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Normal Cousins Lecture

Stress-associated changes in the steady-state expression of latent Epstein–Barr virus: implications for chronic fatigue syndrome and cancer

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Dedicated to the memory of George F. Solomon.

Abstract

Antibodies to several Epstein–Barr virus (EBV)-encoded enzymes are observed in patients with different EBV-associated diseases. The reason for these antibody patterns and the role these proteins might play in the pathophysiology of disease, separate from their role in virus replication, is unknown. In this series of studies, we found that purified EBV deoxyuridine triphosphate nucleotidohydrolase (dUTPase) can inhibit the replication of human peripheral blood mononuclear cells in vitro and upregulate the production of TNF-α, IL-1β, IL-6, IL-8, and IL-10. It also enhanced the ability of natural killer cells to lyse target cells. The EBV dUTPase also significantly inhibited the replication of mitogen-stimulated lymphocytes and the synthesis of IFN-γ by cells isolated from lymph nodes and spleens obtained from mice inoculated with the protein. It also produced sickness behaviors known to be induced by some of the cytokines that were studied in the in vitro experiments. These symptoms include an increase in body temperature, a decrease in body mass and in physical activity. The data provide a new perspective on how an early nonstructural EBV-encoded protein can cause immune dysregulation and produce clinical symptoms observed in patients with chronic fatigue syndrome (CFS) separate from its role in virus replication and may serve as a new approach to help identify one of the etiological agents for CFS. The data also provide additional insight into the pathophysiology of EBV infection, inflammation and cancer.

Keywords: EBV; Viral latency; Chronic fatigue syndrome; Cancer; dUTPase; Stress; Immune dysregulation

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1. Introduction

Work from our laboratory has focused on Epstein–Barr virus (EBV) latency, the expression of the EBV genome in different types of cells latently infected with the virus, and the pathophysiology of EBV-associated disease. We have also explored the role that psychological stress plays in the modulation of the steady-state expression of latent EBV and how this interaction could be a factor in the risk for chronic fatigue syndrome (CFS) and perhaps cancer (Cacioppo et al., 2002; Glaser et al., 1987, 1991, 1993, 1995; Glaser and Kiecolt-Glaser, 1994, 1998).

Chronic fatigue syndrome is a syndrome in which the prevalent clinical symptoms include severe fatigue, myalgia, lymphadenopathy, sore throat, stress, and depression. The fatigue must not be related to exertion and must be present for 6 months or more (Barker et al., 1994; Borish et al., 1998; Cannon et al., 1999; Gerrity et al., 2002; Komaroff, 2000; Mawle et al., 1997; Natelson, 2001; Tan et al., 2002). The most recent case definition of CFS by the Centers for Disease Control and the international committee (that is examining the case definition) agree that CFS is likely to be an umbrella term encompassing subsets that manifest a common symptom complex, as discussed by Reeves et al. in BioMed Central Health Services Research (Reeves et al., 2003). Thus, patients who are diagnosed with CFS based on symptoms are a very heterogeneous group perhaps involving different etiologies and responding differently to different therapies. For simplicity, we refer to CFS patients as a group but emphasize the heterogeneity of that group.

In reviewing the literature to determine if there is any consensus on immune changes/immune dysregulation observed in CFS patients, one finds a range of reports with some studies showing very few changes in the immune system and others showing significant changes in a variety of immune markers. These include differences in subpopulations of lymphocytes and serum levels of proinflammatory cytokines (Patarca et al., 1995a,b, 2000). Some studies show that CFS patients have increases in the number of $CD8^+$ cytotoxic T-cells and activated T-lymphocytes in peripheral blood concomitant with an increase in natural killer (NK) cell numbers (Klimas et al., 1990; Subira et al., 1989). Other studies suggest that the symptoms associated with CFS may be more closely related to pathology in the central nervous system (CNS), for example glial cells, than to circulating cytokines (Vollmer-Conna et al., 1998). Two recent studies by Natelson and colleagues suggest that there may not be a reliable pattern of immune dysregulation that can be observed in CFS patients. In the review of this literature, they conclude that there is a significant degree of variability in the observations made in a large number of studies with little consensus on immune abnormalities, particularly related to cytokines (Natelson and Gudrun, 2002; Natelson et al., 2002). Nevertheless, there are several studies that show that there are increases in serum levels of several proinflammatory cytokines or in levels of cytokines produced by peripheral blood leukocytes (PBLs) after stimulation in vitro. These include: interleukin 1-α (IL-1α), tumor necrosis factor-α (TNF-α), IL-2, IL-6, and IL-10 (Barker et al., 1994; Borish et al., 1998; Gerrity et al., 2002; Gupta et al., 1997; Komaroff, 2000; Mawle et al., 1997; Natelson, 2001; Patarca et al., 1994; Tan et al., 2002).

It is possible that the heterogeneity of patients diagnosed with CFS produces the significant variability obtained in immune and behavioral markers that could account for this literature. Trying to identify a subgroup of patients within the heterogeneous group may be a way to help reduce the variance and explore possible etiologies for at least that group of subjects. One example is acute onset CFS patients who show symptoms compatible with a virus infection. It is also possible that the inconsistent patterns of immune markers found in the literature are related to the present knowledge base of the fields of virology and immunology. New types of immune cells and cytokines are undoubtedly going to be discovered as we learn more about the immune system. It is possible that there may be other links to CFS, yet to be identified and characterized, which may help in the understanding of the pathophysiology and etiology of CFS.

While the literature is inconsistent, some of the data suggest an association with cytokine dysregulation and CFS. There is also a significant literature that links abnormal levels of cytokines to sickness behavior. It is of interest, and relevant to this overview, that similarities exist between cytokine-induced sickness behaviors and clinical symptoms observed in CFS patients (Anisman and Merali, 2003; Dantzer, 2001; Maier and Watkins, 1998; Reichenberg et al., 2001). Because many of the symptoms associated with CFS are also associated with an active virus infection, studies have focused on the hypothesis that reactivation of a latent virus(es) can induce CFS; two herpesviruses, EBV and human herpesvirus 6 (HHV-6) are two candidate viruses that have been linked to CFS.

