Epstein–Barr Virus-Encoded dUTPase Modulates Immune Function and Induces Sickness Behavior in Mice

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INTRODUCTION

In addition to being the etiological agent for infectious mononucleosis (IM), Epstein–Barr virus (EBV), is also closely associated with Burkitt’s lymphoma (BL) and nasopharyngeal carcinoma (NPC). EBV encodes for several groups of proteins in infected cells such as early antigens (EA), virus capsid antigens (VCA), and nuclear antigens (EBNA) [Lidin et al., 1993; Collins and Medveczky, 2002; Levin et al., 2003]. Antibodies to VCA proteins are commonly detected in patients during primary infection and are diagnostic of a primary or persistent infection. Antibodies to several EA proteins have been detected in patients with diseases associated with latent EBV, such as BL and NPC [Henle et al., 1973; Reedman and Klein, 1973; Glaser and Zhang, 1987]. Although the pattern of expression of antibodies to EBV-encoded proteins may be useful in diagnosing a putative etiology of a disease such as IM, NPC, or BL, the role that these EBV-encoded proteins may have in the pathophysiology of EBV-associated diseases is not thoroughly understood. Therefore, understanding why these antibody patterns exist, and the underlying mechanisms that produce them may provide insight into the role that EBV may play in disease syndromes that have been associated with infection with this herpesvirus.

Work in our laboratories has begun to focus on the role that enzymes encoded by EBV might play in the pathophysiology of EBV infection. EBV, like other

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herpesviruses, encodes for several enzymes that are involved in viral DNA replication; all are part of the EA complex. Thus far, six EBV-associated enzymes have been described. These include thymidine kinase (TK), DNA polymerase, alkaline deoxyribonuclease (DNase), deoxyuridine triphosphate nucleotidohydrolase (dUTPase), ribonucleotide reductase, and uracil-DNA glycosylase [Glaser et al., 1973; Miller et al., 1977; Goodman et al., 1978; Henry et al., 1978; Cheng et al., 1980a; Baer et al., 1984; Williams et al., 1985]. We and others have shown that antibodies to EBV-encoded DNA polymerase and DNase can be detected in patients with NPC [Cheng et al., 1980b; Liu et al., 1989]. In addition, patients with IM, and chronic EBV infections, as well as some patients with HIV infections have elevated antibodies titers to EBV dUTPase [Sommer et al., 1996].

Recent observations from our group have shown that the EBV-encoded dUTPase modulates human peripheral blood mononuclear cell activation and cytokine secretion in vitro (unpublished observations). These studies also showed that dUTPase enhanced the secretion of cytokines, such as TNF-α, IL-1β, and IL-6, which have been implicated in the expression of sickness behaviors [Maier and Watkins, 1998; Dantzer, 2001] including fever [Kozak et al., 2000], food intake [Swiergiel et al., 1997], and locomotor activity [Mentkowski et al., 1997]. Taken together, it is possible that one or more of the EBV-encoded enzymes may play a role in EBV associated diseases.

In an attempt to further examine the potential role of the EBV-encoded dUTPase in EBV associated disease, the current study characterized the influence of the purified EBV-encoded dUTPase on the activation of lymphocytes in vivo and examined the effects of the enzyme on sickness behaviors in mice. The blastogenic response of lymphocytes obtained from lymph nodes and spleens from mice treated with EBV dUTPase was inhibited when treated with concanavalin A (Con-A) and lipopolysaccharide (LPS) when compared to the response of lymphocytes obtained from control mice. These lymphocytes were also less able to synthesize interferon-gamma (IFN-γ) when stimulated with Con-A. Additional studies were performed to investigate whether exposure to the EBV-encoded dUTPase influenced or contributed to sickness behaviors associated with pro-inflammatory cytokine production. The data revealed that injection of dUTPase induced fever and reduced locomotor function consistent with altered expression of cytokines.

MATERIALS AND METHODS

Purification of EBV dUTPase

The EBV-encoded dUTPase gene was cloned into a pET3A vector, which was kindly provided by Peter Sommer (Institut fur Mikrobiologic und Hygiene, Abteilung Virolgie). This plasmid (pET3aEBVdUT) was maintained in Escherichia coli BL21 using ampicillin (100 μg/ml) selection. The EBV-encoded dUTPase was purified, using Blue Sepharose affinity chromatography, to apparent homogeneity, based upon analysis by SDS–PAGE using a modification of procedures we have described previously [Williams and Parris, 1987]. For the studies described in this report, the negative control consisted of protein from the induced E. coli BL21EBVdUT that did not bind to the Blue Sepharose matrix. This negative control contains the E. coli dUTPase as well as other E. coli proteins.

