

Antiviral Pathway Deregulation of Chronic Fatigue Syndrome Induces Nitric Oxide Production in Immune Cells That Precludes a Resolution of the Inflammatory Response

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ABSTRACT. Chronic fatigue syndrome (CFS) is a poorly defined medical condition diagnosed by exclusion, which, besides severe chronic fatigue as the hallmark symptom, involves inflammatory and immune activation stigma. Although viral infections are not systematically found in CFS patients, the type I interferon antiviral pathway has been repeatedly shown to be activated in peripheral blood mononuclear cells (PBMC) of the most afflicted patients. An abnormal truncated form of ribonuclease L (37-kDa RNase L) is also found in the PBMC of CFS patients and this protein has been proposed as a biological marker for CFS.

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Recently, the levels of this abnormal protein have been significantly correlated to the extent of inflammatory symptoms displayed by CFS patients. We report here that active double-stranded RNA-dependent kinase (PKR) is expressed and activated in parallel to the presence of the 37-kDa RNase L and to an increase in nitric oxide production by immune cells. However, PKR upregulation results also in a significant increase followed by a decrease in caspase 3 activity for the samples containing the highest levels of 37-kDa RNase L. This caspase 3 downregulation does not result from increased expression of the anti-apoptotic proteins Bcl-2 and Bcl-X_L. These results therefore suggest that chronic inflammation due to excess nitric oxide production plays a role in CFS and that the normal resolution of the inflammatory process by NF- κ B activation and apoptotic induction is impaired. These observations draw new directions for the therapeutic approach of CFS. doi:10.1300/J092v13n04_03 [Article copies available for a fee from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: <docdelivery@haworthpress.com> Website: <<http://www.HaworthPress.com>> © 2006 by The Haworth Press, Inc. All rights reserved.]

KEYWORDS. RNase L pathway, nitric oxide, PKR, chronic inflammation

INTRODUCTION

Chronic fatigue syndrome (CFS) is an illness of unknown etiology characterized by various symptoms, which include severe chronic debilitating fatigue, tender cervical or axillary lymph nodes, headaches, multi-joint and muscle pain, and impaired memory or concentration (1). The illness often begins by a flu-like syndrome (1), but despite the fact that a positive viral serology could be detected in some patients (2), no single etiologic agent has been so far identified. Nevertheless, an upregulation of the type I interferon (IFN) antiviral pathway has been repeatedly observed in CFS peripheral blood mononuclear cells (PBMC) by different laboratories (3,4). These abnormalities are associated with the presence in PBMC of a truncated ribonuclease L of 37-kDa (37-kDa RNase L) (5), resulting from proteolytic cleavage of the normal enzyme (6,7), which has been proposed as a biological marker for CFS (8,9). More recently (10), we have shown that the proteolytic cleavage of RNase L was the consequence of an abnormal upstream activation of the 2',5'-oligoadenylate pathway. The presence of this truncated RNase L

protein is likely to correlate with the intensity of the inflammatory response of CFS (11), previously documented by the presence of chronically elevated plasma α_2 -macroglobulin and tumor necrosis factor- α (TNF- α) levels (12,13). Since the presence of the 37-kDa RNase L is accompanied by an upregulation of the double-stranded RNA-dependent protein kinase (PKR) (4), we investigated the possible relationship between the upregulation of these enzymes in PBMC with apoptotic induction and nitric oxide (NO) production, both key elements of the inflammatory response (14). We report that in CFS, PKR expression and activation correlates with the presence of 37-kDa RNase L in PBMC. This results in the progressive activation of the caspases. In the samples with the highest 37-kDa RNase L and PKR levels, however, the caspase cascade is blocked and the PKR effect is likely to be limited to the induction of NO production through NF- κ B, which maintains chronic inflammation. We conclude that defects in apoptotic induction by activated RNase L and PKR are responsible for the chronicity of inflammation in CFS, which deserves proper therapeutic focus.