One approach commonly used to measure reactivation of a latent herpesvirus is to determine if there is evidence for higher antibody titers to the virus in a patient group compared to a control group. The cellular immune response plays an important role in controlling the activation/replication of latent herpesviruses. When the cellular immune response is impaired, the viruses are reactivated. The increase in viral proteins being synthesized results in an increase in the memory antibody response and in higher antibody titers in serum or plasma. For EBV, we measure IgG antibody titers to the early antigen (EA) and virus capsid antigen (VCA) complex of proteins using the indirect immunofluorescence

(IF) test. A good example of this is a study in which we show that normal older subjects have much higher antibody titers to EBV antigens as compared to normal young adult subjects. While the older subjects were in good health, their immune response was reflective of an average age of 70 as compared to the medical student group whose average age was 21 (Glaser et al., 1985).

EBV encodes for approximately 70 proteins. One approach to determine EBV reactivation is to measure antibodies to EBV proteins in the serum/plasma. A second method used to determine if reactivation has taken place is to use real-time RT-PCR, which shows evidence of EBV DNA in serum circulating lymphocytes or saliva (Payne et al., 1999).

2. A historical perspective on the etiology of CFS

Jones et al. (1985) and Straus et al. (1985) provided evidence that EBV was the etiologic agent for a chronic illness (chronic infectious nucleosis-like syndrome) that is now known as CFS. These early studies showed that patients with CFS-like symptoms had significantly higher antibody titers to EBV than control subjects. Holmes et al. (1987) found higher EBV antibody titers in patients with symptoms compatible with CFS; antibody titers to other herpesviruses such as cytomegalovirus (CMV) and herpes simplex virus (HSV), and measles virus were also observed in these patients. Buckwald et al. (1987) and Hellinger et al. (1988) also found a relationship between higher EBV antibody titers and CFS. In a later study by Buchwald et al. (1996), and studies by Koelle et al. (2002), Reeves et al. (2003), and Mawle et al. (1995, 1997), antibody titers and/or levels of EBV DNA using PCR were measured in CFS patients, including monozygotic twins; no relationship was found between antibody titers to any of the viruses tested, including latent herpesviruses, and no evidence for viral DNA in a variety of specimens was found. Although the clinical evidence supports the hypothesis that CFS is caused by a virus in at least a subset of patients (acute onset), there is still no clear consensus on this issue.

Work from our laboratory has focused on unique antibody patterns to EBV-encoded enzymes, e.g., DNase, DNA polymerase, and dUTPase. Antibody to those enzymes are observed in different groups of patients who have EBV-associated diseases. We initially found that patients with nasopharyngeal carcinoma (NPC) have antibodies to EBV DNase (Cheng et al., 1980a). Out of 101 serum samples from normal EBV seropositive patients (medical students), none were positive for antibody to EBV DNase. Unpublished data from our laboratory show that out of 16 NPC patients' sera tested, 43.8% were positive for antibody to the EBV dUTPase. When serum from CFS patients was tested for antibody to the EBV DNase and DNA polymerase, we found that CFS patients with high VCA antibody titers were positive for antibody to both enzymes (Jones et al., 1988) (Fig. 1). In a follow-up study, Natelson et al. (1994) also found a relationship

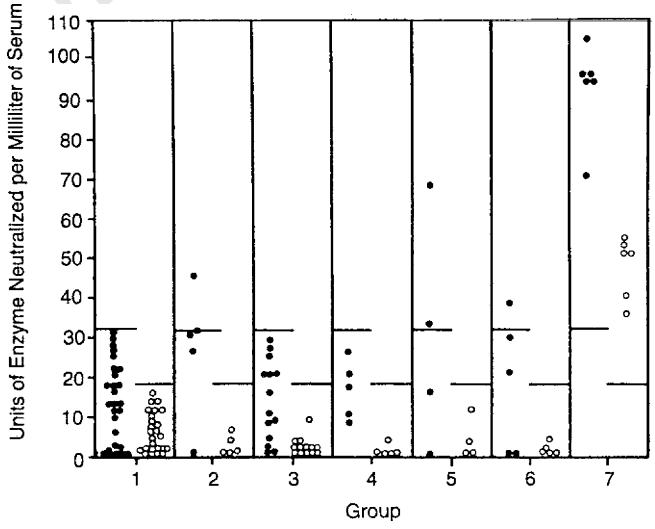

Fig. 1. Antibodies to Epstein–Barr virus-specific DNase and DNA polymerase in the chronic fatigue syndrome. Anti-Epstein–Barr virus (EBV) DNase and anti-EBV DNA polymerase activity (units neutralized per milliliter of serum) in patients according to groups: group 1 indicates control or standardization subjects; group 2, healthy subjects aged 65 years or older; group 3, patients with chronic fatigue syndrome and anti-early antigen antibody liters of 80 or greater; group 4, patients with acquired immunodeficiency syndrome-related complex; group 5, patients with primary infectious mononucleosis; group 6, patients with chronic fatigue syndorme and low or absent anti-EBV antibody levels; and group 7, patients with chronic illness and extraordinarily high anti-EBV antibody liters. Solid circles represent anti-DNA polymerase activity; amd open circles, anti-DNase activity. Horizontal lines in each column represent 95% confidence limit of values derived from group 1, control population; these levels were determined to be levels of positive responses. (From: Jones et al., 1988.)

with antibody to the EBV DNA polymerase and CFS. We recently obtained data that support our earlier findings; 16/33 (41%) of serum from a cohort of CFS patients at the University of Miami and 25/30 (83%) of serum from a University of Texas-Houston group of CFS patients were positive for antibody to EBV DNase. The unique antibody patterns to EBV-encoded proteins may provide a clue linking EBV with CFS (Glaser and Kiecolt-Glaser, 1998).

