Chronic fatigue syndrome is accompanied by an IgM-related immune response directed against neopitopes formed by oxidative or nitrosative damage to lipids and proteins

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Abstract

There is now some evidence that chronic fatigue syndrome (CFS) is accompanied by signs of oxidative stress and by a decreased antioxidant status.
The aim of the present study was to examine whether CFS is accompanied by an immune response to neoepitopes of a variety of modified lipids and proteins indicating damage caused by oxidative and nitrosative stress.
Toward this end we examined serum antibodies to fatty acids (oleic, palmitic and myristic acid), by-products of lipid peroxidation, i.e. azelaic acid and malondialdehyde (MDA), acetylcholine, S-farnesyl-L-cysteine, and N-oxide modified amino-acids in 14 patients with CFS, 14 subjects with partial CFS and 11 normal controls. We found that the prevalences and mean values for the serum IgM levels directed against oleic, palmitic and myristic acid, MDA, azelaic acid, S-farnesyl-L-cysteine, and the N-oxide derivates, nitro-tyrosine, nitro-phenylalanine, nitro-arginine, nitro-tryptophan, and nitro-cysteinyl were significantly greater in CFS patients than in normal controls, whereas patients with partial CFS took up an intermediate position. There were significant and positive correlations between the serum IgM levels directed against fatty acids, MDA and azelaic acid and the above N-oxide-derivates and the severity of illness (as measured by the FibroFatigue scale) and symptoms, such as aches and pain, muscular tension and fatigue.
The results show that CFS is characterized by an IgM-related immune response directed against disrupted lipid membrane components, by-products of lipid peroxidation, S-farnesyl-L-cysteine, and NO-modified amino-acids, which are normally not detected by the immune system but due to oxidative and nitrosative damage have become immunogenic.

ORIGINAL ARTICLE

Introduction

There is now some evidence that chronic fatigue syndrome (CFS) is accompanied by an imbalance between pro-oxidants and antioxidants causing a pro-oxidant status [1]. The presence of increased oxidative stress in CFS is supported by the following findings: a) higher LDL thiobarbituric acid reactive substances (TBARS) [2]; b) increased isoprostane levels and oxidized low density lipoproteins (LDL), two gold standard measures of in vivo oxidative stress [3]; c) elevated protein carbonyl levels, a measure of protein oxidation [4]; d) an increased response to incremental exercise, which is associated with a lengthened and accentuated oxidative stress [5]; and e) increased oxidative stress in animal models of CFS [6]. These findings suggest that - in CFS - lipid peroxidation has occurred, which has generated a variety of oxidatively modified lipids and lipid-protein adducts that are pro-inflammatory and immunogenic [7]. Decreases in anti-oxidants in CFS are reported by Vesschiet et al [2], who found a significant inverse relationship between vitamin E and fatigue and by Maes et al., who found significantly lower serum levels of zinc, a strong antioxidant [8], and dehydroepiendrosterone-sulfate, a hormone with strong antioxidant properties [9].

Besides increased oxidation there is also an immune-inflammatory response in CFS as shown by the following results: a) increased plasma concentrations of the alpha2 globulin fraction obtained by electrophoresis and decreased serum zinc levels [8]; b) immune activation, characterized by an increased expression of T lymphocyte activation markers, such as CD26 and CD38 [10] and alterations in cytokine production [11-13]; and c) signs of decreased cellular immunity, such as decreased mitogen–induced lymphocyte responses and defects in early T cell activation, i.e. a diminished mitogen-induced expression of the early activation marker CD69 [10, 14-16].

It is well established that following inflammatory stimuli, the production of oxygen radicals is increased with an increased production of H_2O_2 (peroxides) and $2O_2$- (superoxide). In inflammatory responses the immune system will - through the production of oxygen radicals - damage lipid membranes and thus brain, muscle, and nerve cells [1]. In addition, inflammation is often accompanied by nitrosative stress whereby endogens nitrogen monoxide (NO) or peroxynitrite ($ONOO^-$) are formed by activated neutrophils and monocytes. Nitration may occur whereby chemical modifications occur of self-proteins with the formation of, for example, nitro-tyrosine, another strongly immunogenic substance [17]. During this process, oxidative and nitrosative stress may have changed the natural structures of otherwise ubiquitous molecules to generate a variety of modified new epitopes (neoepitopes) which are highly immunogenic [18]. The latter can generate an immunoglobulin (Ig)-mediated immune response directed against these neoepitopes. The detection of circulating antibodies to these neoepitopes provides indirect evidence for damage and disruption of endogenous lipids and proteins by oxidation and nitration. However, until now no research in CFS has examined whether an Ig-related immune response is mounted against lipid or amino-acid neoepitopes.