MATERIALS AND METHODS

Study subjects and samples. Blood samples were obtained from individuals selected from a medical practice at the University of Brussels and diagnosed for CFS according to Fukuda's criteria (15). PBMC were separated from heparinized blood (30 mL) by Ficoll-Hypaque density gradient centrifugation as previously described (8,9). Cytoplasmic extracts were prepared in the presence of the protease inhibitors aprotinin, leupeptin, pepabloc-SC and EDTA (Roche Biochemicals). Total proteins in the extracts were quantified using a modified Bradford assay method (Bio-Rad Laboratories) according to the manufacturer's procedure. The samples were classified into four groups according to the levels of 37-kDa RNase L.

Quantification of 37-kDa RNase L in PBMC extracts. Analysis was performed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), using a metaperiodate (10 mM final concentration, pH 4.75) oxidized 2',5'-oligoadenylate (2-5A) trimer radiolabeled at the 3' end with 32 P-pCp as the reporter ligand (8,9). Briefly, the radiolabeled 2-5A trimer was incubated with 200 μ g of cell extract at 2-4°C for 15 minutes and then covalently attached to the binding proteins by the addition of cyanoborohydride (20 mM in 100 mM phosphate buffer, pH 8.0).

After electrophoresis, the 2-5A binding proteins were detected by autoradiography (Bio-Rad Laboratories Molecular Imager Fx) and quantified by density scanning. The results are expressed as the percentage of 37-kDa RNase L present in the sample ($37\text{-kDa}/[80\text{-kDa} + 37\text{-kDa}] \times 100$). We prefer to express here the level of RNase L cleavage as a percentage of total RNase L instead of the simple tenfold 37-kDa/80-kDa protein ratio used in other studies (6,8,9), because the latter expression, but not the former, introduces a logarithmic element which could lead to confusion in evaluating correlations with other biological parameters.

Immunoblotting. PBMC extracts (200 μg proteins) were subjected to standard SDS-PAGE. The separated proteins were then transferred to a 0.2 μm PVDF membrane (Bio-Rad Laboratories) using a semi-dry transfer system (Amersham-Pharmacia Biotech). Transfer was performed at an average current of 0.8 mA per cm^2 for two hours. Proteins were then respectively detected by incubation with anti-PKR, anti-Bcl-2, anti-Bcl-X_L, anti-Bax (Santa Cruz Biotechnology, Inc.), or anti-phosphoserine (Sigma) antibodies, followed by a second horseradish peroxidase labeled anti-species antibody from Sigma. The proteins were detected by the Opti4-CN technique from BioRad Laboratories and quantified by density scanning.

Caspase activity measurements. Caspase activities in PBMC extracts were measured using dye-labeled specific substrates with commercially available kits (Biosource International). The results are reported as mAU generated per μg of protein.

Intracellular NO quantification. PBMC were pelleted (5 min $1,000 \times g$) and resuspended in 750 μl of PBS containing 25 μM of 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (Molecular Probes). After incubation during 45 min at room temperature in the dark, the cells were pelleted, resuspended in 200 μl PBS and further incubated for 20 min. The cells were pelleted, resuspended in 0.5% paraformaldehyde in PBS and analyzed by fluorescence activation and cell sorter analysis (FACS) in a Coulter Altra (Beckman). Intracellular NO production was calculated by integrating the percentage of positive cells with the fluorescence intensity.