HHV-6 has also been associated with CFS. Higher antibody titers to HHV-6 in CFS patients (as compared to controls) are consistent with reactivation of latent virus (Ablashi et al., 2000; Di Luca et al., 1995; Krueger et al., 1994, 2001; Patnaik et al., 1995; Sairenji et al., 1995); the presence of IgM antibodies to HHV-6 in a significant number of patients diagnosed with CFS would indicate a primary infection (Krueger et al., 2001; Patnaik et al., 1995). However, over time, the association between EBV, HHV-6, and/or other herpesviruses with CFS has not resulted in a consensus on the etiology of CFS.

EBV is the etiological agent for heterophile-positive infectious mononucleosis (IM); in Britain, IM is known as glandular fever. Peter White and colleagues performed an interesting longitudinal study. They found that there was a risk for some glandular fever patients presenting symptoms compatible with CFS well after the acute infection. Patients also had behavioral disorders associated with post-glandular fever episodes. The CFS symptoms observed in these studies were not found to be associated with upper respiratory tract infections. These studies continue to support the possibility that EBV may play a role in at least a subset of CFS patients (White, 1997; White et al., 1995, 1998, 2001).

In spite of these studies, it is still not clear why it has been so difficult to consistently identify a virus(es) in CFS patients who have symptoms that are compatible with a virus infection (there is at least some consensus on this point). Indeed, the lack of testable hypotheses in studying the etiology of CFS has been a problem.

3. The effect of psychological stress on the steady-state expression of Latent EBV

It is now well established that the central nervous system (CNS), the endocrine system, and the immune systems interact with each other and that psychological stress can down-regulate/dysregulate the immune response by affecting the interplay of these systems. The interactions are complex, involving both the hypothalamic–pituitary–adrenal (HPA) axis and the autonomic nervous system (Ader et al., 1991; Rabin, 1999). There is also evidence that psychological stress could be a co-factor for the risk of developing CFS (Carter et al., 1999; Theorell et al., 1999)

In a series of studies with medical students, examination stress was associated with changes in the steady-state expression of latent EBV. Higher antibody titers to EBV VCA IgG were observed at the time of examinations as compared with baseline blood samples drawn approximately 1 month before (Glaser and Kiecolt-Glaser, 1994; Glaser et al., 1987, 1991).

In follow-up studies using the academic stress model with medical students, we examined the impact of stress on two different aspects of the EBV-specific memory T-cell response. In the first study, we found a significant decrease in the ability of EBV-specific cytotoxic T-cells (from EBV seropositive students) to kill EBV-infected autologous B-lymphocytes associated with examination stress. In the second study, PBLs obtained from EBV seropositive students (at the time of exams compared to the baseline blood sample) showed a decrease in proliferation when exposed to several purified EBV polypeptides (Glaser et al., 1993). There is a growing literature showing that different types of psychological stressors can reactivate latent EBV and CMV (Esterling et al., 1990, 1992; Lutgendorf et al., 1994; Mehta et al., 2000a,b; Payne et al., 1999; Prosch et al., 2000; Sarid et al., 2002; Stowe et al., 2000).

Our group and others have shown that pharmacological, and most importantly, physiological levels of glucocorticoid hormones can reactivate latent EBV from virus genome-positive cells in vitro (Bauer, 1983; Glaser et al., 1995; Joncas and Leyritz, 1974; Magrath et al., 1979). Glucocorticoid hormones, ACTH, and CRH can also enhance EBV-lytic replication in superinfected cells in vitro (Glaser et al., 1995). However, in one of our studies on the effect of academic stress on the expression of latent EBV, we found no relationship of plasma cortisol levels and VCA antibody titers (Glaser et al., 1994). The data from this study suggested that the association between absolute levels of glucocorticoid hormones and reactivation of latent EBV may be more complicated than we had previously thought. In support of this conclusion are data from a recent study from our laboratory suggesting that fluctuations in glucocorticoid hormone levels, as modulated by autonomic reactivity, rather than absolute levels of plasma cortisol, may modulate the steady-state expression of latent EBV (Cacioppo et al., 2002). Autonomic activity and antibody titers to EBV VCA were measured in 50 elderly women latently infected with EBV. Results revealed that women who were high reactors to a laboratory stressor were characterized by having significantly higher antibody titers to the latent virus than low stress reactors, $p \leqslant .03$. High reactors tended to show larger stress-related increases in cortisol than low reactors, but the differences were not significant. Daily stressors can activate the autonomic nervous system and promote the release of pituitary and adrenal hormones, especially in high reactor subjects. These data could have implications for the role of stress-

associated changes in the steady-state expression of latent EBV in CFS patients.

In one of the medical student examination studies in which we found stress-associated increases of EBV VCA antibody titers (Glaser et al., 1991), we also collected throat-washing samples to probe for the presence of EBV DNA in exfoliated cells in the washings. We found very little evidence for shedding of EBV particles or DNA in the nasopharynx even though we found evidence for virus reactivation as measured by increases in EBV antibody titers in serum samples obtained from the same students (Glaser et al., 1987, 1991). Thus, in this study, increases in EBV antibody titers to latent EBV at the time of academic examinations occurred in the absence of replication of a complete virus (virions containing DNA) in the nasopharynx suggesting that reactivation of latent EBV could be incomplete under some circumstances.

If this were the case, the increase in antibody titers observed by IF and the absence of EBV DNA in throat-washing samples could be the result of incomplete or abortive reactivation of latent EBV. It is possible that only a small number of genes, associated with the early antigen (EA) complex, were expressed; these proteins do not require the synthesis of new viral DNA (Glaser and Kiecolt-Glaser, 1998).

