ORIGINAL ARTICLE

An IgM-mediated immune response directed against nitro-bovine serum albumin (nitro-BSA) in chronic fatigue syndrome (CFS) and major depression: Evidence that nitrosative stress is another factor underpinning the comorbidity between major depression and CFS

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Abstract

BACKGROUND: It has been shown that chronic fatigue syndrome (CFS) and major depression (MDD) are accompanied by signs of oxidative stress and by a decreased antioxidant status. The aim of the present study was to examine whether CFS and MDD are accompanied by an IgM-mediated immune response directed against nitro-serum bovine albumin (BSA), which is a neoepitope of BSA formed by damage caused by nitrosative stress. **AIMS**: Toward this end, we examined serum IgM antibodies to nitro-BSA in 13 patients with CFS, 14 subjects with partial CFS, 16 patients with MDD and 11 normal controls. **RESULTS**: We found that the prevalence and mean values for the serum IgM levels directed against nitro-BSA were significantly greater in patients with partial CFS, CFS and MDD than in normal controls, and significantly greater in CFS than in those with partial CFS and MDD. We found significant and positive correlations between serum IgM levels directed against nitro-BSA and symptoms of the FibroFatigue scale, i.e. aches and pain and muscular tension. There was also a strong positive correlation between serum IgM titers directed against nitro-BSA and an index of increased gut permeability ("leaky gut"), i.e. serum IgM and IgA directed against LPS of different gram-negative enterobacteria. **DISCUSSION**: The abovementioned results indicate that both CFS and MDD are accompanied by a) an increased gut permeability which has allowed an exaggerated passage of BSA through a compromised epithelial barrier; b) increased nitrosative stress which has induced damage to BSA; and c) an IgM-mediated immune response which is directed against the nitro-BSA neoepitopes. Nitrosative stress is one of the factors underpinning the comorbidity and clinical overlap between CFS and MDD.

Abbreviations:

BSA	- bouvine serum albumin
CFS	- chronic fatigue syndrome
iNOS	- inducible NO synthase
LDL	- low density lipoproteins
MDA	- malondialdehyde
MDD	- major depression
NS	- nitrosative stress
O&NS	- oxidative and nitrosative stress
OS	- oxidative stress
TBARS	- thiobarbituric acid reactive substances

INTRODUCTION

There is a high degree of comorbidity between chronic fatigue syndrome (CFS) and major depression (MDD) [1]. One of the key symptoms of MDD is fatigue, while depression or depressive feelings often occur in CFS [2,3].

There are a number of pathophysiological factors which may explain the comorbidity between the two disorders, one of which is oxidative stress (OS) [4]. There is now evidence that CFS and MDD are accompanied by OS and a lowered antioxidant status [4]. Thus, CFS is accompanied by increased isoprostane levels and oxidized low density lipoproteins (LDL) [5]; elevated protein carbonyl levels, a measure of protein oxidation [6]; and increased LDL thiobarbituric acid reactive substances (TBARS) [7]. Recently, we reported that CFS is characterized by increased serum immunoglobulin (Ig)-M levels directed against a number of fatty acids, such as oleic, palmitic and myristic acid; byproducts of lipid peroxidation, i.e malondialdehyde (MDA) and azelaic acid; and phosphatidyl inositol [4,8]. Thus, CFS is characterized by an IgM-related (auto)immune response directed against lipid membrane components, which are disrupted by OS, and against byproducts of lipid peroxidation. These lipid factors are normally not detected by the immune system but due to damage caused by OS they have become immunogenic [4,8]. In CFS, OS is probably caused by an intracellular inflammatory reaction characterized by an increased production of the transcription factor nuclear factor kappa beta (NFκβ), which in turn induces two other inflammatory and oxidative mediators, i.e. cyclo-oxygenase (COX-2) and inducible NO synthase (iNOS) [9, 10]. Indeed, the production of NFκβ, COX-2 and iNOS by peripheral blood lymphocytes is significantly greater in CFS than in normal volunteers, while there are significant and positive correlations between COX-2, iNOS and NFκβ [10]. This intracellular inflammatory mechanism may also explain the many immune disorders which are detected in CFS [11,12]. A reduction in the antioxidant status in CFS is documented by significantly inverse relationships between vitamin E and fatigue [7]; significantly lower serum levels of zinc, a strong antioxidant [13]; dehydroepiendrosterone-sulfate, a hormone with strong antioxidant properties [14]; and omega-3 fatty acids [15]. Also in animal models of CFS, there is evidence that OS plays an important role [16–18].