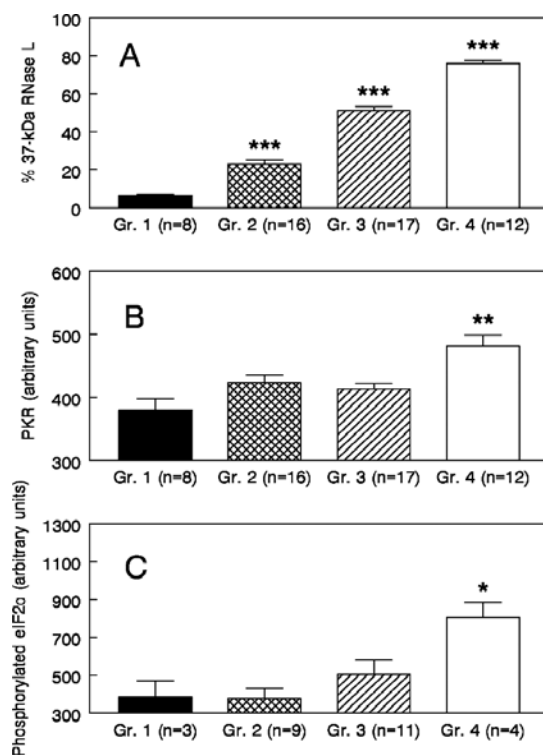
Statistical analysis. Significance of the mean differences of the individual variables (i.e., %RNase L, PKR, phosphorylated eIF-2 α and caspases) between groups were analyzed by one-way variance analysis (ANOVA) with post hoc Bonferroni t-tests. The differences were considered significant at $p < 0.05$ level.

RESULTS AND DISCUSSION

Despite the lack of evidence for a single common etiology, CFS has been repeatedly linked to a dysregulation of the interferon pathway involving the upregulation of RNase L and PKR (3-5). The upregulation of RNase L is associated with the presence in PBMC of a truncated 37-kDa form of the enzyme produced by proteolytic cleavage (6,7) as the consequence of an abnormal upstream activation in the signaling pathway (10), and retaining catalytic activity (16). Beside chronic fatigue as the hallmark symptom, CFS is characterized by a documented chronic inflammation (12,13), which involves oxidative stress (17), excessive NO production (18), and the symptoms of which correlate with the presence of 37-kDa RNase L in PBMC (11). Therefore, we investigated the possible relationship between the IFN antiviral pathway dysregulation observed in CFS and the maintenance of chronic inflammation.

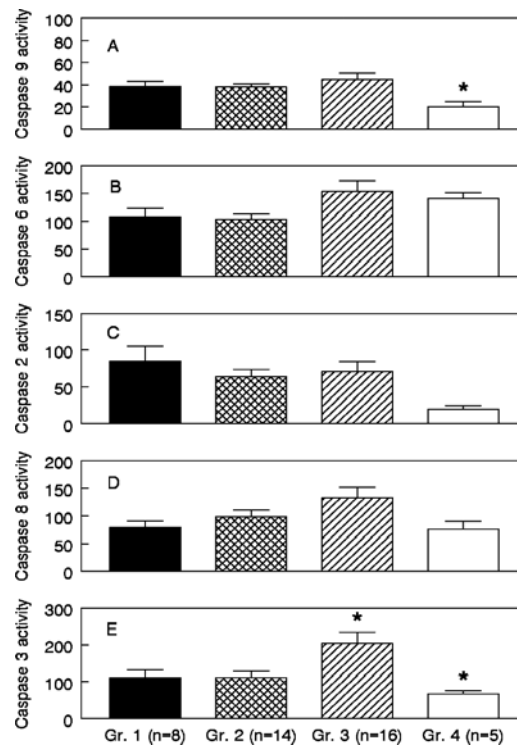
First, we verified that PKR induction and activation parallels the presence of the 37-kDa RNase L. CFS PBMC samples were analyzed for 37-kDa RNase L and were classified in four groups according to the level of abnormal versus normal enzyme, i.e., 0-10% (group 1; normal cutoff), 10-35% (group 2), 35-70% (group 3) and >70% (group 4) (Figure 1A). We used such a classification so as to introduce the notion of an incremental RNase L dysfunction (normal, low, medium, and high), which is supposed to correlate with an increased expression of the inflammatory symptoms (11). As shown in Figure 1B, PKR expression was significantly higher in group 4 and its increased activation was verified by the increase in phosphorylated eukaryotic initiation factor 2 (eIF2)- α (Figure 1C). Both RNase L (19) and PKR (20) are involved in apoptotic induction, a key player in the immune system during the resolution of the inflammatory response (14). Apoptotic induction by RNase L is likely to proceed through the mitochondrial pathway (21) involving caspase 9 and 6 activation (22). PKR induces apoptosis either by translation inhibition through phosphorylation of eIF2- α and subsequent TNF- α susceptibility (20) mediated by caspase 2 activation (22), or by direct Fas-associated death domain signaling (23) involving caspase 8 activation (22). The caspase cascades end in the activation of the executioner caspase 3 (22). As shown in Figure 2A-D, despite a trend to an increase for caspases 9, 6, and 8 activity in the first three groups and a trend to a decrease for caspases 9, 2, and 8 in the fourth group, only the decrease in caspase 9 for this latter group was significant. This general trend resulted, however, (Figure 2E) in a significant activation of caspase 3 in the third group, and a significant downregulation in the fourth group. In order to find the