Because we had shown that there are groups of patients that have antibody to one or more of the six EBV-encoded enzymes, we hypothesized that one or more of these proteins might play a role in the pathophysiology of CFS. These proteins are part of the EA complex and do not require the synthesis of new viral DNA. Proteins of the VCA complex are called late viral proteins and are part of the protein coat that surrounds viral DNA. The synthesis of these late proteins requires the synthesis of new viral DNA. Employing the indirect IF test to measure antibody titers in serum would not have permitted the discrimination between complete and incomplete virus reactivation, because this assay measures essentially all EBV antibodies present in the serum.

4. EBV-encoded enzymes

EBV encodes for several viral enzymes that are part of the EA complex. Thus far, six EBV-associated enzymes have been described. These include thymidine kinase (TK), DNase, dUTPase, ribonucleotide reductase, DNA polymerase (Cheng et al., 1980b; Glaser et al., 1973; Henry et al., 1978; Miller et al., 1977; Williams et al., 1985), and uracil–DNA glycosylase (Baer et al., 1984).

As already mentioned, antibodies to several of these EBV-encoded enzymes have been described in patients with different EBV-associated diseases. Our laboratory was the first to show that antibodies to an EBV-encoded enzyme, DNase, could be found in patients with NPC (Cheng et al., 1980b). These data were confirmed by many researchers, e.g. (Liu et al., 1989). There is also a report that shows that NPC patients may also make an antibody to the EBV TK (de Turenne-Tessier et al., 1989). Patients with IM, chronic active EBV infection, and patients infected with HIV have elevated antibody titers to EBV dUTPase (Sommer et al., 1996). While these unique antibody patterns to these EBV enzymes and other EBV-encoded proteins have been found to be clinically useful (Cheng et al., 1980a; Henle et al., 1971, 1973; Liu et al., 1989; Sommer et al., 1996), the underlying factor(s) that produce these antibody patterns to EBV enzymes and the role these proteins might play in the pathophysiology of EBV-associated disease, separate from their role in the replication of the virus, has not been explored.

5. A new way of linking a latent virus to CFS

A collaborative study with Gary Pearson (Georgetown University) explored the possibility that incomplete virus reactivation could explain the observations described above. ELISA plates were separately coated with four different purified EBV-encoded proteins: the 85 kDa EA-R, ribonucleotide reductase, the 52/50 kDa EA-D, DNase, or two late viral proteins, a 125 kDa VCA structural protein or the gp350/220 membrane glycoprotein. ELISA plates coated with a single purified protein allowed us to probe for a specific antibody against each of these four viral proteins. Previously characterized plasma samples known to have higher antibody titers at the time of examinations (as determined by IF) showed no evidence for changes in the antibody titers to the two late viral proteins tested. In addition, no evidence for changes in antibody titer to the 85 kDa EA-R protein was observed. However, antibody titers to the EA-D 52/50 kDa protein changed across the six blood samples showing evidence for reactivation and the expression of at least one viral protein, the DNase (Glaser et al., 1991). It is possible that other viral enzymes/early proteins were also expressed, but we were not able to measure them.

It is important to point out that these data are preliminary and measured antibodies to just four viral proteins. To determine whether additional antibodies to specific EBV proteins increased in these subjects would require studies testing for virtually all early viral proteins. In addition, the issue of the sensitivity of the assay used must be kept in mind in interpreting the data. However, we believe that these data raise interesting questions on the reactivation of latent EBV and perhaps other herpesviruses in vivo. It is possible that stress and other factors can modulate the steady-state expression of latent herpesviruses, such as EBV, and that under certain

circumstances, only some viral genes may be expressed (or over-expressed), making it difficult to show a consistent relationship between patient groups using routine laboratory methods. However, it also raises the possibility that there may be differences in the immune response to some viral proteins by some patients. Further work needs to be performed to clarify these issues.

Data from a study with mouse CMV (MCMV) are consistent with the results we obtained with EBV. Treating mice latently infected with MCMV with TNF-α induced the expression of the latent virus. Only the immediate-early protein-1 (IE1) transcripts were detected. Allogeneic transplantation also induced the expression of the IE1 transcript (Hummel et al., 2001). This "abortive" or incomplete reactivation of MCMV suggests that our hypothesis for EBV may be true for other herpesviruses (Glaser and Kiecolt-Glaser, 1998).

6. Can viral proteins produce immune dysregulation and induce sickness behavior?

Clinical symptoms associated with a virus infection are due to the combination of the pathology produced by the virus and the immunopathology that results from the immune response to the virus. In a previous report (Glaser and Kiecolt-Glaser, 1998), we hypothesized that some viral proteins, e.g., EBV-encoded DNase, synthesized by reactivated EBV during "complete" lytic replication could induce immunopathology/immune dysregulation. We also hypothesized that EBV could induce clinical symptoms observed in CFS patients in a more subtle way, by synthesizing an early viral protein(s) such as dUTPase and/or DNase, etc., in cells in which limited expression of the latent virus genome occurred (abortive or incomplete replication). These viral proteins, expressed under such conditions, could induce immune dysregulation including the dysregulation of cytokine synthesis and T-cell function. These changes could produce sickness behaviors known to be induced by several cytokines, such as IL-6, IL-1 and TGF-β, that have been observed in some patients diagnosed with CFS. If this hypothesis is correct, there would be an explanation as to why data linking EBV with CFS has been inconsistent. That is to say, it would not be routinely possible to detect significant changes in antibody titers to latent EBV in some CFS patients if antibodies to only a small number of selected (early) EBV proteins were being synthesized. Antibody titers are routinely measured using IF, ELISA, or Western blot, employing either cells expressing a full range of antigens, such as by IF or an ELISA plate coated with a single viral polypeptide—e.g., one of the late EBV VCA proteins (p18) is commonly used in commercial kits as the test antigen. It would not be possible to know which viral polypeptide(s) to use as a probe to measure changes in a specific antibody. Also, if reactivation of latent EBV was abortive and only early viral proteins were synthesized (which our data suggest is possible), it would not be possible to detect differences in the levels of viral DNA by PCR because de novo viral DNA would not be synthesized in this scenario.