There is also evidence that MDD is accompanied by OS. Indeed MDD is accompanied by increased levels of malondialdehyde (MDA); 8-hydroxy-2-deoxyguanosine, indicating damage to DNA by oxygen radicals; peroxidase and catalase activities in blood and saliva [19–23]. Moreover, MDD is accompanied by a decreased antioxidant status, e.g. lowered serum zinc, vitamin E, gluthathione peroxidase and vitamin C levels [24–26]. Reduced glutathione contents in the brain play an important role in animal models of stress-induced depression [27].

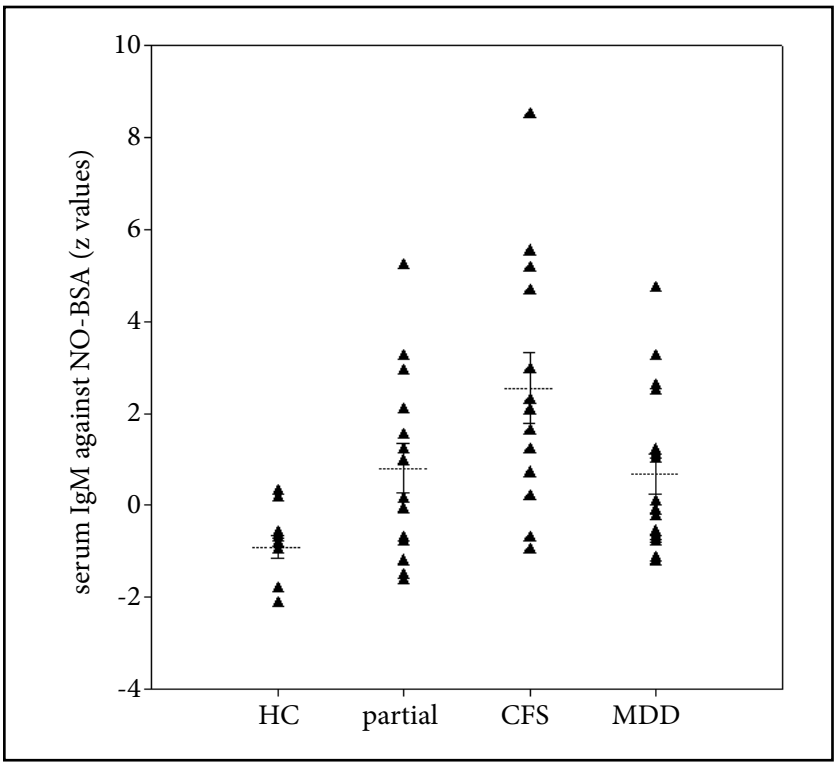
OS and inflammatory reactions are accompanied by a nitration process or nitrosative stress (NS) whereby endogenous nitrogen monoxide (NO) or peroxynitrites (ONOO") are formed. During the latter process, natural protein structures of otherwise ubiquitous molecules may be damaged and changed to generate a variety of modified new epitopes (neoepitopes) which are highly immunogenic [8,28]. One of these self-proteins modified by NS and which is highly immunogenic is nitrotyrosine [29]. These immunogenic neoepitopes are known to generate an Ig-mediated immune response directed against the damaged self-proteins. Moreover, the detection of circulating antibodies to these neoepitopes provides indirect evidence for damage and disruption of proteins by oxidation and nitration [30]. Increased serum IgM levels directed against nitro-tyrosine and a number of other nitro-aminoacids, such as nitro-phenylalanine, nitro-arginine, nitro-tryptophan, and nitrocysteine have been found in CFS [8]. This proves that CFS is accompanied by oxidative and nitrosative stress (O&NS).

One of the proteins which is prone to damage by NS and becomes highly immunogenic after nitrosative damage is bovine serum albumin (BSA) [30]. BSA is a protein which occurs as an allergen in beef and in cow's milk [31–33]. BSA is a tolerogen and allergen in the infant [34,35]. Exposure to BSA may induce food allergies, asthma, wheezing, allergic rhinitis, etc. [36,37]. Increased serum IgM levels directed against nitro-BSA have been detected in inflammatory disorders, such as multiple sclerosis [30].

