Decreased expression of CD69 in chronic fatigue syndrome in relation to inflammatory markers: evidence for a severe disorder in the early activation of T lymphocytes and natural killer cells

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Abstract

There is some evidence that patients with chronic fatigue syndrome (CFS) suffer from immune abnormalities, such as immune activation and decreased immune cell responsivity upon polyclonal stimili. This study was designed to evaluate lymphocyte activation in CFS by using a CD69 expression assay. CD69 acts as a costimulatory molecule for T- and natural killer (NK) cell activation.

We collected whole blood from CFS patients, who met CDC criteria, and healthy volunteers. The blood samples were stimulated with mitogens during 18 h and the levels of activated T and NK cells expressing CD69 were measured on a Coulter Epics flow cytometer using a three color immunofluorescence staining protocol.

The expression of the CD69 activation marker on T cells (CD3+, CD3+CD4+, and CD3+CD8+) and on NK cells (CD45+CD56+) was significantly lower in CFS patients than in healthy subjects. These differences were significant to the extent that a significant diagnostic performance was obtained, i.e. the area under the ROC curve was around 89%. No differences either in the number of leukocytes or in the number or percentage of lymphocytes, i.e. CD3, CD4, CD8 and CD19, could be found between CFS patients and the controls.

Patients with CFS show defects in T- and NK cell activation. Since induction of CD69 surface expression is dependent on the activation of the protein kinase C (PKC) activation pathway, it is suggested that in CFS there is a disorder in the early activation of the immune system involving PKC.

ORIGINAL ARTICLE

INTRODUCTION

Chronic Fatigue Syndrome (CFS) is a medically unexplained disease, characterized by profound fatigue and accompanied by rheumatological, infectious and neuropsychiatric symptoms [1]. In order to receive a diagnosis of CFS, a patient must satisfy two criteria, defined in 1988 and revised in 1994 by the U.S. Center for Disease Control and Prevention [1]: a) Have self–reported severe chronic fatigue lasting six months or longer duration with all other medical conditions being excluded; b) Four or more of the following symptoms should be present: substantial impairment in short–term memory or concentration; sore throat; tender cervical and axillary lymph nodes; muscle pain; multi–joint pain without selling or redness; headache of new type; unrefreshing sleep; and post exertion malaise lasting more than 24 hours. The symptoms must have persisted or recurred during six or more consecutive months of illness and must not have predated the fatigue.

The trigger factors of CFS are, amongst others, viral and bacterial infections, stressful life events, physical stress, exposure to toxins, and increased gut permeability [2]. Changes in the immune system play a role in the pathophysiology of CFS [2–4]. The abnormalities in the immune system in CFS consist of: a) immune activation, e.g. elevations in the numbers and proportions of T lymphocytes expressing surface activation markers, such as CD26 and CD38 [5] and impaired circulating levels of cytokines [6–8]; b) poor cellular function, with low natural killer (NK) cell cytotoxicity and poor in vitro lymphocyte response to mitogens [4,5,9]; c) signs of activation of the inflammatory response system (IRS), such as decreased serum zinc levels and increases in the alpha-2 fraction obtained by electrophoresis [10]; and d) signs of increased oxidative stress and a decreased antioxidant status [11,12].

Under physiological conditions, T-cell activation is initiated when the T-cell receptor for antigen (TCR) binds the antigen, expressed on the surface of Antigen Presenting Cells (APC) in the context of MHC molecules. This interaction is followed by a number of biochemical and molecular events, leading to initiation of the cell cycle and proliferation [13]. The activation of T lymphocytes in vivo can be mimicked in vitro by activating these cells with mitogens [14]. Activation of T cells results in an increased expression of some existing surface molecules, and, the de novo appearance of others, called activation markers. CD69 is the earliest leukocyte activation antigen, expressed by activated T, B and NK cells upon stimulation with different activators, including mitogens [15–17]. CD69 is also designated as: activation induced molecule (AIM), early activation antigen (EA-1), Leu23 and MLR3 antigen. During the cell cycle, the expression of CD69 precedes the synthesis of DNA and the expression of other activation markers, e.g. CD25, CD71 and HLA-DR [18]. Induction of the CD69 surface expression occurs within 1 to 2 h after triggering of the protein kinase C (PKC) activation pathway and calcium mobilization by engagement of the TCR/CD3 complex [19]. Moreover, there is some experimental evidence indicating that the CD69 molecule is capable to transmit activation signals resulting in gene expression and cellular proliferation [17]. Therefore, the rapid expression of CD69 following activation and its measurement by flow cytometry allows to detect lymphocyte activation abnormalities.