EBV dUTPase activity was determined as described previously using the DE81 filter disc assay [Williams et al., 1985]. Protein concentrations were determined by the Coomassie blue dye-binding assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as the standard. SDS–PAGE was performed as described previously [Williams and Parris, 1987]. EBV dUTPase preparations were negative for endotoxin as determined by the Limulus Amebocyte Lyssate Kit (BioWhittaker, Walkersville, MD), which can detect an endotoxin concentration of 0.06 IU/ml.

Mice

Six to eight-week-old male CD-1 outbred mice were obtained from Charles River, Inc. (Wilmington, MA) and randomly segregated into cages of five animals. All mice were allowed free access to food and water. Prior to experimentation, all animals were acclimated to their surroundings for a minimum of 7 days.

Experiment 1: Influence of dUTPase on Immune Function

Treatment of mice with dUTPase. In order to determine if EBV dUTPase could modulate the activity of immune cells in vivo, mice were inoculated with the protein. For the first series of studies, mice were divided into two groups with 11 total mice/group/day over the course of three independent experiments. Group 1 received a 200 μl intramuscular injection in the hind leg containing 10 μg of EBV dUTPase daily for 3 days. Group 2 received 200 μl injections of the control vehicle (10 mM Tris HCl, pH 7.5, 1 mM MgCl2, 2 mM 2-mercaptoethanol) or the negative control, which consisted of bacterial dUTPase in the vehicle. At days 1, 3, and 5 after the last injection, spleens and inguinal lymph nodes were harvested for subsequent analyses. Although it would have been relevant to select a protein concentration to be delivered based on the concentration of dUTPase found during EBV infections in humans, such information does not exist in the literature. Furthermore, the quantity of an EBV-encoded protein would undoubtedly fluctuate depending on the state of infection (i.e., primary infection, latency, or reactivation). Therefore, instead of arbitrarily choosing a dose, timing, and delivery method, these experiments were based on those that have been previously published [Mathes et al., 1979; Nair et al., 1988; Dukers et al., 2000].

Isolation of Lymphoid Cells and Proliferation Assays

Single cell suspensions from spleens and inguinal lymph nodes were prepared as previously described
Clifton, NJ) at 17:00 hr. Food intake was monitored daily between 10:00 and 12:00 hr. Locomotor activity was assessed by placing animals in a Digiscan activity monitor for a 10 min test period with EBV dUTPase, time post inoculation) on quantitative (proliferation, cytokine production) dependent variables. Analyses of effects on dependent variables were conducted using standard methods for analysis of variance (ANOVA). Significant effects were examined using post hoc comparisons of pairs of groups to isolate specific group differences. For all results, an alpha level of 0.05 was used as the criterion for statistical significance.

For the sickness behavior studies, data in each of the experiments were analyzed using a mixed model [SAS Institute, 2001] that consisted of enzyme treatment as the independent variable, day as the repeated measure, and individual animal as a random variable. Dependent variables included: body mass, activity, food intake, and temperature. Treatments were compared by day and significance was ascertained using one-way t-tests. Mean differences were considered significant where \( P < 0.05 \).

**RESULTS**

**EBV dUTPase Inhibits Con-A-Stimulated Proliferation**

EBV dUTPase was purified to a single band as described in the methods. CD-1 mice were inoculated by intramuscular injection in the hind leg with purified EBV dUTPase. Spleens and inguinal lymph nodes that drain the upper leg (i.e., the site of inoculation) were harvested 1, 3, and 5 days after the last injection with dUTPase. Proliferation of lymphocytes isolated from spleens and lymph nodes in response to Con-A was measured. A significant reduction in proliferation was observed in cells obtained from spleens day one post inoculation (P.I.) which persisted until day 3, \( P < 0.05 \) (n = 11 animals/group/day). There was also a significant effect for time, \( P < 0.001 \) (Fig. 1a). An even greater reduction in proliferation was observed using cells obtained from inguinal lymph nodes, \( P < 0.001 \) (n = 11 animals/group/day) with a concomitant effect for time, \( P < 0.001 \) (Fig. 1b).