The aim of the present study is to examine whether CFS and MDD are accompanied by an augmented IgM-related immune response directed against nitro-BSA and whether increased O&NS, as indicated by increases in anti nitro-BSA IgM levels, is another factor which may explain the comorbidity and the symptomatic overlap between CFS and major depression.

Subjects and Methods Subjects

Fifty-four subjects participated in the present study, 11 unrelated controls (staff or their family members), 14 patients with partial CFS, 13 patients with CFS and 16 major depressed patients. The patients were admitted to the M-Care4U Outpatient Clinics, Belgium. The



diagnosis of CFS was made by means of the Centers for Disease Control and Prevention (CDC) criteria [38], and the diagnosis MDD according to the DSM-IV criteria [39]. The diagnostic criteria for CFS are: 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 [40] was used to compute the severity of chronic fatigue. 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, e.g. anxiety disorders, schizophrenia, substance use disorders and organic mental disorders; b) subjects with CFS who ever had major depressive episodes; and patients with major depression who also suffered from concurrent CFS; c) subjects with other medical illness, such as diabetes, inflammatory bowel disease, essential hypertension, and arteriosclerosis; d) subjects who ever had been treated with anti-psychotic drugs, anticonvulsants and subjects who had been taking psychotropic drugs during the last year prior to the studies; e) 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) subjects with infectious 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 ethical committee.

METHODS

The serum IgM values directed against nitro-BSA were analyzed by means of enzyme-linked immunosorbent assay (ELISA) methods as described before [30]. 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%.

Statistics

Pearson's product moment correlation coefficients and Spearman's rank order correlations were used to analyse the relationships between variables. Analysis

of variance (ANOVA) or covariance (ANCOVA) were used in order to examine differences among treatment means. Post-hoc contrasts between multiple group means were ascertained by means of the least significant difference (LSD). The independence of classification systems was ascertained by means of analysis of contingence tables (x^2-test) and Fisher's exact probability test. The diagnostic performance was checked by means of ROC (receiver operating characteristics) analysis with computation of the area under the ROC curve, sensitivity, specificity and predictive value of a positive test result (PV+) and with kappa statistics. Data reduction was obtained by means of principal component (PC) analysis. The significance was set at a=0.05 (two tailed).

RESULTS

We found no significant differences (F=0.3, df=3/50, p=0.8) in age between the study groups, i.e. normal controls (mean ±SD = 41.4 ± 8.7 years), partial CFS (41.4 ±10.9 years), CFS (44.8 ± 12.6 years) and MDD (42.9 ± 10.2 years) patients. We found no significant differences (X^2=l.l, df=3, p=0.8) in the male / female ratio between normal controls (3/8), partial CFS (4/10), CFS (4/9), and MDD (7/9) patients. There were no significant correlations between age and the serum IgM levels directed against nitro-BSA (r=0.01, p=0.9) and no significant differences between males and females (F=0.00, df=l/52, p=0.98).

Figure 1 shows the serum IgM levels directed against nitro-BSA in the normal controls, patients with partial CFS, CFS and MDD. By means of ANCOVA with age and gender as covariates, we found significant differences between the study groups (F=7.0, df=3/48, p=0.0008). LSD showed that the serum IgM values directed against nitro-BSA were significantly higher in partial CFS (t=2.67, p=0.01), CFS (t=4.56, p=0.0001) and MDD (t=2.74, p=0.008) than in normal controls. No significant differences were detected between partial CFS and MDD patients (t=0.02, p=0.98). The IgM values were significantly greater in CFS patients than is those with partial CFS (t=2.01, p=0.06) and MDD (t=2.13, t=0.03).

Using a cut-off point for the anti-IgM antibody titers directed against nitro-BSA > 2 Z values, we found a significantly (Fisher's exact probability test: i|/=0.31, p=0.017) greater number of patients with partial CFS (4/14: 28.5%), CFS (7/13: 53.8%) and MDD (4/16: 25.0%) with abnormally increased IgM antibodies than in the normal controls (0/11: 0%).

