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A first study of cytokine genomic polymorphisms in CFS: Positive association of TNF-857 and IFNγ 874 rare alleles

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ABSTRACT

Objective. *In the past two years we have developed a biological bank of genomic DNA, cDNA, serum and red blood cells of Italian patients with certified CFS from the two Italian referral centers for the syndrome. Recent studies have shown an imbalance in cytokine production in disease states similar to Chronic Fatigue Syndrome (CFS), such as sickness behavior, both in animals and in humans. However we notice that serum cytokine concentrations are often inconstant and degrade rapidly. With this in mind, we investigated cytokine gene polymorphisms in 80 Italian patients with CFS in order to ascertain whether in this group of patients it is possible to describe a genetic predisposition to an inflammatory response.*

Methods. *We analyzed the promoter polymorphisms of IL-10, IL-6 and the IFNγ 874 T/A polymorphism in intron 1 with a PCR-SSP method (Cytogen One Lambda Inc. Canoga Park, CA, U.S.A) in 54 patients and TNF-308 G/A and –857 C/T promoter polymorphisms with a PCR-RFLP method (in 54 and 80 patients respectively).*

Results. *There is a highly significant increase of TNF –857 TT and CT genotypes (p = 0.002) among patients with respect to controls and a significant decrease of IFN gamma low producers (A/A) (p = 0.04) among patients with respect to controls.*

Conclusions. *We hypothesize that CFS patients can have a genetic predisposition to an immunomodulatory response of an inflammatory nature probably secondary to one or more environmental insults of unknown nature.*

Introduction

Chronic fatigue syndrome (CFS) is a disease characterized by debilitating fatigue that lasts for more than six months and reduces the patient's daily activities by at least 50% (1). All other possible causes of fatigue must be excluded. Besides these two major case definition criteria, at least four of eight minor criteria including neurological symptoms such as headaches, mental confusion, concentration difficulties, as well as fever, post-exertional fatigue, unrefreshing sleep, swollen cervical or axillary lymph nodes and pharyngitis should be present (2). CFS is often associated with fibromyalgia and irritable bowel syndrome (3). Its etiology remains unknown. At the moment no treatment exists for this syndrome and there is no established predictive marker. Lab testing is normal. Immune function has been widely studied with inconsistent results in $CD4^+$ cell counts, $CD8^+$ cell counts, CD4/CD8 ratio, B cell, monocyte and NK cell numbers, total IgG, IgA and IgM, immune complexes and ANA positivity (4). The only immunological alteration in which there is agreement is a reduced activity in NK cell lytic activity (5).

Because the immune system and genetics are very closely connected via the HLA system, there is good reason to suspect genetic involvement in immune disorders, although not necessarily of a Mendelian nature. There are a relatively growing number of studies on the genetics of CFS. Data on familial aggregation (6), twin studies (7) and DNA microarray studies (8, 9) indicate a genetic involvement in the pathogenesis of the syndrome.

Recently, a new disease "sickness behavior" in which symptoms very similar to chronic fatigue syndrome are described and which has a homeostatic function in infectious diseases, has been defined as a cytokine imbalance both in the periphery and CNS with a marked increase in pro-inflammatory cytokines. This has been demonstrated in animals, treated with LPS, which developed symptoms of sickness behavior (10).

An involvement of cytokines in the pathogenesis of chronic fatigue syndrome has been studied but the results of serological studies have been contradictory and a precise pattern of cytokine secretion has not been found (4, 11). Circadian rythmns of cytokine secretion remain unknown; however the most often cited cause for the discrepancy in these findings is that cytokines degrade very rapidly in serum after blood withdrawal. To overcome this limitation we decided to study cytokine gene polymorphisms which, to our knowledge, have not yet been investi-

gated in CFS in any ethnic group. We therefore analyzed the promoter polymorphisms of TNF, IL-10, IL-6 and the IFNγ 874 T/A polymorphism located in intron 1. Our aim was to investigate whether a genetic difference in pro-inflammatory or anti-inflammatory cytokines could be described in CFS patients with respect to controls.