The present study has been conducted in order to examine whether CFS is accompanied by disorders in the mitogen-induced expression of the CD69 molecule on T (CD3+, CD3+CD4+, CD3+CD8+) and NK (CD45+CD56+) cells and whether CD69 expression is related to markers of IRS activation in CFS.

SUBJECTS AND METHODS

Subjects

Forty subjects participated in the present study, 30 patients with CFS and 10 unrelated controls (staff or their family members). The patients were admitted to the M-Care4U Outpatient Clinics, Antwerp, Belgium. We made the diagnosis of CFS by means of the diagnostic criteria of the Centers for Disease Control and Prevention (CDC).

The diagnostic criteria 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 symptoms listed in the Introduction. In order to measure the severity of CFS we used the the FibroFatigue scale (the fibromyalgia and chronic fatigue syndrome rating scale) [20,21]. The FibroFatigue scale is an observer's rating scale with 12 items measuring pain, muscular tension, fatigue, concentration difficulties, failing memory, irritability, sadness, sleep disturbances, autonomic disturbances, irritable bowel, headache, and subjective experience of infection. The FibroFatigue scale is a reliable and valid instrument with a capacity to monitor symptom severity and change during treatment of CFS and fibromyalgia [20].

In the present study, we excluded subjects with DSM-IV axis-I diagnoses (life-time), such as depression, anxiety disorders, schizophrenia, substance use disorder, and organic mental disorder. Also, patients who ever were treated with antipsychotics, anticonvulsants or mood stabilizers were excluded. No one of the subjects had been taking psychotropic drugs for at least 12 months. No one of the subjects had abnormal results for alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), calcium, creatinine, electrolytes, thyroid stimulating hormone (TSH), total protein, and iron or transferrin saturation. All subjects were free of medical disorders and drugs known to be accompanied by alterations in endocrine and immune function. All subjects gave written informed consent after the study protocol was fully explained. The study has been approved by the local ethical committee.

Methods

Blood samples were collected in lithium heparinized tubes. In the 30 control subjects and 10 normal controls, white blood cell (WBC) and lymphocyte counts were performed by means of the Bayer ADVIA120 Hematology System. Measurements of the CD3+, (pan T) CD4+ (T helper), CD8+ (T suppressor) and CD19+ (B) cells were performed using a two color immunofluorescence staining protocol (Becton Dickinson Immunofluorescent mAB). The mitogen-induced CD69 expression on T and NK cells was measured in 17 of the CFS patients and in the normal controls. Mitogen solutions were prepared in RPMI 1640 medium with L–glutamin (300 mg/ml, Gibko). 200:l of the whole blood samples were stimulated with 200:l of the mitogen ConA 125 g/ml (Sigma), at a final concentration of 62.5 g/ml; PHA 10 g/ml (Sigma) at a final concentration of 5 g/ml; and PWM 10:g/ml (Sigma) at a final concentration of 5 g/ml. All samples, including 200:l unstimulated blood, which served as a negative control, were incubated 18 hours in humidified atmosphere at 37 °C with 5% CO_2. Fifty microliters of stimulated and unstimulated samples were triple stained with different combinations of immunofluorescent mABs (FastImmune Becton Dickinson), specific for CD3, CD4, CD8, CD69, CD45, and CD56. The combinations were ready for use (Fastimmune Becton Dickinson). Samples were incubated with 10:l of CD3-PerCP/CD4-FITC/CD69-PE, 10:l of CD3-PerCP/CD8-FITC/CD69-PE or 10:l of CD45-PerCP/CD56-FITC/CD69-PE during 30 minutes in a dark place. The negative control consisted of 50:l unstimulated blood and 10:l of gamma1-PE/gamma1-FITC/CD3-PerCP isotype control. All samples were proceeded on the multi Q-prep workstation and analyzed on a Coulter Epics XL flow cytometer using a tree color immunofluorescence staining protocol. The analysis determined the percentage of cells expressing CD69 within T lymphocytes (CD3+), T helpers (CD3+CD4+), T suppressors (C3+CD8+), and activated NK cells (CD45+CD56+). The day-to-day coefficient of variation was 6.5%. The serum alpha-2 protein fraction, which was used as an inflammatory marker, was measured by means of serum protein electrophoresis (SEBIA Benelux, Raketstraat, Brussels). The inter-assay CV values was <2.0%.