ANOVA showed that the total score on the FibroFatigue scale was significantly greater (F=13.2, df=2/33, p=0.0001) in CFS patients (mean ±SD score: 48.6 ±6.0, t=5.05, p=0.00008) and MDD patients (41.3 ±9.8; t=3.08, p=0.004) than in those with partial CFS (32.6 ±5.3). The Fibrofatigue score was significantly greater in CFS patients than in those with MDD (t=2.24, p=0.03). There was no significant correlation between the total score on the FibroFatigue scale and the serum IgM levels directed against nitro-BSA (r=0.20, p=0.2). There were significant and positive correlations between the serum IgM levels directed against nitro-BSA and two symptoms of the FibroFatigue scale, i.e. aches and pain (r=0.48, p=0.001) and muscular tension (r=0.41, p=0.007).

Previously, we have reported that CFS is accompanied by increased IgM and in particular IgA serum levels directed against the LPS of gram-negative enterobacteria, such as Hafnia Alvei, Pseudomonas Aeruginosa, Morganella Morganii, Proteus Mirabilis, Pseudomonas Putida, Citrobacter Koseri, and Klebsielle Pneumoniae [41,42]. In order to examine the relationships between serum IgM levels directed against nitro-BSA and the immune response against the LPS of gram-negative bacteria in partial CFS, CFS and MDD we computed the correlations between anti-BSA antibodies and the first PC subtracted from the serum IgA and IgM levels directed against the 7 above enterobacteria. This first PC explained 40.6% of the variance in the 14 variables, which were all highly loaded on this PC (> 0.5). There was a significant and positive correlation between serum IgM directed against nitro-BSA and the first PC subtracted from the serum IgA and IgM data directed against 7 gram-negative enterobacteria (r=44, p=0.007). The first PC was significantly higher (F=4.4, df=2/31, p=0.019) in patients with CFS than in those with partial CFS whereas patients with MDD occupied an intermediate position and did not differ significantly either from patients with CFS or those without CFS.

DISCUSSION

The main findings of this study are that CFS, partial CFS and MDD are accompanied by an increased IgM-mediated immune response directed against nitro-BSA, that the increased IgM values are strongly related to two symptoms of the FibroFatigue Scale, i.e. aches and pain and muscular tension, and that the immune response against nitro-BSA was significantly correlated to immunological signs of an increased gut permeability.

The results show that in MDD, CFS and partial CFS there is an IgM-mediated immune response directed against nitro-BSA. The latter may more easily be recognized by the immune system since NO en peroxynitrites, released during nitrosative stress, have modified BSA resulting in the formation of nitro-neoantigens, i.e nitro-BSA. Thus, in CFS and MDD, BSA epitopes are damaged by NS and changed into antigens which are immunogenic [30]. These findings show that in both (partial) CFS and MDD there is an increased NS with damage to BSA epitopes and that this effect is more pronounced in CFS than in MDD and partial CFS. Increased serum IgM levels directed against nitro-BSA have also been detected in autoimmune disorders, such as multiple sclerosis [30].

In the present study we found that two symptoms are related to the increased anti nitro-BSA IgM levels, i.e. aches and pain and muscular tension. The latter are two of the key symptoms of CFS but may occur in MDD too. Our results extent previous reports showing that joint pain and postexertional malaise in CFS correlated well with isoprostane levels [5].

Moreover, administration of N-acetylcystein, a strong antioxidant, may delay muscle fatigue during repetitive handgrip exercise, supporting the hypothesis that O&NS is a causal factor in human muscle fatigue [43]. Jammes et al. [44] detected that in CFS the response to incremental exercise associates a lengthened and accentuated OS together with marked alterations of the muscle membrane excitability. These objective signs of muscle dysfunction are sufficient to explain muscle pain and postexertional malaise reported by the CFS patients [44]. Thus, it may be hypothesized that the O&NS has induced symptoms, such as aches and pain and muscular tension, which are typical for CFS but may also occur in MDD.

The results of the present study are in agreement with previous research in CFS and in MDD that both disorders are accompanied by O&NS. For example, CFS as well as MDD are characterized by increased antibody titers to epitopes of oxidized LDL and increased levels of MDA, a byproduct of lipid peroxidation [5,7,19,26]. Other findings show a decreased antioxidant status in CFS and MDD, e.g. lower serum zinc and vitamin E [7,13,25,26]. IgM antibodies directed against various lipids, byproducts of lipid peroxidation, such as MDA and azelaic acid, and against phosphatidyl inositol have also been detected in CFS [4,8].