**EBV dUTPase Inhibits Con-A-Stimulated IFN-\( \gamma \) Production**

From similarly prepared cultures of spleen and lymph node cells, supernatants were collected 72 hr after Con-A stimulation. The data showed that dUTPase treatment of CD-1 mice reduced Con-A-stimulated production of IFN-\( \gamma \) by cells obtained from both the spleens (Fig. 2a) and lymph nodes (Fig. 2b), \( P < 0.001 \) (n = 11 animals/group/day). For example, for cells obtained from lymph nodes, dUTPase treatment reduced Con-A-stimulated IFN-\( \gamma \) production by 27% at day 1. This significant inhibition was also observed 3 days post inoculation but had diminished by day 5 in cells from both organs. In contrast to the production of IFN-\( \gamma \), dUTPase treatment had no measurable effect on the production of IL-10 from mitogen-stimulated cells obtained from either spleens or lymph nodes (Fig. 2c,d).

**EBV-dUTPase Inhibits Lipopolysaccharide-Stimulated Proliferation**

Cells from lymph nodes and spleens were then examined for their ability to proliferate in response to LPS on days 1, 3, and 5 P.I. In contrast to the results
obtained following Con-A stimulation, no measurable effect was observed on the response of cells treated with LPS, on day 1 after treatment. However, cells obtained from mice 3 and 5 days P.I. showed a significant decrease in the proliferative response to LPS, \( P < 0.05 \) (n = 11 animals/group/day) (Fig. 3a,b). For example, at 5 days P.I., proliferation of lymph node cells from dUTPase treated animals was only 73% of LPS-stimulated control cultures (Fig. 3a). An effect for time was also observed with cells obtained on days 3 and 5 P.I., \( P < 0.05 \).

**EBV dUTPase Influences Sickness Responses**

In order to determine if the immune dysregulation observed in EBV dUTPase-treated mice induces sickness behavior(s), mice were inoculated with either EBV dUTPase or the vehicle control. Altered behaviors that are associated with abnormal cytokine levels were observed in the dUTPase-treated mice. Body mass of vehicle-treated mice did not change over 2 weeks; whereas, mice treated with dUTPase lost significant body mass (\( P < 0.05 \)). Surprisingly, food intake was unaffected by EBV dUTPase treatment (\( P > 0.05 \)) (data not shown). Reduced body mass may reflect elevated metabolic rates given that body temperatures were significantly elevated in dUTPase-treated mice (\( P < 0.05 \)) (Fig. 4a). Furthermore, EBV dUTPase treatment significantly decreased locomotor activity (\( P < 0.02 \)) (Fig. 4b).

**DISCUSSION**

We have hypothesized that the reactivation of latent EBV, under some circumstances, may be incomplete resulting in the limited expression of a limited number of EA proteins and that some, if not all, of the EA proteins can induce immune dysregulation in vitro and in vivo [Glaser et al., 1991; Glaser and Kiecolt-Glaser, 1998]. Based on work from our laboratory and others, we also hypothesized that some EBV-encoded early proteins can induce immune dysregulation in vitro and in vivo [Mathes et al., 1979; Glaser et al., 1991; Glaser and Kiecolt-Glaser, 1998]. The data obtained in this study support this hypothesis. The results of this study show that the EBV dUTPase induces suppression of the proliferative response of cells (to Con-A and LPS) obtained from spleens and lymph nodes removed from mice inoculated with the purified viral protein. The data also show that Con-A-stimulated cells from mice inoculated with EBV dUTPase were less able to synthesize IFN-\( \gamma \) when compared to cells obtained from control mice. In contrast, IL-10 responses were intact when assayed in vivo.

As a mitogen, Con-A non-specifically cross-links the T-cell receptor complex and typically induces a polyclonal T cell cytokine response that includes both IFN-\( \gamma \) and IL-10 [Ruscetti and Chervenick, 1975]. The in vivo mouse data suggest that EBV dUTPase treatment may diminish the activation of those cells that produce IFN-\( \gamma \) (presumably Th-1 cells) while not affecting those cells that produce IL-10 (presumably Th-2 cells). Finally, EBV dUTPase can induce sickness behaviors in mice consistent with the increased expression of specific cytokines. Data obtained in an in vitro study with human PBMCs also support the hypothesis that EBV dUTPase can induce the production of proinflammatory cytokines (unpublished data).

The data are consistent with previous reports showing that structural viral proteins can induce immune dysregulation. For example, purified p15e protein of feline leukemia virus suppressed Con-A induced cell proliferation of normal feline lymphocytes [Mathes et al., 1979]. In another study, HIV-ENV-GAG protein suppressed IgG synthesis while enhancing the proliferative response of peripheral blood mononuclear cells (PBMC) obtained from normal subjects [Nair et al., 1988]. Furthermore, EBV LMP-1, which is expressed in latently infected cells, suppressed T-cell and NK cell responses as well [Dukers et al., 2000]. Each of these virus-encoded proteins is a capsid or envelope...
component or is expressed on the membranes of latently infected cells. In contrast, the EBV-encoded dUTPase is a non-structural protein. EBV dUTPase is an enzyme involved in hydrolyzing dUTP and preventing its incorporation into viral DNA [Williams and Parris, 1987]. Thus, these current studies extend the immune regulatory findings concerning structural proteins to virus-encoded enzymes.