The aim of the present study was to examine serum IgM antibodies to a panel of membrane fatty acids, i.e. oleic, palmitic, and myristic acid, byproducts of lipid peroxidation, i.e. malondialdehyde (MDA) and azelaic acid, acetylcholine (Ach), S-farnesyl-L-cysteine and; and NO modified amino-acids in order to determine whether CFS is accompanied by an IgM-related immune response directed against modifications in the structure of the above substances caused by oxidative stress and nitrosative stress.

Subjects and Methods

Subjects

Thirty-nine subjects participated in the present study, 11 unrelated controls (staff or their family members), and 28 patients admitted to the M-Care4U Outpatient Clinics, Belgium. We made the diagnosis of CFS by means of the Centers for Disease Control and Prevention (CDC) criteria [19]: a) the patient must have a severe chronic fatigue of six months or longer, while there is no other known medical condition which can explain the fatigue; and b) the patient must have four or more of the following symptoms: substantial impairment in short-term memory or concentration, sore throat, tender lymph nodes, muscle pain, multi-joint pain without swelling or redness, headache of a new type, pattern or severity, unrefreshing sleep, and post-exertional malaise lasting more than 24 hours. Patients presenting with criterion a) but who did not fulfill criterion b) were rated as partial CFS. The total sum of the FibroFatigue scale, i.e. the Fibromyalgia and Chronic Fatigue Syndrome Rating Scale [20, 21] was used to compute the severity of illness. This scale measures 12 items reminiscent for CFS (and fibromyalgia): pain, muscular tension, fatigue, concentration difficulties, failing memory, irritability, sadness, sleep disturbances, autonomic disturbances, irritable bowel, headache, and subjective experience of infection.

We have excluded: a) subjects with life-time diagnosis of psychiatric DSM IV disorders, such as bipolar disorder, depression, anxiety disorders, schizophrenia, substance use disorders and organic mental disorders; b) subjects with other medical illness, such as diabetes, inflammatory bowel disease, essential hypertension, and arteriosclerosis; c) subjects who ever had been treated with anti-psychotic drugs, anticonvulsants or mood stabilizers and subjects who had been taking psychotropic drugs during the last year prior to the studies; d) subjects with abnormal values for routine blood tests, such as alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), calcium, creatinine, electrolytes, thyroid stimulating hormone (TSH), total protein, and iron or transferrin saturation; and f) sub-

jects with acute inflammatory and allergic reactions for at least 2 months prior to the study. Patients and controls gave written informed consent after the study protocol was fully explained. The study has been approved by the local ethical committee.

Methods

The serum IgM values directed against a number of neoepitopes were analyzed by means of enzyme-linked immunosorbent assay (ELISA) methods as described before [22, 23]. Each plasma sample was measured in duplicate and tested simultaneously with three standard solutions. The optical densities (OD) of the three standards are expressed as Z values and from this the reference linear curve is calculated as Z = f(OD) with Z = a OD + b. Thus, the Z value of the lowest standard can be negative. This curve allows to deduce the mean values of the duplicate measurements of the OD values. The biological interassay CV values were < 10%. Table 1 shows the circulating antibodies identified in the present study: a) autoantibodies directed against lipid membrane components, such as oleic, palmitic and myristic acid, byproducts of lipid peroxidation, i.e. azelaic acid and MDA, S-farnesyl-L-cysteine and acetylcholine; and b) autoantibodies to nitric oxide (NO)-modified amino acids and creatine.

Statistics

Relationships between variables were assessed by means of Spearman's rank order correlation coefficients. Group mean differences were examined by means of analysis of variance (ANOVA) or covariance (ANCOVA) and by means of the Kruskal Wallis test. Post-hoc contrasts between multiple group means were ascertained by means of the Dunn test. The independence of classification systems was ascertained by means of analysis of contingence tables (χ^2-test) and Fisher's exact probability test. The significance was set at α=0.05 (two tailed).