There is evidence in the literature to support the hypothesis that viral proteins, by themselves, can induce immune dysregulation and sickness behavior. In one study, the 15 kDa polypeptide (p15e) of the feline leukemia virus (FeLV) was shown to significantly inhibit replication of mitogen-stimulated feline lymphocytes in vitro. In addition, capping of receptors for Con A on normal feline lymphocytes was also inhibited by the p15e protein (Mathes et al., 1979). Most importantly, the p15e protein induced immune suppression in vitro and in cats. It has also been shown that a recombinant peptide, HIV-1 env-gag, suppressed the synthesis of IgG by pokeweed mitogen-treated human B-lymphocytes. The same recombinant peptide significantly increased the proliferative response of peripheral blood mononuclear cells (PBMCs) as compared with control cultures (Nair et al., 1988). Pugh et al. showed that HIV gp120 can induce the release of IL-1β in rats. Memory impairment was associated with treatment with gp120 (Pugh et al., 2000). Data from a recent study showed that purified EBV latent membrane protein-1 (LMP-1), which is expressed in latently infected cells, was able to suppress T-cell and NK cell responses (Dukers et al., 2000). Another study using purified EBV envelope glycoprotein (gp-350/220) showed that this late structural viral protein was able to upregulate TNF-α gene expression in human monocytes (Addario et al., 2000).

If individual FeLV and HIV-1 proteins can modulate different aspects of T-cell responses, it is likely that cytokines and other cellular factors produced by subpopulations of leukocytes may also be affected by EBV early viral proteins. Putting these findings in the context of the preliminary data from our laboratory showing evidence for partial reactivation of latent EBV and the MCMV study by Hummel et al. (2001), it is possible that one or several early proteins expressed by a latent virus during abortive reactivation or expressed during full lytic replication could induce immunomodulation that results in abnormal levels of several cytokines. These cytokines could influence the steady-state expression of other latent viruses, e.g., CMV and induce the clinical symptoms associated with CFS in, for example acute onset patients. Furthermore, this link would not be obvious using the standard laboratory procedures already discussed.

Putting all of these factors together, a hypothesis was proposed to explain how a virus, like EBV, HSV-6 (or another latent virus), could be the etiological agent for CFS (Glaser and Kiecolt-Glaser, 1998). It was proposed that one or more of the EBV early proteins, which are

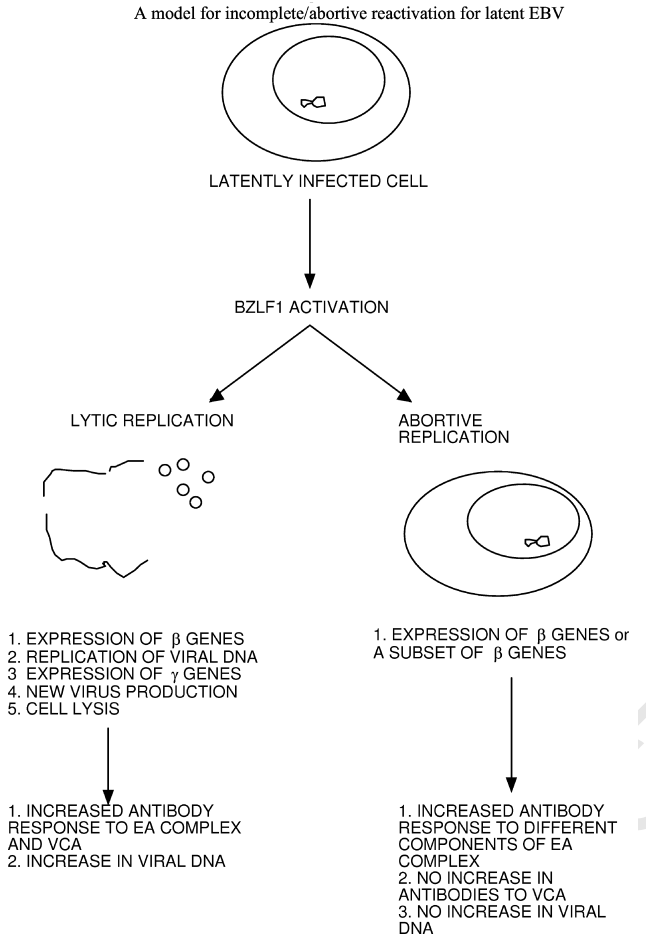

Fig. 2. A model for incomplete/abortive reactivation for latent EBV.

synthesized after the latent virus is reactivated, could play a role in the pathophysiology of EBV-associated disease, including CFS. A second alternative, as already discussed, is related to data from our group that suggests that one or more of the EBV-encoded early proteins (such as DNase or dUTPase) could be expressed as part of the incomplete reactivation of latent EBV in the absence of virus DNA synthesis and the expression of late viral proteins encoded by the newly synthesized viral DNA. A proposed model is shown in Fig 2.

6.1. The EBV dUTPase can induce immune dysregulation in vitro and in vivo

We have started to explore the hypothesis that one or more EBV-encoded enzymes could induce immune dysregulation in vitro and/or in vivo. The EBV-encoded dUTPase was chosen because antibody to the enzyme has been shown in patients with chronic active EBV infection (Sommer et al., 1996). Furthermore since the in vivo studies would require milligram quantities of the protein, of all the EBV-encoded enzymes, the procedure used to purify and concentrate the dUTPase is the least problematic. Thus, there was also a practical consideration as well.

