Inhibiting reactivation of Epstein-Barr virus

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Statement of the Problem:

Epstein-Barr virus (EBV) infects 90-95% of humans. EBV does not cause major illness when primary infection occurs in young children, but can cause infectious mononucleosis in adolescents. Though infection is common, EBV infection is very rarely associated with human cancers. EBV infection can contribute to cancers, such as Burkitt lymphoma. Despite 50 years since the discovery of EBV, we do not know why only a small number of people who are infected with EBV develop cancer.

EBV is a member of the herpesvirus family. Like all herpesviruses, EBV has two distinct life cycle phases. EBV persists as a latent, or dormant, infection for the lifetime of the host. During latency few viral genes are expressed and no new viruses are made. EBV periodically activates into its lytic phase to produce more virus. Lytic activation is required for EBV to spread among cells and among hosts. The lytic phase is also important during the development of cancer because people have higher levels of virus before the cancer is detected (1-3). These observations highlight the importance of the lytic phase of the EBV life cycle in human disease.

Significance of the Project:

My research focuses on the control of the switch from the latent into the lytic phase of the EBV life cycle. Human Burkitt lymphoma cells that are infected with EBV can be grown in the lab. Under typical growth conditions, these cell lines have maintained a latent viral infection for decades. However, the lytic cycle of EBV can be induced in cell culture by a variety of drugs, many of which have been discovered serendipitously. For example, treatment of EBV-infected cells with butyrate (Fig 1) results in activation of the EBV lytic cycle. Years after its use in the lab as an EBV reactivator, butyrate was found to inhibit histone deacetylases (HDAC), a class of cellular enzymes most noted for their role in regulating cellular gene expression. Therefore, it was hypothesized that butyrate activates viral gene expression by affecting HDACs (4-7). To test this

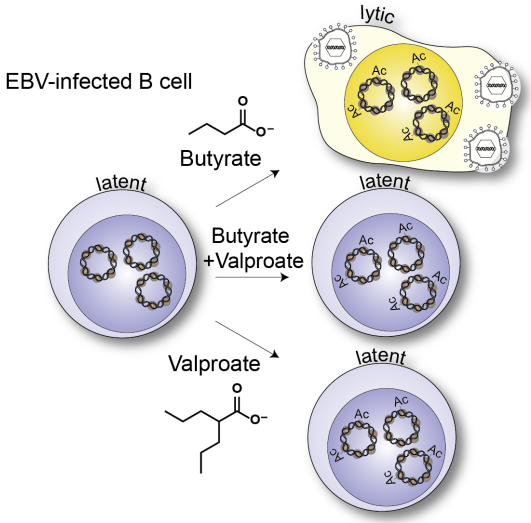

Figure. 1. B cells infected with EBV with the viral latent phase (left). Butyrate causes the virus to enter the active lytic phase (top right). Valproate does not induce the virus lytic cycle (bottom right) and inhibits viral reactivation by butyrate (middle right).

hypothesis, a molecule with a chemical structure similar to butyrate that also inhibits HDACs, valproate, was investigated (Fig 1). Surprisingly, valproate did not activate EBV. In fact, valproate actually blocked reactivation of EBV in the presence of activating drugs (8). Therefore, valproate is a novel inhibitor of EBV lytic reactivation.

To explore the minor structural differences between the short-chain fatty acid butyrate and the medium-chain fatty acid valproate, with the goal of explaining differences in their functional effects on EBV reactivation, I investigated the capacity of 16 structurally related fatty acids to promote or prevent lytic cycle reactivation (9). A few short-chain fatty acids, like butyrate, induced EBV reactivation, though butyrate was the most effective. However, isobutyrate, a molecule with a structure very similar to butyrate (same number of carbon atoms, but with a branched chain), did not cause EBV reactivation. Two medium-chain fatty acids, like valproate, inhibited lytic activation of EBV. A property of the fatty acids that partly explained whether it activated or inhibited viral reactivation was the length of the fatty acid chain. There were some notable exceptions, which greatly informed identification of chemical structures that are important for function (9). Since some of the fatty acids tested are naturally occurring molecules, these results also suggest a possible mechanism by which fatty acids in the diet influence the EBV life cycle in humans.