Statistics

Group mean differences were examined by means of analysis of variance (ANOVA) or covariance (ANCOVA) and by means of an automatic linear discriminant analysis. Relationships between variables were assessed by means of Pearson's product moment or Spearman's rank order correlation coefficients or by means of an automatic multiple regression analysis (with an F-to-enter of p=0.05). 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. The significance was set at α=0.05 (two tailed).

RESULTS

Table 1 shows that there were no significant differences in age or gender between both study groups. Table 1 gives the results of the cell counts in CFS and in the normal controls. No significant differences in the number of WBC, lymphocytes, CD3+, CD4+, CD8+ and CD19+ cells could be found between both study groups. There was no significant difference in the CD4/CD8 ratio between both study groups. Table 2 shows the flow cytometric results of the CD69 expression. We found that the mitogen-induced expression of the CD69 molecule on the CD3+, CD3+CD4+, CD3+CD8+ and CD45+CD56+ cells was significantly lower in CFS patients than in controls (except for the PHA-stimulated expression of CD69 on the CD3+CD4+ cells). Covarying for age and gender did not change these results. There were no significant relationships between either age or gender or any of the CD69 data.

ROC analysis performed on the PWM-induced CD69 expression on the CD3+CD8+ cells showed that the area under the ROC curve (AUC) was highly significant (AUC=89.7%). Using the Con-A-induced CD69

Table 1. Measurements of white blood cells (WBC), lymphocytes (Lymph) and CD3+, CD4+, and CD8+ T cells, the CD4+/CD8+ T cell ratio and CD19+ B cells in patients with chronic fatigue syndrome (CFS) and in healthy controls.

Variables	Controls (n=10)	CFS (n=30)	F-value	p-value
Age	40.9 (6.6)	39.3 (11.4)	0	0.98
Sex (M/F)	3/7	8/22	0.04	0.8
WBC	6.31 (1.03)	6.09 (2.10)	0.09	0.7
Lymph%	29.7 (4.6)	33.3 (7.7)	1.89	0.2
LymphAN	1.86 (0.29)	1.96 (0.69)	0.23	0.6
CD3%	67.9 (7.5)	70.8 (9.6)	0.76	0.6
CD3AN	1.26 (0.27)	1.34 (0.32)	0.5	0.5
CD4%	44.8 (7.6)	44.7 (10.1)	0	0.97
CD4AN	0.82 (0.18)	0.85 (0.31)	0.07	0.8
CD8%	27.1 (4.1)	27.1 (7.4)	0	0.98
CD8AN	0.51 (0.13)	0.52 (0.99)	0.02	0.9
CD4/CD8	1.70 (0.47)	1.82 (0.85)	0.19	0.7
CD19%	11.6 (5.3)	11.6 (3.8)	0.11	0.7
CD19AN	0.21 (0.09)	0.21 (0.09)	0.03	0.9

All results are shown as mean (SD) and as percentages (%) or absolute numbers (109 / L). All results of ANOVAs (df=1/39), except for gender (results of χ^2 test, df=1).

Table 2. Mitogen (Con-A, PHA and PWM)-induced expression of CD69 on CD3+, CD3+CD4+, CD3+CD8+, and CD45+CD56+ cells in patients with chronic fatigue syndrome (CFS) and normal controls.

Mitogens used	the induced CD69 expression	Controls (n=10)	CFS (n=17)	F-value	p-value
Con-A	CD3+CD69+	11.41 (4.21)	6.89 (2.82)	7.8	0.009
	CD3+CD4+CD69+	7.66 (2.23)	4.68 (1.95)	13.3	0.002
	CD3+CD8+CD69+	1.64 (1.15)	0.43 (0.30)	17.3	0.0006
	CD45+CD56+CD69+	3.60 (2.24)	1.22 (1.13)	13.7	0.001
PHA	CD3+CD69+	16.97 (4.28)	10.69 (4.17)	14	0.001
	CD3+CD4+CD69+	7.90 (2.73)	6.28 (3.36)	1.7	0.2
	CD3+CD8+CD69+	1.92 (0.93)	1.19 (8.31)	4.4	0.04
	CD45+CD56+CD69+	5.60 (2.27)	2.97 (2.36)	8	0.009
PWM	CD3+CD69+	7.84 (5.12)	3.03 (1.43)	13.5	0.001
	CD3+CD4+CD69+	3.05 (1.73)	1.66 (0.97)	7.1	0.01
	CD3+CD8+CD69+	1.05 (0.73)	0.29 (0.29)	15.1	0.0009
	CD45+CD56+CD69+	5.99 (2.80)	2.31 (1.81)	17.3	0.0006