Materials and methods

Patients

A biological bank of DNA, RNA, and serum for Italian patients with chronic fatigue syndrome is being developed by our group in this last year and patients have been recruited through the two referral centers in Italy for chronic fatigue syndrome (CRO-Aviano, Prof. U. Tirelli; G. D'Annunzio University Hospital Chieti, Prof. G. Pizzogallo) and with the collaboration of the Rheumatology ward of the S. Chiara Hospital, Pisa (Dr. L. Bazzichi). The development of this bank has required considerable effort since the precise epidemiology of CFS is unknown in Italy. All of these centers use the Fukuda critreria (2) for the diagnosis of CFS. Patients were contacted by phone and invited to participate in the study.

First study

Fifty-four patients with certified chronic fatigue syndrome from Northern Italy agreed and filled out a questionnaire on the onset of the disease, symptoms, the classification criteria used, any altered blood tests (including hepatitis testing, infectious disease serology and ANA testing), other imaging tests (X-rays, ultrasound, MRI scanning), muscle biopsy, EEG done in the past. The purpose of this questionnaire was to exclude an alternative diagnosis, and a brief family and personal history of autoimmune or allergic disease was also investigated. If there were difficulties in filling out the questionnaire, patients brought test results the day of the blood withdrawal and the questionnaire was completed by the author after examining patient's files. All particpants signed a written informed consent. Permission for this study was obtained from the University of Pavia's ethics comittee. Patients' characteristics can be seen in Table I. One hundred and forty healthy controls, matched for mean age and sex were selected from a pool of healthy blood donors who donate in the same ward used for patients' blood withdrawal (the Transfusion Center of the Policlinico San Matteo Hospital, Pavia). These subjects come mostly from Northern Italy, and attention was paid so that patients and controls came from the same area (patients from central and southern Italy were excluded). Blood donors underwent a thorough history and physical examination and were not be accepted with symptoms of long-standing fatigue.

Table I. Patients' characteristics.

Sex	
F	42/54 (78%)
M	13/54 (22%)
Age (years)	
Mean	43
Range	11-68
Duration (years)	
Mean	10.6
Range	1-30
Onset	
Acute	27/54 (50%)
Chronic	27/54 (50%)
Allergies	
Present	18/54 (33%)
absent	37/54 (67%)
Arthralgia/Myalgia	
Present	50/54 (93%)
Absent	5/54 (7%)

Methods

15 ml of blood (7 of blood in EDTA and 8 of whole blood) were drawn from patients for the biological bank which includes DNA, cDNA, serum and red blood cells. DNA was isolated from leukocytes with a DNA extraction kit (GFX Genomic Blood DNA Purification Kit, Amersham Biosciences, Piscataway NJ, U.S.A.) according to manufacturer's instructions.

The TNF promoter polymorphisms were studied using the PCR-RFLP (Polymerase Chain Reaction- Restriction Fragment Length Polymorphism) technique. Briefly, extracted DNA was amplified using 2 μl DNA, 2.5 μl reaction buffer, 1 μl MgCl2, 2,5μl dNTPs, 1 μl each of forward and reverse primers, 0.1 μl Taq polymerase and enough sterile water to reach a reaction solution of 25 μl. The forward primer for TNF –308 was 5'-AAAGTTGGGGACACACAA-3'. The reverse primer used was 5'-AAATGGAGGCAATAGGTTTTGAGGGCC-3'. For TNF-857 the forward primer used was 5'-TTTCATTCTGACCCGGAGACTCA-3' and the reverse primer was: 5'-GTCGAGGTATGGGGACCCCCAGTTA-3'. PCR conditions were as follows:

the amplification cycle for TNF-308 polymorphism used was: a denaturing step of 95°C for 5 min, 35 cycles of 95°C 15 sec, 60°C 15 sec, 72°C 30 sec; a final incubation at 72°C for 7 min. The amplification cycle for TNF-857 polymorphism used was: a denaturing step of 95°C for 5 min, 5 cycles of 95°C 15 sec, 65°C 15 sec 72°C 1 min; 10 cycles of 95°C 15 sec, 60°C 15 sec, 72°C 1 min; 20 cycles of 95°C 15 sec, 55°C 15 sec, 72°C 1 min; a final incubation of 72°C for 5 minutes.