The life cycle of EBV, like every virus, is interwoven with the biology of its host cell. For example, expression of the viral lytic genes depends on activation of the host cellular transcription machinery. Also, the virus responds to the differentiation status of the host cell (10, 11). Conversely, the EBV genome encodes proteins to modify the host cell to enhance virus replication (12-14) and evade the immune system (15, 16). To understand how the virus responds to environmental stimuli, the response of the host cell must also be studied. Therefore, I will explore the cellular pathways impacted by molecules that activate (butyrate) or repress (valproate) the EBV lytic cycle. These experiments will uncover the mechanisms that control the virus and identify potential drug targets.

Objectives:

I will study the control of reactivation of EBV from its latent to lytic phase. Small molecules induce the lytic cycle in cell culture, but in vivo stimuli are not known. Butyrate, a naturally occurring fatty acid, activates EBV. Viral reactivation is blocked by valproate (8), a drug used clinically to treat neurological disorders. I will study how these fatty acids, as well as other metabolites and drugs, alter the host cell and ultimately induce or block viral reactivation.

Aim 1: Are valproate analogs better than valproate as inhibitors of EBV lytic reactivation? To improve the inhibitory properties of valproate, I will test commercially available valproate derivatives designed based on my previous data that identified portions of the molecule that can and cannot be modified (manuscript in preparation). The goal is to retain the inhibitory effect on EBV, but to lower the effective concentration and to reduce potential side effects.

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Aim 3: Do drugs that alter EBV reactivation work by affecting intracellular calcium? Signals outside the cell can trigger an influx of calcium into the cell. The rise in calcium concentration is detected inside the cell and a variety of responses ensue. In EBV-infected cells, the lytic cycle is activated in response to calcium. I have shown that reactivation of EBV by butyrate requires extracellular calcium. I will examine whether valproate affects calcium levels as part of its mechanism of blocking EBV reactivation.

Overall, the proposed research investigates the interplay among the virus, the host cell, and molecules in the cellular environment. Two questions my work addresses are: 1) How do small molecules control the latent–lytic switch of Epstein-Barr virus? 2) How does the human host cell respond to EBV lytic activators and repressors? Insight into these questions from the proposed work has the potential to impact the treatment of viral-associated cancers.

Research Methods:

Experimental system: The EBV switch from latency into the lytic cycle is tightly regulated by the expression of two key viral transactivator genes, Z and R (17-19). Once expressed, these two viral transcription factors initiate subsequent steps in the lytic cascade. In the laboratory, EBV lytic reactivation is studied in established cell lines that are latently infected with EBV. These EBV-positive Burkitt lymphoma cells (Biosafety level 2) grow quickly and are tolerant to varied growth conditions. The virus is reactivated by growing the infected cells in the presence of small molecules activators, such as butyrate. Reactivation of the virus is detected by measuring molecular products of the viral lytic life cycle using common techniques in biochemistry and virology: 1) Expression of viral lytic genes, such as Z and R (Fig 2 top), by quantitative PCR, 2) Expression of viral lytic proteins by Western blot (Fig 2 bottom),

or 3) Replication of the viral DNA by quantitative PCR. Inhibitors of viral reactivation will be discovered by treated EBV-positive cells with an activator and the potential inhibitor.

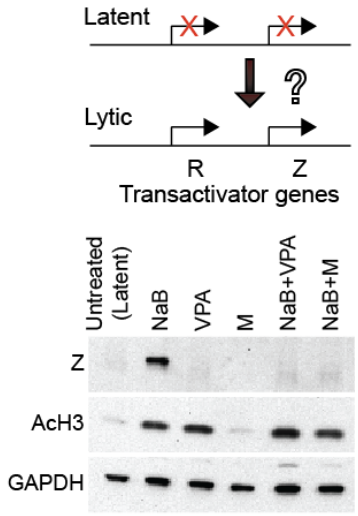

Figure 2. The EBV lytic cycle is reactivated by expression of viral lytic genes, Z and R. Reactivation is induced by butyrate (NaB), but blocked by valproate (VPA) and a non-HDAC inhibitor M.

Aim 1: Are valproate analogs better inhibitors of EBV lytic reactivation?

To develop more potent and specific inhibitors of EBV lytic reactivation I will test derivatives of valproate (VPA), a known inhibitor. I identified one analog of VPA, VPM (M), that inhibits EBV reactivation by blocking expression of the EBV Z protein (Fig 2 Z). However, M does not cause an increase in acetylated histone H3 like VPA (Fig. 2 AcH3), which suggests inhibitor M will have fewer negative side effects. M has enhanced potency and induces fewer changes in cell gene expression than VPA (unpublished data). I will test more VPA derivatives designed based on my previous work that has defined chemical moieties of valproate important for its antiviral activity.