FIGURE 1. The progressive presence of the truncated 37-kDa RNase L in the PBMC of CFS patients (part A) is accompanied by an increased expression (part B) and activation (part C) of PKR. PBMC samples are classified in four groups according to the level of 37-kDa RNase L, that is, 0-10% (group 1, black bars), 10-35% (group 2, crossed bars), 35-70% (group 3, hatched bars) and >70% (group 4, white bars). The number of samples in each group is given into brackets. Enhanced expression of PKR (part B) results in enhanced kinase activity as verified by the increase in phosphorylated eIF2- α (part C). Levels of significance by ANOVA and post hoc Bonferroni t-tests are, respectively, < 0.05 (*), < 0.01 (**), and < 0.001 (***).



origin of this downregulation, we measured anti- and pro-apoptotic proteins of the Bcl-2 family in PBMC samples characterized by progressively increased 37-kDa RNase L levels. A previous report (24) had indeed suggested that apoptotic downregulation in CFS immune cells could result from an increased expression of Bcl-2. As shown in Figure 3, we were, however, unable to confirm this observation. Our results conversely show

FIGURE 2. Increases in 37-kDa RNase L and PKR result in a progressive induction of mitochondrial caspases 9 (part A) and 6 (part B) in the first three groups of CFS PBMC samples (symbols and groups as in Figure 1). No change is observed for the death receptor caspase 2 in these groups (part C), while caspase 8 (part D) is progressively activated. Despite the lack of statistical significance, this trend to the increase results in a significant activation of caspase 3 in the third group (part E). In the fourth group of PBMC samples, most of the caspase activities are down-regulated, resulting in a significant decrease in caspase 3 activity.

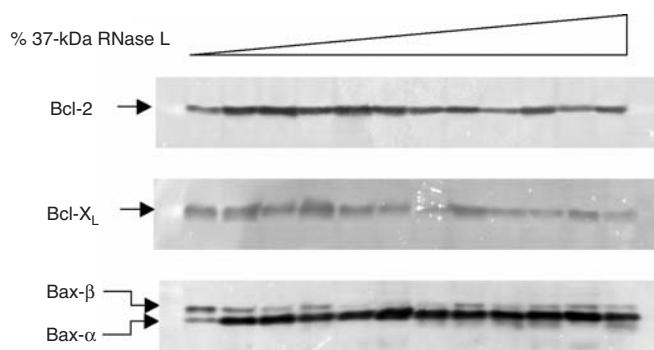


an increased expression of the pro-apoptotic Bax- α in samples containing the 37-kDa RNase L (compare with Lane 1 in Figure 3), which is, however, paralleled by a decrease in Bax- β expression. The respective roles of these two splice variants of Bax are, however, presently unknown (25). In some samples, the increase in Bax is matched by increases in the anti-apoptotic proteins Bcl-2 and Bcl-XL expression (Figure 3). Consequently,

our results rule out the possible involvement of an increase in the ratio between anti- and pro-apoptotic proteins of the Bcl-2 family in the apoptotic downregulation we observed in the fourth group of samples. These results are likely to suggest that immune cell apoptotic induction is impaired in the most afflicted CFS patients.