For both of these studies, we used purified EBV dUTPase (31 kDa); the preparations were tested for the presence of endotoxin and found to be negative. In the first study, human PBMCs were treated with an anti-CD3 monoclonal antibody (mAb) to stimulate T-cell blastogenesis then exposed to either 7.5 or 15 μg/ml of the dUTPase for 72 h. Separate cultures of PBMCs were treated with buffer or human γ-globulin as negative controls. The EBV dUTPase significantly inhibited the replication of mAb-treated PBMCs in a dose–response manner. Neither buffer nor human gamma-globulin showed a significant effect on T-cell proliferation (unpublished data).

Studies were performed to determine if the EBV dUTPase could induce immune dysregulation in PBMCs resulting in the production of cytokines capable of producing sickness behavior observed in CFS patients. The same concentrations of the EBV dUTPase were used to treat resting human PBMCs. Cell supernatants were tested for the production of IL-2, IL-4, IL-5, IL-1β, IL-6, IL-8, IL-10, IL-12 p70, and TNF-α concentrations at 24, 48, and 72 h after exposure as measured by ELISA or by flow cytometry. The results are summarized in Table 1. We found that the treatment of resting PBMCs with the viral protein induced a rapid release of TNF-α that peaked at 24 h and quickly declined over time. A significant increase in IL-10 and IL-1β was observed, which reached a maximum at 24 h then slowly declined over time. IL-6 and IL-8 production was also found to be upregulated and sustained over time as well. No changes in IL-2, IL-4, IL-5, and IL-12 p70 were observed.

Cytokine mRNA levels were examined at 24 h after treatment. An increase in IL-10, IL-1β, IL-6, and IL-8 mRNA was observed in PBMCs treated with EBV dUTPase. While an upregulation of TNF-α was observed using flow cytometry beads, changes in TNF-α mRNA levels were not detected possibly because the samples that we used to measure mRNA were taken at 24 h, and gene expression occurred prior to this or that

Table 1
EBV dUTPase effects on cytokine production in vitro

Time (h)	TNF-α (pg/ml)	IL-10 (pg/ml)	IL-1β (pg/ml)	IL-6 (ng/ml)	IL-8 (ng/ml)
24	29.55	37.58	46.35	30.29	5.49

PBMCs were treated with buffer (control) or 15 μg/ml EBV dUTPase for the indicated time. Results are shown as the fold increase in the dUTPase treated cells when compared to controls.

the elevated TNF-α levels detected by ELISA resulted from release of the membrane-associated TNF-α precursor. Because we did not see an upregulation of IL-2, IL-4, IL-5 or IL-12 p70, we did not measure mRNA levels of these cytokines (unpublished data).

Finally, we also performed a study to determine if the EBV dUTPase could influence the ability of NK cells to lyse K562 target cells. Preliminary data suggest that the protein can stimulate NK cell lysis in PBLs obtained from normal individuals (Fig 3). The data need to be confirmed, but they are consistent with increases in NK cell activity observed in some CFS patients already discussed (Klimas et al., 1990; Subira et al., 1989).

We were interested in determining the EBV dUTPase target subpopulation(s) of PBMCs responsible for the production of these cytokines. A series of depletion studies using magnetic cell sorting microbeads (MACS) was performed. Based upon the increase in several proinflammatory cytokines observed in the studies using PBMCs, we focused on macrophage/monocytes (CD14-positive cells). The data show that by removal of CD14-positive cells, it was possible to totally eliminate the cytokine pattern upregulated by PBMCs treated with the viral protein. In fact, treatment with EBV dUTPase of the adherent fraction, which was enriched for monocytes/macrophages, showed a profile similar to the response of PBMCs treated with the viral protein. Thus, monocytes/macrophages appear to be the primary cell type affected by the EBV dUTPase and responsible for the upregulation of the proinflammatory cytokines observed (unpublished data).

The induction of IL-10 by EBV dUTPase may help explain the inhibition of anti-CD3-mAb-induced blastogenesis and down-regulation of anti-CD3-mAb-induced IFN-γ production (de Waal Malefy et al., 1991; Taga et al., 1993). IL-10, which is produced by activated monocytes and T- and B-cell lymphomas, can inhibit the production of IL-1, IL-2, IL-6, and IFN-γ (Mosmann, 1994). The EBV also encodes for a viral IL-10 (vIL-10), which can inhibit the production of IFN-γ and IL-1; these cytokines favor the survival of EBV (Moore et al., 1993). Our finding that the EBV dUTPase can also induce the production of cellular IL-10 suggests that EBV can regulate host cellular IL-10, as well as vIL-10, to induce immune dysregulation, which would favor the survival of EBV, and perhaps, even stimulate the proliferation of B-cells latently infected with EBV (Emilie et al., 1992). This result has implications for EBV-associated tumors. Both cytokines will negatively affect the induction of EBV-specific cytotoxic T-lymphocytes to EBV latently infected growth and malignantly transformed cells (Kanno et al., 1997; Rickinson et al., 1992).

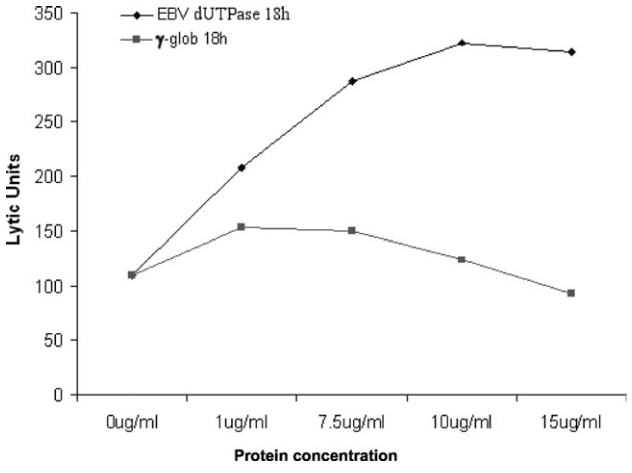

Fig. 3. NK cell activity measured in 4 h ^{51}Cr-release assays following 18 h incubation of a normal donor's PBMC with EBV dUTPase or γ-globulin as control at various protein concentrations.