Results

There were no significant differences in age (F=0.4, df=2/36, p=0.7) between normal controls (mean age±SD=41.4±8.7 years), patients with partial CFS (41.4±10.9 years) and patients with CFS (44.6±12.2 years). There were no significant differences (X^2=0.00, df=2, p=0.99) in gender distribution between normal controls (male/female ratio = 3/8), patients with partial CFS (4/10) and patients with CFS (4/10). There were no significant correlations (even at the p=0.05 level) between age and any of the IgM values. There were no significant differences between men and women (even at the p=0.05 level) in any of the serum IgM levels.

Table 2 shows that the anti-oleoyl, palmitoyl and myristolyl IgM antibodies were significantly different between the study groups. Dunn tests showed that the anti-oleoyl (t1=5.81, p=0.00002, t2=4.44, p=0.0002), anti-palmitoyl (t1=5.48, p=0.00004; t2=4.16, p=0.0004) and anti-myristoyl (t1=5.23, p=0.00005, t2=3.79, p=0.0006) antibodies were significantly greater in CFS patients than in normal controls (t1) and patients with partial CFS (t2). The anti-azelaoyl antibodies were significantly higher (t=2.86, p=0.007) in CFS patients than

Table 1. The circulating IgM antibodies identified in the present study.

antigens/haptens	pathological significance
palmitic acid	autoepitopes on cell membranes that are normally hidden from the immune system;
oleic acid	they are recognized after damage or disruption of lipid membrane components by oxidative processes thus suggesting that autoepitopes have become immunogenic
Myristic	
Acetylcholine	
azelaic acid	An aliphatic, dibasic acid, derived from a fatty acid such as oleic acid by oxidation. Azelaic acid is a saturated dicarboxylic acid found naturally in wheat, rye, and barley. Azelaic acid markedly decreases neutrophil-generated ROS leading to a reduction in oxidative tissue injury at sites of inflammation
malondialdehyde (MDA) residu	final product of lipoperoxidation
S-farnesyl-L-cysteine	farnesylation of ras-encoded proteins is a key process that apparently leads to membrane association of proteins, which perform a function in cell growth-promoting activity.
NO amino acids	endogenous constituents modified by NO and peroxynitrites resulting in the formation of neoantigens; the detection of circulating antibodies to these epitopes provides indirect evidence for nitrosative stress.

Table 2. Measurements of fatty acids, azelaic acid, malondialdehyde (MDA), phosphatidyl inositol (Pi), acetylcholine, S-farnesyl-L-cysteine, and N-oxide (NO)-modified amino-acids and creatinine in 14 patients with CFS, 14 subjects with partial CFS and 11 normal controls.

IgM levels against:	Normal Controls	Partial CFS	CFS	F	p-values
oleic acid	-0.68 (1.04)	0.43 (1.52)	3.25 (2.11)**	19.1	0.00003
palmitic acid	-0.91 (0.90)	0.26 (1.42)	3.15 (2.51)**	16.8	0.00005
myristic acid	-0.95 (0.60)	0.32 (1.60)	3.46 (2.96)**	15.3	0.00008
azelaic acid	-0.66 (0.84)	0.22 (1.37)	1.64 (2.84)*	4	0.02
MDA	-0.68 (1.28)	0.27 (0.97)	1.94 (3.00)**	5.6	0.008
Acetylcholine	-0.49 (0.38)	-0.18 (0.81)	0.63 (1.71)	2.8	0.07
S-farnesyl-L-cysteine	-0.84 (0.84)	0.25 (1.27)	2.29 (1.92)**	15.5	0.00007
NO-tyrosine	-0.34 (0.61)	-0.14 (1.34)	1.65 (2.78)**	3.9	0.03
NO2-tyrosine	0.38 (2.44)	-0.09 (1.14)	0.78 (1.44)	0.9	0.6
NO-phenylalanine	-0.64 (0.59)	0.30 (1.15)	1.37 (2.62)**	4.8	0.01
NO-histidine	-0.02 (0.70)	0.18 (1.47)	1.26 (2.26)	2	0.15
NO-arginine	-0.63 (0.59)	0.30 (1.85)	1.86 (3.13)*	3.9	0.03
NO-tryptophan	-0.30 (0.60)	0.06 (1.54)	1.80 (2.45)**	5.1	0.01
NO-methionine	-0.50 (0.50)	0.05 (1.40)	1.47 (2.83)	3.2	0.051
NO-cysteine	-0.36 (0.46)	-0.19 (1.21)	2.06 (3.16)**	5.5	0.008
N-asparagine	-0.71 (0.56)	0.04 (1.98)	1.45 (3.71)	2.3	0.11
NO-creatinine	-0.32 (0.64)	-0.01 (1.66)	1.26 (2.94)	1.9	0.2