TNF-308 was digested with NcoI enzyme according to producer's instructions (New England Biolabs Inc.) at a constant temperature of 37°C overnight. TNF –857 was digested with Hinc II enzyme according to producer's instructions (New England Biolabs Inc) at a constant temperature of 37°C overnight.

The resulting digests were run on an ethidium bromide stained 1% Nu-sieve agarose gel and visualized with a U.V. transilluminator and genotyping was defined.

IL-10, IL-6 and IFN-γ polymorphisms were studied with a PCR-SSP (Polymerase Chain Reaction-Sequence Specific Primer) DNA typing kit (Cytogen One Lambda Inc. Canoga Park, CA, U.S.A.) using an amplification cycle which consisted of: a single two-step first cycle: 96°C 130 sec, 60°C 60 sec; 9 times a second two-step cycle: 96°C 10 sec, 60°C for 60 sec; twenty times a third three-step cycle:96°C for 10 sec, 59°C for 50 sec and 72°C for 30 sec. The amplification products were run on a 2% agarose gel and read with a U.V. transilluminator and allelic variants were defined.

Statistical analysis

Direct count of alleles was undertaken

and allelic frequencies were calculated. Chi- square test and Fisher's exact test were used as appropriate to analyse the data. Statistical significance was considered as $p< 0.05$. Bonferroni's correction was not used because a second study was undertaken to confirm the first one.

Results

The results are summarized in Table II. Our results demonstrate a statistically significant difference ($p = 0.0049$) between case subjects and controls for the TNF-857 gene polymorphism in which a greater presence of homozygous and heterozygous subjects for the rare allele can be seen. We also demonstrate a lower number of low IFN-γ producers (874 A/A phenotype) among patients with CFS with respect to controls ($p = 0.044$). In both populations the allele distributions respect the Hardy-Weinberg equilibrium equation.

Second study

To confirm the data, especially on the TNF-857 gene polymorphism we recruited 26 more patients (for a total of 80 patients) and 224 healthy blood donors (a number of them having been used for the first study) to be used as controls and recruited from the same Transfusion Center ward of the Policlinico San Matteo Hospital in Pavia with the same criteria. The analysis by PCR-RFLP was repeated as cited above. The results (shown in Table III) repeat the genotype frequency distribution described in Table II for the TNF-857 gene polymorphism with a p value equal to 0.002. The Hardy-Weinberg equilibrium equation is maintained.

Table II. Cytokine gene polymorphism distribution in CFS patients and controls.

Gene Polymorphism	Patients		Controls		P value
TNF-308					0.65
AA	1/46	2%	2/143	1%	
AG	9/46	20%	37/143	26%	
GG	36/46	78%	104/143	63%	
TNF-857					**0.0049**
TT	4/54	8%	11/224	5%	
CT	27/54	50%	64/224	29%	
CC	23/54	42%	149/224	66%	
C	73/108	68%	362/448	81%	
T	35/108	32%	86/448	19%	**0.0028**
IL-10 –1082					0.096
G/G	8/48	17%	17/140	12%	
G/A	19/48	40%	76/140	54%	
A/A	21/48	43%	47/140	34%	
L-10 –819					0.75
T/T	4/48	8%	8/140	6%	
C/T	22/48	46%	62/140	44%	
C/C	22/48	46%	70/140	50%	
IL-10 –592					0.75
A/A	4/48	6%	8/140	6%	
A/C	22/48	46%	62/140	44%	
C/C	22/48	46%	70/140	50%	
IL-6 –174					0.1
C/C	8/44	18%	13/140	9%	
G/C	15/44	34%	70/140	50%	
G/G	21/44	48%	57/140	41%	
IFN-γ-874					**0.044**
A/A	6/47	13%	42/140	30%	
A/T	30/47	64%	66/140	47%	
T/T	11/47	23%	32/140	23%	
A	42/94	45%	150/280	54%	
T	52/94	55%	130/280	46%	0.14

Table III. TNF-857 gene polymorphism distribution for 80 patients and controls.