EBV-encoded dUTPase shares greater than 50% homology in its catalytic domain with that of *E. coli* dUTPase. However, because the *E. coli* dUTPase did not affect lymphocyte proliferation or cytokine production (data not shown), we surmised that the immunosuppressive effects of the EBV-encoded dUTPase may be related to its primary structure rather than to its enzymatic activity. Innate immune recognition detects conserved microbial products essential to microbial function and conserved amongst closely related species. EBV dUTPase is one such protein, highly conserved and essential to the replication of progeny EBV. Such conserved microbial antigens are recognized by receptors on host cells known as pattern-recognition receptors (PRRs) [Armant and Fenton, 2002; Barton and Medzhitov, 2002] or Toll-like receptors (TLRs) [Heine and Lien, 2003]. Ligation of TLRs activate signal transduction pathways leading to the induction of various genes including those encoding for inflammatory cytokines, chemokines, MHC molecules and co-stimulatory molecules [O’Neill, 2002]. Future experiments will test the hypothesis that dUTPase is recognized by a mammalian TLR which subsequently activates the expression of immune regulatory cytokines and growth factors which are immune modulatory in nature.

Such a theory does raise one important question. If EBV-encoded viral dUTPase is not a structural protein and is not typically expressed on the outside of virus-infected cells, how would it cause immune dysregulation through a TLR? This is indirectly answered by the detection of antibodies to EBV dUTPase in clinical populations [Sommer et al., 1996]. To mount an antibody response to a protein, it typically must be present in the extracellular space as immunoglobulin recognizes antigen in its free form. Thus, the detection of anti-dUTPase antibodies suggests that intact dUTPase is recognizable by the adaptive immune response. Therefore, we hypothesize that free dUTPase may be available for TLR binding in EBV-infected individuals; this hypothesis is being addressed in ongoing studies.

In the current studies, dUTPase-treatment of mice resulted in symptoms associated with a viral infection, including an increase in body temperature and a decrease in activity (fatigue). More specifically, mice inoculated with dUTPase exhibited sickness behaviors known to be induced by cytokines such as TNF-α, IL-1β, IL-6 [Maier and Watkins, 1998; Dantzer, 2001]. These sickness behaviors include an increase in body

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Fig. 2. T-cell cytokine production by lymph node and spleen derived lymphocytes: The data represent the concentration of interferon-gamma from (a) spleen and (b) lymph node and interleukin-10 from (c) spleen or (d) lymph node produced after 72 hr of Con-A stimulation as measured by ELISA. Solid bars represent cells from animals treated with EBV dUTPase and open bars represent cells from vehicle treated control animals. Data represent the mean ± SD of samples obtained from 11 animals run in triplicate at each time point. *P < 0.001.
temperature, signs of fatigue/lethargy, and loss of body mass. Surprisingly, food intake was unaffected by EBV dUTPase treatment. The reduction in body mass may have reflected elevated metabolic rates associated with increases in body temperature.

Although the temperature differences between dUTPase-treated and control mice were only statistically significant on four specific days, the mean temperature of dUTPase-treated mice was numerically higher on each day, and the overall ANOVA for all temperature differences was statistically significant. The variation of colonic temperature among mice is relatively high as indicated by our error bars in Figure 4. Regardless, the significant decrease in body mass of dUTPase-treated mice was analyzed over the same duration of time in which temperature was measured. Due to the overlap in these data, the data suggest that the increased energy requirements necessary for maintaining elevated body temperature may have resulted in the decrease in energy storage (i.e., body mass). Taken together, administration of the viral protein, dUTPase, was sufficient to induce sickness behaviors associated with viral infections. These behaviors are not simply manifestations of a viral infection, but can be regarded as adaptive strategies to efficiently rid the host of the pathogen, thereby increasing survival [Kluger and Rothenburg, 1979; Bilbo et al., 2002].

In conclusion, the data demonstrate that at least one of the proteins in the EBV–EA complex induces immunosuppression/dysregulation. Therefore, it is possible that a viral protein such as EBV dUTPase, which is expressed in cells infected with EBV during lytic replication and in some cells during reactivation, may be involved in the pathophysiology of EBV associated disease.

REFERENCES