All results are shown as mean (±SD). All results of ANCOVA with age and sex as covariates (df=2/34).
* Significantly different from normal controls (p<0.05)
** Significantly different from normal controls and partial CFS (p<0.05)

in normal controls. The anti-MDA (t1=3.17, p=0.003; t2=2.15, p=0.04) IgM titers were significantly higher in CFS patients than in normal controls and patients with partial CFS, respectively. No significant differences were found in acetylcholine IgM antibodies between normal controls and CFS patients or those with partial CFS. The anti-S-farnesyl-L-cysteine (t1=5.31, p=0.00004; t2=3.71, p=0.001) IgM titers were significantly higher in CFS patients than in normal controls and patients with partial CFS, respectively.

Table 2 shows that the serum IgM antibodies directed against nitro-tyrosine (t1=2.59, p=0.01; t2=2.48, p=0.017), nitro-phenylalanine (t1=2.78, p=0.009; t2=2.46, p=002), nitro-tryptophan (t1=2.88, p=0.007; t2=2.54, p=0.01), and nitro-cysteinyl (t1=2.87, p=0.007; t2=2.85, p=0.007) were significantly greater in CFS than in normal controls (t1) and patients with partial CFS (t2). We found that the serum IgM antibodies directed against nitro-arginine (t=0.009) were significantly higher in CFS patients than in normal controls.

By means of Fisher's exact probability test we found a significantly greater number of CFS patients (9/14) with abnormally increased IgM antibodies directed against oleic acid, palmitic acid, myristic acid, azelaic acid or MDA (i.e. anyone of the IgM values > 3 Z values) than in controls (0/11; ψ=0.66, p=0.0009) and patients with partial CFS (2/14; ψ=0.51, p=0.009), while there were no significant differences between normal controls and patients with partial CFS (ψ=0.26, p=0.3). By means of Fisher's exact probability test we found a significantly greater number of CFS patients with abnormally increased IgM antibodies directed against the N-oxide-aminoacids (i.e. anyone of the IgM values > 3 Z values) (8/14; ψ=0.50, p=0.02) than in normal controls (1/11) but not between CFS patients and those with partial CFS (5/14; Θ=0.21, p=0.2) and between normal controls and those with partial CFS (Ψ=0.31, p=0.1).

As an integrative index for the IgM responses to the fatty acids and the byproducts of lipid peroxidation, we computed the peak values of the serum IgM antibodies directed against oleic acid, palmitic acid, myristic acid, MDA and azelaic acid (peak OS), and as an index for the damage caused by nitrosative stress we computed the peak values for the IgM antibodies directed against nitro-tyrosine, NO_2-tyrosine, nitro-phenylalanine, nitro-histidine, nitro-arginine, nitro-tryptophan, nitro-methionine, nitro-cysteinyl, nitro-asparagine, and nitro-creatine (peak NS). We found that the peak OS values were significantly correlated to the total score on the FibroFatigue scale (r=0.59, p=0.001), aches and pain (r=0.65, p=0.0003), muscular tension (r=0.55, p=0.003), and fatigue (r=0.53, p=0.004). The peak NS values were

correlated to the total score on the FibroFatigue scale (r=045, p=0.02), aches and pain (r=0.64, p=0.0004) and muscular tension (r=52, p=0.005).