All results are shown as mean (±SD). All results of ANOVAs (df=1/25).

expression on the CD3+CD8+ cells we found that the area under the ROC curve (AUC) was highly significant (AUC=87.3%). The PHA-induced CD69 expression on the CD45+CD56+ cells resulted in a somewhat lower area under the ROC curve (AUC=77.9%). Accordingly, the diagnostic performances computed were highly significant. For example, at a cut off value for the PWM-stimulated CD69 expression on CD3+CD8+ cells <0.35%, the diagnostic performance was: sensitivity=76.5%, specificity = 100%, and PV+ = 100% (κ=0.71, t=0.522, p=0.00009). By means of automatic linear discriminant analysis (automatic stepdown method with both study groups as dependent variables and all mitogen-induced CD69 expression data as discriminatory variables) a highly significant discrimination (Wilk's lambda=0.46, Bartlett χ^2=18.5, df=2, p=0.00009; distance between the centroids of both groups = 2.1 standard deviations) was obtained. The two most significant discriminatory variables were PWM-induced CD69 expression on the CD3+ and CD45+CD56+ cells.

In order to examine the relationships between the expression of CD69 and the symptom profiles of the FibroFatigue scale, we performed automatic multiple regression analyses (with an F-to-enter of p=0.05) with the PWM, PHA and ConA-induced CD69 expression as dependent variables and the symptoms of the FibroFatigue scale as independent variables. We found that the PWM-induced expression of CD69 on CD45+CD56+ cells (F=8.8, df=1/15, p=0.009) and the PHA-induced CD69 expression on CD45+CD56+ cells (F=13.8, df=1/15, p=0.002) was significantly and negatively correlated to concentration difficulties, whilst the Con-A-induced expression of CD69 on CD45+CD56+ cells was significantly (F=17.5, df=2/14, p=0.0003) and negatively correlated to concentration difficulties (F=5.7, p=0.03, negatively loaded) and irritable bowel symptoms (F=8.2, p=0.01, negatively loaded).

In order to examine whether the expression of the CD69 molecule is related to signs of the inflammatory response system in CFS, we examined the relationships between the PHA, Con-A or PWM-stimulated expression of CD69 and the serum alpha-2 fraction obtained by means of electrophoresis. The alpha-2 fraction was significantly higher in CFS patients (mean ± SD = 8.6 ± 1.5%) than in the normal controls (mean ± SD = 7.1 ± 1.1%) (results of ANCOVA with age and sex as covariates: F=9.7, df=1/40, p=0.004). There were significant and negative correlations between the alpha-2 fraction and PHA-

induced CD69 expression on the CD3+CD8+ (r=–0.58, p=0.006), and CD45+CD56+ (r=–0.48, p=0.02) cells, the Con-A-induced CD69 expression on the CD45+CD56+ cells (r=–0.46, p=0.03), and the PWM-induced CD69 expression on the CD3+CD8+ (r=–0.44, p=0.04) and CD45+CD56+ (r=–0.53, p=0.01) cells. There was a trend towards a negative correlation between the serum alpha-2 fraction and the Con-A-induced CD69 expression on the CD3+ cells (r=–0.41, p=0.06).

DISCUSSION

The main finding of this study is that the expression of the CD69 activation marker on the T and NK cell surface of patients with CFS is significantly lower than in normal volunteers, whereas the absolute number and percentage of CD3+ (T cells), CD4+ (T helper cells), CD8+ (T suppressor cells) and CD19+ (B cells) was normal in CFS patients. The latter results are in agreement with some previous reports in CFS showing no significant difference in CD4+ and CD8+ expression or in the CD4/CD8 ratio [9, 22]. Other authors, however, found a decreased number of both CD4+ and CD8+ cells [23]; a reduced number of CD8+ and an increased CD4/CD8 ratio [24]; and elevated numbers of CD8+ cells with a decreased CD4/CD8 ratio [5].

To the best of our knowledge, this is a first study showing a significantly decreased in vitro expression of activation markers in CFS. If the test was not so expensive it could be employed as an external validating criterion for the clinical diagnosis of CFS. In particular, the stimulated expression of the CD69 molecule on the CD45+CD56+ and also on the CD3+CD8+ T cells yielded a good diagnostic performance (better than the CD69 expression on the CD3+ and CD3+CD4+ T cells). The decreased expression of the CD69 is, however, not specific for CFS. Thus, the number of T cells expressing activation markers, such as CD69, is decreased in patients with fibromyalgia [25], type I diabetes [26], systemic lupus erythematosus [27] and HIV infection [28].