As a member of the I κ B kinase complex (26), PKR induces apoptosis by activation of the nuclear factor NF- κ B (27). Beside apoptotic induction, the phosphorylation of I κ B by PKR and the subsequent NF- κ B activation results in the enhanced expression of the inducible NO synthetase (iNOS) (28). NF- κ B activation is likely to play a central role in the inflammatory process (14). On the one hand, NF- κ B activation is held responsible for the increased iNOS expression and NO production by immune cells during the induction of the inflammatory response. On the other hand, NF- κ B activation induces apoptosis in immune cells during the resolution of inflammation. Therefore, we looked for a possible correlation between the IFN antiviral pathway dysregulation and NO production in CFS. As demonstrated in Figure 4, a significant positive correlation was observed between the level of 37-kDa RNase L and NO production in CFS PBMC samples. Since NO inhibits apoptosis by several mechanisms including a reduction in ceramide generation (29) and nitrosylation of the critical cysteine in caspase catalytic sites (30), our re-

FIGURE 3. Expression of Bcl-2, Bcl-X_L, and Bax as a function of the level of 37-kDa RNase L in PBMC samples. The samples containing the 37-kDa RNase L (compare with 1st lane) are characterized by an increased expression of the pro-apoptotic protein Bax- α . This is matched by a parallel increase in the anti-apoptotic Bcl-2 and Bcl-X_L proteins. Therefore, an increased ratio of anti- over pro-apoptotic proteins of the Bcl-2 family cannot be held responsible for the caspase downregulation.

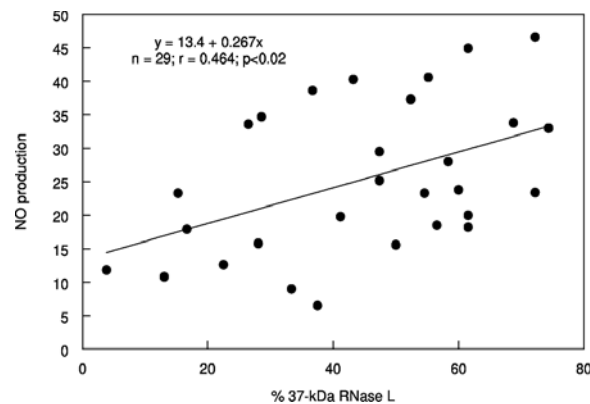


sults suggest that increased NO production could be held responsible for the apoptotic downregulation observed in PBMC samples characterized by the highest IFN antiviral pathway dysregulations.

CONCLUSION

Despite the lack of a single etiologic infectious agent in CFS, we confirm the presence in CFS immune cells of a strong dysregulation of the interferon pathway, which involves the progressive production of a truncated 37-kDa RNase L concomitant with the induction and activation of PKR. The activation of the IFN antiviral pathway by non-infectious agents receives progressive attention and PKR has been shown to be activated by specific intracellular activators such as its protein activator PACT (31) and Alu RNA (32). The dysregulation results in caspase 3 activation, indicative of a resolution of the inflammatory response, followed by a downregulation in the PBMC samples showing the highest levels of IFN antiviral pathway dysregulation. The dysregulation is further likely to result in a strong production of NO as demonstrated by the significant correlation between NO production in immune cells and the presence of 37-kDa RNase L. Taken together, our results seem to indicate

FIGURE 4. Correlation between NO production and percentage of 37-kDa RNase L in CFS PBMC samples. A positive and significant correlation was observed, indicating that the progressive dysregulation in the interferon pathway induces iNOS expression through NF- κ B activation by PKR.



that the chronic inflammation of CFS results from a preferential activation of NF- κ B by PKR toward increased NO production by immune cells, rather than toward apoptotic induction, which precludes a normal resolution of the inflammatory process (14). This observation should deserve further investigation in order to be properly addressed at the therapeutic level (33). Indeed, NO scavengers such as vitamin B12 (34) and N-acetylcysteine (35) have been advocated as CFS treatments (35) and show some efficacy in relieving the symptoms. Targeted therapies with PKR or iNOS inhibitors (36) might, however, prove more relevant in the future as they become available.

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