Discussion

The results of the present study show that CFS is accompanied by increased mean levels and increased prevalences of IgM antibodies directed against fatty acids, by-products of lipid peroxidation (MDA and azelaic acid), and anti-S-farnesyl-L-cysteine, and NO derivates, such as nitro-tyrosine, nitro-phenylalanine, nitro-arginine, nitro-tryptophan and nitro-cysteine. The above disorders are highly pronounced in full blown CFS, while patients with partial CFS take up an intermediate position. The are significant and positive correlations between serum IgM levels and the severity of illness as measured by the FibroFatigue scale. The intergroup differences in serum IgM directed against the fatty acids and the by-products of lipid peroxidation are far more significant than those directed against the N-oxide derivates of the amino-acids. Thus, in CFS there is an IgM-mediated immune response directed against autoepitopes, which are normally hidden from the immune system. The latter may be recognized since a) oxidative stress may have damaged or disrupted the lipid membrane components and formed by-products of lipid peroxidation; and b) nitrosative stress (NO and peroxynitrite) may have modified endogenous proteins resulting in the formation of neoantigens. Phrased differently, the latter may have changed otherwise inactive autoepitopes to antigens which have acquired immunogenicity and thus may serve as a trigger to impair or bypass immunological tolerance leading to autoantibody production.

Thus, the results of the present study confirm those of previous research (see Introduction) that CFS is accompanied by increased oxidative stress [2-5] and a decreased anti-oxidant status [2, 8, 9]. The results also support previous reports that CFS is characterized by signs of damage to lipid components or proteins [2, 3]. However, the present study shows that in CFS an autoimmune response is mounted, which appears to be directed against multivarious lipid and protein neoepitopes.

The increased IgM antibodies directed against fatty acids (anti palmitoyl, myristoyl and oleoyl) indicate that in CFS the natural lipid structures have been modified to generate a variety of modified lipids with immunogenic determinants. Previously, it has been found that autoantibody titers to epitopes of oxidized LDL are correlated to the severity of disorders, such as atherosclerosis [18]. Antibodies directed against lipids have also been detected in remitting-relapsing multiple sclerosis (MS) [22, 24]. The circulating IgM antibody titers in MS appear to be related to the presence of inflammation because they increase during relapses and decrease during remissions [25].

We also detected increased serum IgM directed against by-products of lipid peroxidation, i.e. MDA and azelaic acid. MDA is one of the most frequently used indicators of lipid peroxidation [26, 27]. MDA is a highly reactive three carbon dialdehyde produced as a byproduct of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism. MDA readily combines with several functional groups on molecules including proteins, lipoproteins, and DNA, which in turn may show an altered physico-chemical behavior and antigenicity and may acquire immunogenic properties. The antibody titre against MDA-protein adducts may be considered as a marker of lipoperoxidative protein damage in vivo. Lipid peroxidation with increased IgM antibody formation to MDA can be observed in other disorders, such as systemic lupus erythematosus (SLE) [28]. Azelaic acid is a saturated C9 dicarboxylic acid, which can be derived from fatty acids, such as oleic acid, by oxidation. Azelaic acid possesses significant biologic properties, e.g. it markedly decreases neutrophil-generated ROS leading to a reduction in oxidative tissue injury at sites of inflammation [29]; and it inhibits both bacterial DNA polymerase and multienzyme complexes isolated from cultured melanoma cells and keratinocytes [30]. Increased antibody titers against azelaic acid indicate increased oxidation of fatty acids [22] and may be observed in patients with acute relapsing MS [31].

In patients with CFS we also found increased IgM levels to S-farnesyl-L-cysteine. S-farnesyl-L-cysteine plays a key role in regulating cell growth, differentiation and apoptosis through RAS protein activity. The latter depends on their anchorage to the inner surface of the plasma membrane, which is promoted by their common carboxy-terminal S-farnesyl-cysteine [32]. The presence of antibodies to S-farnesyl-L-cysteine suggest that RAS functions may have undergone damage by oxidative / nitrosative stress, causing disturbed functional activity in the regulation of cell growth.