Our results are also in agreement with previous reports that the ex vivo proliferative response of lymphocytes to mitogens is significantly decreased in CFS [5,9,22,29]. The commonly used method for the determination of cellular immune functions is quantification of lymphocyte responses to stimuli by quantification of DNA synthesis, which is determined by the addition of tritiated thymidine. It has been demonstrated that measuring the CD69 surface expression on T lymphocytes is an adequate method to assess T-lymphocyte activation and function [30]. CD69 expression by CD3 cells generally parallels DNA synthesis in response to PHA, PWM and staphylococcal enterotoxin B [30]. In HIV Type 1, CD69 expression reliably predicts the anti-CD3-induced proliferative response of lymphocytes [30]. Moreover, the sensitivity and specificity of CD69 assays are greater than those of DNA synthesis assays [30]. Since after its expression, CD69 may act as a costimulatory molecule for T-cell activation and proliferation, the decreased levels of CD69 in CFS could result in poor cell function and thus in the decreased lymphoproliferative responses, which has been established in CFS.

Another finding of this study is that – in CFS – the stimulated expression of the CD69 molecule is significantly and negatively related to signs of inflammation, i.e. to the increases in the serum alpha-2 protein fraction. As discussed in the Introduction, there are more signs of IRS activation in CFS [2,5,10,11]. In the latter, an increased in vivo expression of activation markers has been reported [5]. At first sight these findings may be contradictory to those of the present study. However, it is well known that in many conditions, such as autoimmune disorders and major depression, signs of in vivo activation and in vitro suppression of T cell immunity are intertwined phenomena [31,32]. As discussed previously, this may be attributed to the immunosuppressive effetcs of increased levels of pro-inflammatory cytokines and some acute phase proteins, such as haptoglobin; cytokine-induced exhaustion of T cells; and cytokine-induced depletion of serum tryptophan and zinc [31,32]. In addition, activation of the IRS is accompanied by signs of increased oxidative and nitrosative stress, which may be detected in many patients with CFS [11]. Increased oxidative stress may decrease lymphocyte proliferation and CD69 expression. For example, nanomole amounts of hydrogen peroxide cause a suppression of Con-A-stimulated human lymphocyte proliferation [33]. Exercise-induced oxidative stress decreases the proportion of activated-lymphocyte subsets (CD4+ and CD8+) expressing CD69 and decreases lymphocyte response to Con-A and PHA [34]. The relationship between decreased CD69 expression and activation of the IRS are further underscored by the present findings that the decreased expression of the CD69 molecule is significantly related to the occurrence of specific symptoms of CFS, i.e. concentration disorders and gastro-intestinal problems (irritable bowel). Concentration disorders are symptoms that may occur as a consequence of activation of the IRS [31]. Gastro-intestinal symptoms are key symptoms of CFS corresponding to an increased gut permeability with an increased IgM response against gram negative enterobacteria [2]. In another study, we detected significant correlations between the lowered CD69 expression and a lowered omega-3 status in CFS [35].

Another point which needs discussion is the relationship between CD69 expression and NK cells in CFS. One of the most consistently reported immune abnormalities in CFS is a diminished NK cell activity or a reduced number of NK cells [5,36,37]. Also, in the present study we found that the defect in the expression of CD69 was most pronounced on the CD45+CD56+ cells. NK cells express CD69 after activation by different stimuli and CD69 is implicated in NK cell activation. In humans, the CD69 gene is located on chromosome 12 in a region known as the NK gene complex, associated with the lectin-like receptor genes that control NK-cell activity [38]. CD69

has indeed a very particular significance in NK-cell activation and functions, such as cytolytic activity [39]. Therefore, it may be hypothesized that – in CFS – the diminished ability of NK cells to kill targets in vitro may be attributed to a decreased expression of CD69 on NK cells.

In summary, the above results reveal a defect in T and NK cell activation in patients with CFS. In addition, we may suggest that a defect in PKC activation may underlie the decreased expression of the CD69 molecule in CFS. On T cells, the expression and function of CD69 is dependent on the activation of PKC, but not PTK [18,40–42]. On NK cells, CD69 expression is in part mediated by the PKC pathway, although stimulation of PTK is a common step in signal transduction leading to CD69 Ag induction [43]. Since PKC-dependent pathways appear to modulate CD69 expression in T and NK cells it is probable that defects in PKC may underlie the expression of the CD69 Ag in CFS. Future research in CFS should focus on the role played by PKC and PTK.

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