TNF-857 p = 0.002	Patients		Controls	
TT	4/80	5%	11/224	5%
CT	40/80	50%	64/224	28%
CC	36/80	45%	149/224	67%
T p = 0.004	48/160	30%	86/448	19%
C	112/160	70%	362/448	80%

Discussion

There are several advantages of studying cytokine gene polymorphisms rather than or in support of evaluating circulating cytokine serum levels or cytokine secretion by immune cells. First of all protein concentrations vary with circadian rhythms of the subject which could be disrupted in CFS patients. Cytokine gene polymorphisms have been identified and can significantly influence cytokine production levels. More importantly, they can express a host predisposition to disease, in this case an excessive inflammatory reaction in CFS. Finally they can predict a patient's cytokine/inflammatory profile making him a candidate for specific anti-inflammatory therapy. Cytokine gene polymorphisms have not been studied to our knowledge in Chronic Fatigue Syndrome in any other population.

The number of patients used in this study is not very high. In Italy, the prevalence of CFS has not been studied and CFS is considered rare. It is unknown whether the disease is actually rare or underdiagnosed in Italy because the syndrome is relatively unknown to family doctors and there are only two referral centers in the country.

The TNF-857T allele seems to be associated with an increased production of this cytokine (12). This polymorphism has been studied in a few rheumatological diseases (13, 14). TNF expression is evident in CFS patients at the mRNA level, which suggests de novo synthesis (15). TNF serum concentration is increased in CFS patients although this datum has not been found by all the groups studying cytokine serum concentrations (16, 17). An explanation for this discrepancy could be that TNF lev-

els decrease precipitously if the serum is not frozen within 30 minutes from collection (18). Elevated levels of TNF have been associated with fatigue in CFS patients whose blood has been stimulated *in vitro* with LPS mimicking the studies done in sickness behavior (19). We would therefore suppose that the increased TNF cytokine levels found by certain groups are indeed trustworthy and depend upon a genetic susceptibility due to the increased frequency of the TNF-857T allele in this patient population.

The IFN-γ 874 AA genotype is associated with a low production of this cytokine. In this study, we show that the IFN-γ AA genotype is less frequent in patients with CFS than in controls. This is important because IFN-γ is induced in the brain not only during acute infectious diseases but is also released during extended periods of time after viral clearance (20) and during viral latency (21, 22) and therefore could affect the brain even when the acute inflammatory reaction has subsided. Because of its receptor's topographical distribution in neurons IFN-γ could play a role in limiting the spread of infections in the CNS (23). At the molecular level IFN-γ exerts its antiviral effect on the cell through the transcriptional induction of three genes, namely double-stranded RNA activated protein kinase (PKR), 2'-5' oligoadenylate synthetase and dsRNA-specific adenosine deaminase. DeMeirleir *et al.* (24) have shown that the 2'-5' oligoadenylate synthetase pathway is disrupted in CFS patients. IFN-γ may also elicit certain aspects of sickness behavior; patients treated for example with this cytokine experience somnolence, fatigue and overall mental and motor slowing as well as pain (25).

In conclusion, patients with CFS show a proinflammatory cytokine genetic profile that influences the immune response to environmental stress and justifies the chronicity of the disease.

Furthermore, we can hypothesize that the reduction in NK cell activity documented in most CFS patients could have elicited an evolutionary adaptive mechanism consisting in the selection of those alleles of cytokine gene polymorphisms that are responsible for a non cytolitic viral elimination and control of persistent infection of the CNS (26). This could result in a symbiotic state in which the innate immune system does not eliminate the pathogen (an event which could also damage the host), but compensates by producing pro-inflammatory cytokines that keep viral proliferation under control. However this mechanism produces as side-effects the symptoms experienced by these patients.

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