Interestingly, patients with chronic active EBV infection have high levels of circulating IL-10; they also make antibody to IL-10 as well (Tanner et al., 1997). This increase in IL-10 may be part of the pathophysiology of some EBV-associated diseases, including acute onset CFS patients (Tanner et al., 1997). Data from these studies suggest that the increase in IL-10 in those patients could be related to the synthesis of EBV dUTPase synthesized by lytically replicating EBV or by the expression of the EBV genome to synthesize dUTPase in latently infected cells.

6.2. dUTPase-induced immune dysregulation: Implications for malignant disease

The EBV has been associated with several malignant diseases including NPC, chronic lymphocytic leukemia (CLL), and several different B-cell malignancies including Burkitt's lymphoma (BL) and post-transplant B-cell lymphomas (Ansell et al., 1999). Of interest is that in patients with some of these cancers, there is evidence linking serum IL-10 and IL-6 levels with prognosis. For example, serum from patients with NPC have elevated levels of IL-10 that are related to late-stage disease. This suggests that IL-10 may have a potential role in the progression of NPC tumors (Budiani et al., 2002). Furthermore, examining tumor biopsies showed that NPC tumor cells were positive for IL-10.

Patients with CLL also have high serum levels of IL-6 and IL-10. In fact, serum levels of these cytokines are predictive of survival of CLL patients (Fayad et al., 2001; Lai et al., 2002). There may also be a link for IL-10 in the pathogenesis of EBV-associated B-cell lymphomas (Khatri and Caliguiri, 1998). As already discussed, IL-10 may act to the advantage of selecting for EBV-genome-positive B-cells latently infected with EBV and enhance the proliferation of the cells by down-regulating the host-specific cytotoxic T-cell response to them. The data from our studies on the EBV dUTPase suggest that

the production of IL-6 and IL-10 in these patients could be related to the synthesis of EBV dUTPase during lytic replication of latent EBV or by cells latently infected with the virus similar to the case for NPC (Fleischmann et al., 2002). Further studies will have to be performed to explore this possibility. However, the data that we have obtained in our studies in which both of these cytokines are upregulated by PBMCs exposed to the EBV dUTPase suggest that there could be a connection. Furthermore, the increased levels of IL-6 and IL-10 observed in the patients with poor prognosis could relate to the increase in EBV replication/reactivation and an increase in synthesis of the EBV dUTPase.

7. A possible mechanism to explain the interactions between the EBV dUTPase and target CD14$^+$ cells

The results obtained from the depletion/add-back experiments with CD14$^+$ monocytes/macrophages suggest a possible mechanism to explain the interaction between the EBV dUTPase and the PBMCs. It is possible that the innate immune response may play a role in these interactions. Innate immune recognition detects conserved microbial products through toll-like receptors (TLRs).

Ligation of TLRs activates signal transduction pathways leading to the induction of proinflammatory cytokines, chemokines, and MHC molecules. TLRs activate both NF-κB and MAP kinases (O'Neill, 2002). It is possible that EBV dUTPase, or a peptide fragment of the protein yet to be determined, may be recognized by an intracellular toll/IL-1 receptor (TIR) and may subsequently drive the expression and upregulation of the proinflammatory cytokines observed in this study.

8. The ability of the EBV dUTPase to induce immune dysregulation in vivo: an animal model for EBV and CFS?

Except for cotton top tamarins, which are an endangered species and are essentially unavailable for studies with EBV, there are no animal species that can be successfully used as an animal model for EBV infection. Nonetheless, experiments were performed to explore the possibility that the immune dysregulation associated with EBV dUTPase in vitro might produce immunological changes in a mouse. To explore this possibility, CD-1 outbred mice were inoculated intramuscularly (IM) with purified EBV dUTPase in the hind limb; 11 mice/group/day in three independent experiments. Three daily doses of 10 μg of EBV dUTPase were used in these studies. The control group received inoculations of the vehicle control. One, three, and five days after the last injection of the viral protein, lymphoid cells were prepared from spleens and inguinal lymph nodes. The cells were treated with either Con A or LPS (or media as a control) to induce cell proliferation. A significant reduction in the proliferation of the cells prepared from spleens was observed Day 1 post-inoculation (PI), which persisted until Day 3, $p \leqslant .05$. There was also a significant effect for time, $p \leqslant .001$. An even greater reduction in cell proliferation was observed in cells prepared from the inguinal draining lymph nodes, $p \leqslant .001$, again with an effect for time, $p \leqslant .001$.

Cell supernatants were obtained from cultures of spleen and lymph node cells 72 h after Con A stimulation and assayed for the production of IFN-γ. The data show that the EBV dUTPase treatment reduced the level of Con A-stimulated production of IFN-γ by cells, $p \leqslant .001$. A significant inhibition was also observed three days PI but this effect diminished by Day 5 in cells from both lymphoid organs. Treatment with EBV dUTPase did not have a measurable effect on the production of IL-10 by the mitogen-stimulated cells obtained from either spleens or lymph nodes (Padgett et al., in press). These data are in contrast to the data in the study with human PBMCs. The differences could be related to different conditions used in the studies or the difference in species.

When the cells from EBV dUTPase-inoculated mice were treated with LPS, in contrast to the results obtained with Con A, no measurable effect was observed on cell proliferation on Day 1 PI. However, cells obtained from dUTPase-treated mice 3 and 5 days PI showed a significant decrease in the response to LPS, $p \leqslant .05$. Once again, an effect for time was also observed with cells obtained on Days 3 and 5 PI, $p \leqslant .05$.