[redacted]

Aim 3: Do drugs that alter EBV reactivation work by affecting intracellular calcium?

Environmental signals outside the cell trigger an uptake of calcium into the cell. In EBV-infected cells, the lytic cycle is activated in response to calcium influx (20). I have shown that reactivation of EBV by butyrate requires extracellular calcium. I will investigate calcium influx (Fig. 3) in response to the molecules that either activate or inhibit EBV reactivation.

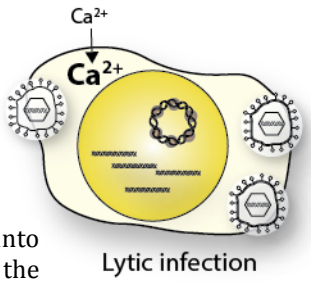

Figure 3. Calcium (Ca^{2+}) moves into the EBV-infected cell when the virus activates into its lytic cycle.

I will measure intracellular calcium levels using Indo-1, a molecule that is taken up by cells and changes in fluorescence upon binding calcium. I will compare results to cells treated with ionomycin, which is known to induce a calcium influx. In preliminary experiments, calcium uptake induced by ionomycin was enhanced in butyrate-treated cells, but reduced in cells treated with our recently discovered EBV reactivation inhibitor M (Fig. 4). I will determine the dose and length of treatment of M that is required for decreasing calcium uptake. I will determine the effects of other EBV inhibitors on calcium uptake. I will examine the effects of calcium perturbations during various stages of the EBV life cycle. These experiments will characterize the role of calcium in EBV reactivation.

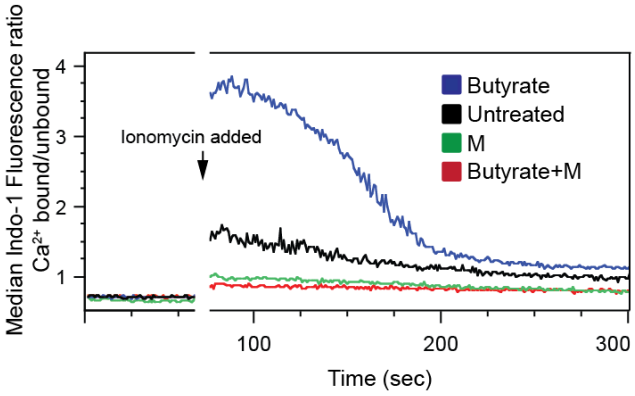

Figure 4. Calcium influx induced by ionomycin was measured by Indo-1 fluorescence in EBV+ Burkitt lymphoma cells pretreated with butyrate or inhibitor M.

Research Implications: The results of my work will improve understanding of the molecular mechanisms of EBV reactivation and contribute to the development of therapeutic strategies for viral-associated cancers. The presence of EBV in tumor cells provides a target for therapy. Oncolytic therapy intentionally induces the EBV lytic cycle leading to death of the infected cells (21-24). On the other hand, repression of the viral lytic cycle could treat infectious mononucleosis and lower the risk of developing cancer. Either therapeutic approach requires drugs that efficiently activate or repress the virus. Results of my work will provide 1) characterization of chemical features that improve the efficacy of inhibitors, and 2) discovery of molecules that either induce or block the activation of EBV, and 3) identify molecular targets in the cell that control EBV lytic reactivation. The proposed work provides molecular details for how the EBV latent-to-lytic switch is controlled.

Timeline:

Each of the aims is based on preliminary data obtained during my postdoctoral work. The goal of Aim 1 and Aim 2 is to identify inhibitors of EBV reactivation. These projects will be performed by undergraduate students. During the school year the students will learn to culture the EBV-positive cells and quantitative PCR to analyze viral reactivation. Students will also decide which chemicals to test as inhibitors by generating a hypothesis based on my preliminary data. During the summer, the students will run the experiment testing a chemical as an inhibitor, repeating the experiment at various inhibitor

concentrations. Any EBV inhibitors identified will be published in peer-reviewed journals with undergraduates as coauthors. If none of the chemicals inhibit EBV, then an analysis of the chemical functional groups in the non-inhibitors will be compared to known inhibitors to characterize which functional groups are critical for inhibiting EBV. This information will inform the design of future inhibitors. The effects of anti-epileptic drugs on EBV reactivation, whether or not they inhibit the virus, will be compared to the types of epilepsy the drugs are used to treat and their proposed mechanisms. This work will be publishable since it will determine which of the cellular pathways targeted by valproate and other anti-epileptic drugs either are or are not required for EBV reactivation.

