are likely to contribute to increased concentrations of elastase, which in turn cleaves 80 kDa RNase L into 37 kDa. We have explored another potential pathway that could explain the observed association between the presence of *Mycoplasma spp.* and deregulated RNase L antiviral activity. High molecular weight RNase L has been shown to induce apoptosis [or programmed cell death] (19,20). Conversely, the level of low molecular weight RNase L, characteristic for the deregulated pathway in CFS patients, is related to deregulation of apoptotic activity in peripheral blood mononuclear cells of CFS patients (21). Initial up-regulation of apoptotic activity in peripheral blood mononuclear cells due to deregulation of the 2.5A synthetase RNase L antiviral pathway, is followed by down-regulation due to the accumulation of its proteolytic cleavage products (21). The latter implicates a suppressed ability to eliminate intracellular antigens.

Although compelling, these results should be interpreted with caution. First, this patient sample consisted of only CFS-patients. A deregulated 2,5A synthetase RNase L antiviral pathway (10) and *Mycoplasma* infections (4) appear to be rare among healthy subjects. Therefore, no attempt was made to search for an analogue mechanism in healthy controls. Second, this sample was not randomly selected. The aim of randomisation is to prevent selection-bias. All CFS patients visiting the Chronic Fatigue Clinic between January and June 1999, who fulfilled all inclusion and exclusion criteria, participated in this trial. Consequently, selection-bias can only be due to appointment-allocation or the unblinded nature of patients' screening. Our secretaries however, were blinded to patients' medical records when they allocated the appointments. Moreover, a sample consisting of consecutive patients visiting a clinic is more likely to represent routine clinical practice. Third, most of the patients were tertiary referrals [from general physicians, rheumatologists, internists, etc.], resulting in selection of highly disabled patients. This might explain the high prevalence observed in this sample. Fourth, the validity of PCR techniques for *Mycoplasma* detection has been questioned. In our studies however, the sensitivity and specificity of the PCR method for *Mycoplasma* detection were determined by examining serial dilutions of purified

DNA from *M. fermentans*, *M. pneumoniae*, *M. hominis* and *M. genitalium*. The primers produced the expected amplification product size in all test species, which was confirmed by hybridisation using the appropriate ³²P-labeled internal probe. Amounts as low as a few fg of purified DNA were detectable for all species with the specific internal probes. There was no cross-reactivity between the internal probes of one species and the PCR product from another species. In other experiments *Mycoplasma spp.* were added to whole blood at various concentrations. Specific PCR products down to 10 ccu / ml of blood could be detected (2, 3).

Future research should establish whether these observations are associated with a particular stage of the illness or if they fluctuate over time. Moreover, one can argue other pathogens may also deregulate the antiviral pathway in patients with CFS. Candidate pathogens that have also been found in CFS patients are Cytomegalovirus (22-23), Chlamydia pneumoniae (24) or Adenovirus (25). Finally, data highlighting the disease-specificity of these interactions are not currently available. Deregulation of the 2,5A synthetase RNase L antiviral pathway has been studied in a small number of patients with primary Fibromyalgia Syndrome and Depression (10), however this has not been studied in Rheumatoid Arthritis. *Mycoplasma spp.* have been suggested to contribute to patients' morbidity in Rheumatoid Arthritis (4, 6, 26, 27), while others refute this association (28). Deregulation of the 2,5A synthetase RNase L antiviral pathway and the presence of *Mycoplasma spp.* in Rheumatoid Arthritis warrants further investigation.

The present study provides new insight into the complex nature of CFS. These data clearly suggest a co-morbid pathobiological pathway between *Mycoplasma spp.* and a deregulation of the 2,5A synthetase RNase L antiviral pathway in subsets of CFS. Patients fulfilling the 1994 CDC case definition for CFS that present with a *Mycoplasma* infection are more likely to show a deregulated RNase L antiviral pathway compared to non-infected patients. Future research should address possible disease- and infection-specificity of this association.

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TABLE 1: Demographic features of the sample.

N	Mean age [years]	Standard Deviation	Range	Number of males	Males [%]	Number of females [%]	Females [%]
186	35.8	9.9	[18 – 64]	23	12.4	163	87.6

TABLE 2: Prevalence of *Mycoplasma spp.* among these CFS-patients [N = 186].

Subgroup of <i>Mycoplasma</i> -genus	Number of CFS-patients [± SD ¹]	%
None	55 [± 7.4]	29.6
<i>M. fermentans</i> **	25 [± 5.0]	13.4
<i>M. fermentans</i> *	48 [± 6.9]	25.8
<i>M. hominis</i> **	45 [± 6.7]	24.2
<i>M. hominis</i> *	66 [± 8.1]	35.5
<i>M. pneumoniae</i> **	30 [± 5.5]	16.1
<i>M. pneumoniae</i> *	53 [± 7.3]	28.5
<i>M. penetrans</i> **	0 [± 0.0]	0.0
<i>M. penetrans</i> *	0 [± 0.0]	0.0
Multiple infection	31 [± 5.6]	16.7
Single infection	100 [± 10.0]	53.8
<i>M. fermentans</i> + <i>M. hominis</i>	8 [± 2.8]	4.3
<i>M. fermentans</i> + <i>M. pneumoniae</i>	10 [± 3.2]	5.4
<i>M. pneumoniae</i> + <i>M. hominis</i>	8 [± 2.8]	4.3
<i>M. fermentans</i> + <i>M. pneumoniae</i> + <i>M. hominis</i>	5 [± 2.2]	2.7

* alone or in combination with another type of *Mycoplasma spp.*

** single infection

¹ Poisson distribution

TABLE 3: Comparisons between CFS-patients with and with no *Mycoplasma spp.* detected.

	<i>Mycoplasma</i> detected	No <i>Mycoplasma</i> detected	P-value
N	131	55	
Mean age in years [SD]	35.7 [9.6]	36.2 [10.6]	0.746*
Mean log RNase L ratio [SD]	.3959 [.6932]	.1327 [.6282]	0.016*
Number of females	113	50	0.470**
Females [%]	86.3	90.9	
Number of males	18	5	
Males [%]	13.7	9.1	

* 2-tailed and equal variances assumed [Levene's test]

** Fisher's exact test

TABLE 4: Comparison of mean log RNase L among different subgroups of CFS-patients [subgrouping according to type of *Mycoplasma* detected].

Subgroup of <i>Mycoplasma</i> -genus	Number of CFS-patients	Mean log RNase L [SD]	p-value [in comparison to patients with no <i>Mycoplasma spp.</i> detected]
No <i>Mycoplasma</i> detected	55	.1327 [.6282]	
<i>M. hominis</i> ¹	45	.3845 [.7695]	.075 *
<i>M. fermentans</i> ¹	25	.4175 [.5599]	.056 *
<i>M. pneumoniae</i> ¹	30	.3386 [.6226]	.151 *
<i>M. fermentans</i> + <i>M. hominis</i>	8	.2307 [.4601]	.673 *
<i>M. fermentans</i> + <i>M. pneumoniae</i>	10	.4362 [.9234]	.198 *
<i>M. pneumoniae</i> + <i>M. hominis</i>	8	.6401 [.9020]	.048 *
<i>M. fermentans</i> + <i>M. pneumoniae</i> + <i>M. hominis</i>	5	.5288 [.6852]	.185 *

* 2-tailed and equal variances assumed [Levene's test]