We then explored the possibility that the purified EBV dUTPase, alone, could induce sickness behavior in mice. Mice were inoculated with either purified EBV dUTPase or a vehicle control. Changes in behaviors known to be induced by cytokines were observed in the dUTPase-treated mice. No changes in food intake or body mass of the mice treated with the vehicle were observed over the two-week study. Food intake was also unaffected by the EBV dUTPase treatment, $p \leqslant .05$. However, mice treated with the EBV dUTPase showed a significant loss in body mass, $p \leqslant .05$ in spite of no change in their food intake. The results could be explained by the increase in body temperatures observed in the EBV dUTPase-treated mice, $p \leqslant .05$. A particularly exciting finding is that the viral protein induced fatigue in the mice as measured by a significant decrease in locomotor activity, $p \leqslant .02$. Locomotor activity was measured by the number of photobeam gridlines crossed by the mice in the monitoring chamber (Padgett et al., in press). The results from these mouse studies are summarized in Table 2. Thus, we have developed a mouse model to study the immune modulatory properties of EBV-encoded proteins.

Table 2

	Spleen	Lymph node
In vivo influence of dUTPase on immune function in mice		
Con-A-stimulated proliferation	Suppressed	Suppressed
Con-A-stimulated IFN-γ production	Suppressed	Suppressed
Con-A-stimulated IL-10 production	Not affected	Not affected
Lipopolysaccharide-stimulated proliferation	Suppressed	Suppressed
In vivo influence of dUTPase on sickness behavior in mice		
Body mass	Caused significant loss of body mass	
Locomotor activity	Significantly diminished locomotor activity	

9. A summation

The data support the hypothesis that at least one or more protein(s) of the EBV EA complex can induce immune dysregulation. The EBV dUTPase and perhaps other viral proteins, expressed in cells during lytic replication and/or after the latent viral genome is reactivated (perhaps associated with psychological stressors) alone or in concert with other EBV-encoded enzymes, may be involved in the pathophysiology of EBV-associated disease, including CFS. The unique antibody patterns to EBV enzymes observed in, for example, NPC and CFS patients might be related to over-expression of these proteins with a concomitant increase in the memory antibody response to the protein(s). It is also of interest that patients with chronic active EBV infection and NPC make antibody to vIL-10 and cellular IL-10 (Tanner et al., 1997). The antibody patterns may reflect the role that viral proteins play in the disease process.

It is possible that reactivation of latent EBV in resting cells could be abortive in some individuals leading to the expression of only certain early viral proteins, such as the dUTPase. This could occur in nonreplicating cells in the absence of EBV DNA synthesis and in the absence of the production of late viral proteins. Data from a recent study support the conclusion that EBV dUTPase can be expressed in resting cells (Fleischmann et al., 2002). The fact that some individuals latently infected with EBV have antibody to some EBV enzymes shows that there must be a population of latently infected cells synthesizing the protein to a level sufficient to induce an effective antibody response.

As already discussed, we recently examined serum from two groups of CFS patients: one from the University of Miami and the other from the University of Texas, Houston. A significant percentage of these subjects were positive for the EBV-encoded DNase continuing to confirm the relationship described in our earlier study (Jones et al., 1988). We also assayed the same serum samples for the presence of neutralizing antibody to the EBV dUTPase. It has been shown that sera from normal EBV-seronegative or seropositive individuals do not have antibody to EBV dUTPase (Sommer et al., 1996). Of the sera obtained from the University of Texas–Houston group of patients, 6/34 samples (18%) were positive for antibody to EBV dUTPase. Four of the 33 sera samples (12%) from the Miami cohort had antibody to the EBV dUTPase. The data consistently show a relationship between antibody to EBV DNase and CFS, but the percentage of serum from CFS patients tested thus far for antibody to the dUTPase is much lower than that observed for the DNase. It is still not clear if the relationship between the presence of neutralizing antibody to one or more EBV-encoded enzymes is related to the pathogenesis of the disease. Therefore, it is difficult to interpret these differences until more data are obtained. Further work will be necessary to understand why antibodies to one or more EBV-encoded enzymes are present in only some patients with EBV-associated disease, including some CFS patients. Is it possible that patients who are negative for neutralizing antibody against EBV dUTPase might actually have low detectable levels of the protein in their serum? Does this have implications for disease/symptoms? The fact that there are antibodies in some patients' serum demonstrates that there is enough protein being synthesized to induce an antibody response. We are attempting to clarify and explore these relationships.

These studies also provide data that could explain why it has been difficult to link EBV with CFS by commonly used diagnostic procedures, e.g., antibody titers to EBV antigens and increased levels of EBV DNA in PBMCs, serum or saliva from CFS patients.

Stress is thought to be a factor in CFS. Stress has also been shown to induce reactivation of latent EBV, and other herpesviruses (Glaser and Kiecolt-Glaser, 1994), and could be a co-factor for risk for developing CFS. There are now several papers, including work from our laboratory, that show that EBV-encoded proteins, by themselves (and independent of their role in virus replication) can produce immune dysregulation. Other viral proteins can cause immune suppression, e.g., the FeLV p15e protein (Mathes et al., 1979). We will continue to explore these relationships.

There are still several important questions to be explored: (1) Can other EBV-encoded early proteins, such as the EBV DNase, TK, DNA polymerase, etc., induce immune dysregulation? (2) Can combinations of these proteins produce different affects than when used alone? (3) Can proteins encoded by other latent herpesvirus, such as HHV-6 or CMV, induce similar patterns of immune dysregulation? (4) Since many individuals are latently infected with HHV-6, CMV, and EBV, could

stress or other factors induce complete or incomplete reactivation of these latent viruses, such that in combination, the end result is a syndrome known as CFS? Could therapy, utilizing drugs designed to inhibit EBV dUTPase (or another viral protein(s)), be effective in reducing sickness behavior? Further studies using this approach may help clarify the etiology of at least a subset of CFS patients and help us understand the complexities of the pathophysiology of diseases associated with latent herpesviruses.

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