The results of the present study are also in agreement with previous reports showing that CFS is accompanied by significantly increased antibodies directed against antigens bearing nitrated epitopes, such as nitro-tyrosine, nitro-phenylalanine, nitro-arginine, nitro-tryptophan, and nitro-cysteine [8]. The latter are biomarkers of nitrosation / nitrosylation and the presence of antibodies against those compounds provide indirect evidence for NO-mediated tissue damage [45–47]. Moreover, the post-translational modification of these self-proteins by reactive nitrogen species, such as ONOO and nitrogen dioxide ($N0_2$), causes the generation of nitro-amino-acid epitopes which may serve as a trigger to impair immunological tolerance [46].

BSA is a protein that has the capacity to bind serum-specific IgE, while antigenic determinants on BSA can be recognized by IgM, IgG and IgA antibodies. The proliferative responses of peripheral blood mononuclear cells to BSA are significantly greater in children with atopic dermatitis who are sensitive to cow's milk than in normal children [48]. In a case report, high serum levels of circulating immune complexes containing BSA were found in a woman with severe recurrent angioma and urticaria [49]. Elimination of bovine products from the diet resulted in the disappearance of immune complexes within 2 days and reintroduction of bovine products resulted in reappearance of the immune complexes. Severe allergic reactions to BSA have been reported in a number of studies during artificial insemination (IVF) as a result of the presence of BSA in the medium (Menezo's medium) employed for rinsing follicles [50]. Women who develop a symptom complex compatible with serum sickness during IVF show specific antibodies to BSA and were positive to intradermal skin testing with BSA [51]. Increased IgM levels are also found in hepatitis E virus infection [52]. Many patients with autoimmune disorders have increased anti-BSA antibodies [53]. The latter authors [53] conclude that anti-BSA antibodies reflect a general defect in the process of immunologic tolerance associated with a predisposition to autoimmunity. It has been suggested that early exposure to cow's milk may be an important determinant of subsequent diabetes mellitus, while the latter patients display increased anti-BSA antibodies [54].

In the next paragraph we discuss that there is also a relationship between increased anti-BSA or NO-BSA antibodies and an increased gut permeability ("leaky gut"). Thus, many patients with celiac disease show BSA antibodies [55]. Patients with an altered epithelial permeability are more prone to producing a number of antibodies, one of these being anti-BSA. For example, in patients with type-I diabetes a significantly higher prevalence of serum BSA antibodies has been found in those with celiac-disease related antibodies than in diabetes patients lacking these celiac-related antibodies [55]. Thus, serum BSA antibodies appear in patients with a compromised epithelial permeability. In this respect, we found a strong correlation in our study sample between the IgM-mediated immune response against nitro-BSA and an index of increased gut permeability, i.e. serum IgA and IgM levels against LPS of gram-negative enterobacteria, such as Hafnia Alvei; Pseudomonas Aeruginosa, Morganella Morganii, Proteus Mirabilis, Pseudomonas Putida, Citrobacter Koseri, and Klebsielle Pneumoniae [41,42,56]. As explained elsewhere, increased IgA and IgM serum levels against LPS of the above enterobacteria indicate a failure of gut barrier function whereby enlarged spaces between the cells of the gut wall cause a loss of the protective barrier [41,42,56]. This may cause an increased translocation of bacterial LPS which may trigger an IgM and IgA-related immune response to LPS [57]. Recently, we found that the prevalences and median values for serum IgA and IgM against the LPS of the above gram-negative enterobacteria are significantly greater in patients with CFS and MDD than in normal volunteers [41,42,56].

Based on the above we may conclude that the increased IgM levels directed against nitro-BSA in CFS and MDD indicate a) an increased gut permeability which allowed an exaggerated passage of BSA through a compromised epithelial barrier; b) increased NS which has induced nitrosative damage to BSA; and c)

an IgM-mediated immune response directed against the nitro-BSAneoepitopes.

The results of the present study show that NS may be one factor underpinning the comorbidity between CFS and MDD, and the high prevalence of fatigue in MDD and of depressive symptoms in CFS. Differences in other pathophysiological factors may differentiate both syndromes into two different illnesses, e.g. an exaggerated inflammation-induced catabolism of tryptophan with formation of neurotoxic TRYCATs (tryptophan catabolites along the indoleamine - pathway) in MDD [58–60].

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