In the present study we found that CFS was accompanied by significantly increased serum IgM antibodies to nitrated products of amino-acids, such as tyrosine and other amino-acids. Nitro-tyrosine has been revealed as a relevant inflammatory biomarker of NO-dependent nitrosative stress and NO-mediated tissue damage [17, 33, 34]. Tyrosine nitration is mediated by RNS such as peroxynitrite anion ($ONOO^-$), and nitrogen dioxide (NO_2), and nitryl chloride formed as secondary products of oxidants including superoxide radicals, hydrogen peroxide (H_2O_2) [33]. The extent of protein nitro-tyrosine formation provides an index of the production of reactive nitrogen species (RNS) and potential cell damage over a period of time. For example, in coeliac disease elevated nitro-tyrosine levels are parallelled by an increase in plasma concentrations of NO-oxidation products (NOx), nitrite and nitrate [34]. Both nitro-tyrosine and NOx levels decline when the patients are on a gluten-free diet, suggesting a relation between intestinal inflammation and plasma nitro-tyrosine and NOx levels [34]. The post-translational modification of self-proteins gives rise to the generation of new nitro-tyrosine (or other

amino-acids) epitopes to which T and B lymphocytes are not rendered tolerant and thus may serve as a trigger to impair or bypass immunological tolerance [17].

Another question is why CFS is accompanied by oxidative and nitrosative stress with eventually causes damage to lipid and protein structures and which in turn may mount an IgM-related autoimmune response to the neoepitopes. As explained elsewhere, there are many trigger factors which may induce CFS, e.g. causes of inflammatory reactions, such as bacterial and viral infections; trauma and injuries; food allergies type IV; heavy metal allergies type IV; and gut hyperpermeability [1, 35]. In addition, also psychological stress and sustained strenuous exercise, which are other established trigger factors for CFS [1] may generate oxidative and nitrosative stress. Emotional stress not only induces inflammatory reactions with an increased production of pro-inflammatory cytokines [36], but also a prooxidant state and lipid peroxidation. There is a vast Russian literature on the effects of emotional stress on lipid peroxidation [37-39]. Sivonova et al. [40] found that, in university students, examination stress was accompanied by oxidative damage to DNA, sensitivity to lipid oxidation and a significantly decreased plasma antioxidant activity. In female subjects, the levels of 8-OH-desoxyguanosine, another marker of oxidative damage to DNA, were significantly related to the perceived workload, the perceived psychological stress, and the impossibility of alleviating stress [41]. Also, exercise training may cause increased urinary excretion of lipid, protein and DNA oxidation products [42]. In soccer players, high titres of antibodies against oxLDL could be accounted for by their higher in vivo susceptibility of LDL to structural modification under conditions of intensive training-induced oxidative stress [43]. Thus, the trigger factors which may cause CFS can also induce oxidative stress and lipid peroxidation.

Another major finding of this study is that the IgM response mounted to the above neoepitopes is significantly correlated to the key symptoms of CFS, i.e. aches and pain, muscular tension and fatigue. These results extent those of Vecchiet et al. [2] who found that - in CFS - increased oxidative stress and decreased antioxidant defences are related to the extent of fatigue. The results also extent those of Kennedy et al. [3] who found that CFS symptoms, such as joint pain and postexertional malaise correlated well with isoprostane levels. Moreover, other research implicates a causal role for oxidative stress in those symptoms. Thus, Jammes et al. [5] found that the response of CFS patients to incremental exercise associates a lengthened and accentuated oxidative stress together with marked alterations of the muscle membrane excitability. These two objective signs of muscle dysfunction are sufficient to explain muscle pain and postexertional malaise reported by CFS patients. Matuszczak et al. [44] report that administration of NAC, a strong antioxidant, supports glutathione homeostasis in exercising humans and may delay muscle fatigue during repetitive handgrip exercise, supporting the hypothesis that oxidative stress is a causal factor in human muscle fatigue.

In summary, we may hypothesize that CFS is an immune disorder with increased oxidative and nitrosative stress, which has caused damage to lipids and proteins which, in turn, a) may cause disturbed functional activity in a number of important functions (cell-membrane related, cell proliferation through S-farnesyl-L-cysteine); and b) may become immunogenic and consequently may mount an IgM-related autoimmune response to neoepitopes. Oxidative and nitrosative stress are probably etiopathophysiological factors in the key symptoms of CFS, i.e. fatigue, muscular tension and aches and pain, through damage to important cell components and a subsequent autoimmune response.

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