Aim 3 investigates the role of the host cell in EBV reactivation. I have the plasmids and reagents needed to do these experiments. During this school year I will establish the cell cultures and test the instruments needed for analysis. Next summer I will begin performing the experiments with the assistance of undergraduate students. The training the students will receive in the summer will allow them to continue the projects during the following school year. These proposed projects will generate data that will be incorporated into a publishable manuscript and provide preliminary data I can use in applications to external funding sources, such as the National Institutes of Health AREA grant (R15). Undergraduates contributing to these projects will present their results at the UWL Undergraduate Research Day.

Past Faculty Research Grant and/or International Development Fund Awards:

None. I am new to UW-L Fall 2015.

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PROFESSIONAL EXPERIENCE

Assistant Professor of Biochemistry	University of Wisconsin-La Crosse	2015-present
Postdoctoral Research Fellow	Yale University, New Haven, CT	2010-2015

EDUCATION

Ph.D. in Biochemistry
Delta Certificate in Research, Teaching, and Learning
University of Wisconsin–Madison, 2009

B.A. in Chemistry and Biochemistry, with High Distinction and with Honors
University of Minnesota, Morris, 2003

RESEARCH EXPERIENCE

Postdoctoral Research Fellow, Yale University, New Haven, CT 2010–2015
- Investigated the role of short-chain fatty acids in Epstein-Barr virus life cycle and Kaposi sarcoma-associated herpesvirus in lymphoma cells

Postdoctoral Research Associate, Washington University, St. Louis, MO 2009–2010
- Examined the ubiquitin–proteasome-mediated degradation of nuclear hormone receptors

Graduate Research, University of Wisconsin–Madison, Madison, WI 2003–2009
Dissertation: Substrate Recognition by Prolyl 4-Hydroxylase
- Identified substrate conformational preferences of prolyl 4-hydroxylase
- Developed a direct and continuous assay for prolyl 4-hydroxylase
- Characterized a novel protein disulfide isomerase from *Bacillus subtilis*

Chemical-Biology Interface Research Intern, Beckman Coulter, Inc., Chaska, MN Spring 2006
- Purified an isoform of Prostate Specific Antigen for clinical diagnosis of prostate cancer

National Institutes of Health Study Group, Colgate University at the NIH, Bethesda, MD Fall 2002
- Developed proteins derived from HIV-1 Env gp41 as HIV fusion inhibitors

Laboratory Intern, Minnesota Valley Testing Laboratories, New Ulm, MN Summers 2002, 2003
- Analyzed sugars and vitamins in food samples by HPLC or GC

Research Experience for Undergraduates, North Dakota State University, Fargo, ND Summer 2001
- Screened Lewis acid-mediated reactions of *N*-acyl oxazolidinones

PUBLICATIONS

Gorres K, Daigle D, Mohanram S, Miller G. 2014. Activation and repression of Epstein-Barr Virus and Kaposi's sarcoma-associated herpesvirus lytic cycles by short- and medium-chain fatty acids. *J. Virol.* 88:8028–8044. (Cover Photo)

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HONORS and AWARDS

NIH Ruth L. Kirschstein Award (F32 CA165705) Postdoctoral Fellowship	2012-2015
American Cancer Society Postdoctoral Fellowship (awarded, but declined)	2012
American Society of Virology Postdoctoral Fellow Travel Award	2011
NIH Chemistry–Biology Interface Training Program (T32 GM008505)	2004–2007
Barry M. Goldwater Scholarship	2002

TEACHING EXPERIENCE

Assistant Professor, University of Wisconsin-La Crosse

CHM 325: Fundamental Biochemistry lecture and laboratory	Fall 2015
CHM 489: Independent Study	Fall 2015

Postdoctoral Teaching Scholar, Yale University

Biology 101: Biochemistry and Biophysics	Fall 2012, Fall 2013, Spring 2014
Biology 102: Cell Biology and Membrane Physiology	Fall 2012
Molecular Biophysics & Biochemistry 105: Issues Approach to Biology	Spring 2013
MCDB 485: Undergraduate Research in Molecular Biophysics & Biochemistry	2010-2015

Graduate Teaching Assistant, University of Wisconsin–Madison

Delta Program for Teaching and Learning Intern: Issues in Biomedical Engineering	Spring 2007
Biochemical Methods Laboratory	Fall 2004, 2005

Undergraduate Teaching Assistant, University of Minnesota, Morris

Introduction to Research	Spring 2003
Science Sensations: Performing chemistry demonstrations for the public	2001–2002