Neutralizing Antibodies to Epstein Barr Virus in the Rhesus Macaque Animal Model and in Humans

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Neutralizing Antibodies to Epstein Barr Virus in the Rhesus Macaque Animal Model and in Humans

A dissertation presented

by

Marissa Ann Herrman

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

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Neutralizing Antibodies to Epstein Barr Virus in the Rhesus Macaque Animal Model and in Humans

Epstein-Barr virus (EBV) is associated with a number of human diseases and does not have a vaccine. It is believed that neutralizing antibodies are an important immune effector for an EBV vaccine, but it is unknown whether serum neutralizing antibodies can alter EBV infection through the oral mucosa. The studies presented in this dissertation were designed 1) to adapt the rhesus macaque animal model to allow testing of neutralizing antibodies in a biologically relevant system and 2) to better define the neutralizing antibody response of EBV infected humans.

Infection of rhesus macaques with the EBV related lymphocryptovirus, rhLCV, provides an accurate model system for studying EBV, but there were two hurdles that needed to be overcome before neutralizing antibodies could be tested in this model. First, there are no neutralizing antibodies specific to rhLCV and we found that a potent EBV neutralizing antibody, 72A1, did not cross-neutralize rhLCV. Second, murine monoclonal antibodies are inherently immunogenic in macaques and induce anti-antibody responses that limit their utility. To create a virus sensitive to 72A1 neutralization, the major membrane glycoprotein of rhLCV with replaced with EBV gp350. The data presented here show that this chimeric virus can use EBV gp350 to support entry into macaque cells in vitro and following oral inoculation of rhesus macaques. To reduce the immunogenicity of the murine antibodies, “rhesized” antibody variants were generated and shown to retain antigen specificity. The combination of this novel, chimeric virus
and the “rhesusized” antibodies will now allow testing of neutralizing antibodies in the macaque model.

Although multiple EBV glycoproteins have been shown to induce neutralizing antibodies in mice, studies of the human neutralizing antibody response have been narrowly focused on a single antibody binding epitope on gp350. Here we show that antibodies binding to this epitope do not represent all EBV neutralizing activity in human sera. Additionally, these data suggest that the neutralizing response is much broader than appreciated, with multiple glycoproteins inducing EBV neutralizing antibodies. Accurately defining the repertoire of viral glycoproteins targeted by human neutralizing antibodies can inform us of the naturally immunogenic proteins that may make good vaccine immunogens.
Acknowledgements

These past five years have probably been the toughest years of my life, both personally and professionally, and there are so many people that have helped me get through it, whether they know it or not.

I have to start by acknowledging Fred. In my opinion, Fred was both the best and the worst person that I could have chosen to go on this journey with. There was never a day where Fred didn’t try to push me to be better, or faster, or more critical, or more correct. He was always my toughest critic. But he was also probably the most understanding and caring mentor that I could have asked for. During my graduate career I had to deal with many, serious and unforeseen personal problems and I was never afraid to let Fred know when I had reached my limit or when something new was causing problems. Granted, he never let me off easy, but at least he knew how to temper himself from a 10 to maybe a 5 on those really bad days. He has made me both a better scientist and a better person.

I also have to acknowledge all the people on the 8th floor of Channing, particularly Janine, Ina, Carol, Molly, and Karl. They all made me look forward to coming to work, and have in one way or another made me a stronger, more outgoing person. That is something I will always remember.

Graduate school probably would have been unbearable without my friends and my husband, and for that I am truly grateful. Having people around to talk (i.e. complain) to was probably the only thing that has kept me sane. I hope that I was half the friend that they all were for me.
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Chapter One

Introduction
Herpesvirus classification

There are three families of herpesviruses: the alphaherpesviruses, such as herpes simplex virus (HSV) and varicella zoster virus (VZV), the betaherpesviruses, such as cytomegalovirus (CMV) and the gammaherpesviruses, such as Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV). All herpesviruses share a number of common characteristics. They are enveloped viruses with large, linear, double stranded DNA genomes. They share a common set of conserved proteins that are necessary for viral entry/exit and DNA replication. One defining feature is a viral life cycle that includes both lytic and latent stages of infection. During lytic infection, the viral genome is replicated and packaged through the coordinated expression of immediate early, early, and late genes to produce new viral progeny. During latent infection, the viral genome is maintained as a circular episome and a very limited number of viral genes, if any, are actively expressed. Notably, latent infection allows the virus to escape detection by the immune system and results in a life-long persistent infection.

The gammaherpesviruses can be further subdivided into two distinct genera: the gamma-1 herpesviruses, or lymphocryptoviruses (LCVs), and the gamma-2 herpesviruses, or rhadinoviruses. EBV is a human gamma-1 virus and KSHV is a human gamma-2 virus. Gamma-1 viruses exclusively infect primates and almost all primate species are naturally infected by their own LCV (1). In contrast, gamma-2 viruses have a broader host range and include viruses, such as murine herpesvirus-68 (MHV-68), that naturally infect mice (2). The fact that LCVs cannot be found in any non-primate species suggests that this genus of herpesvirus has evolved more recently than any other.

EBV and Humans: Infection, Disease, and Vaccines
EBV life cycle in a human host

EBV infects nearly all individuals by adulthood. Primary infection often occurs during early childhood, is asymptomatic, and resolves into a steady, life-long infection. EBV infection of a human host can be divided into two stages: 1) acute, which occurs in the first few weeks after initial virus exposure and 2) persistent, which occurs many months to years later, after the virus has established itself in latently infected memory B cells.

EBV is periodically shed in the saliva of infected individuals and EBV naïve individuals acquire the virus through the oral mucosa (3). It is currently unknown how EBV crosses the oral mucosa. The most commonly accepted model is that EBV infects the epithelial cells of the oral mucosa, replicates, and then moves on to infect B cells. An alternative model is that EBV gains direct access to B cells though microfissures within the mucosal barrier and epithelial cells are not infected during the acute phase. There is evidence to suggest that the virus shed in the saliva of infected individuals would preferentially infect B cells, and not epithelial cells, due to high levels of the viral glycoprotein gp42 (discussed below) (4).

EBV must eventually infect B cells in order to persistently infect its host (5). Most infected B cells will enter directly into latency and expression of the latent genes drives infected B cells to proliferate. Through the combination of virus entering new B cells and infected B cells being driven to proliferate and pass on the EBV episome to each daughter cell, EBV infected B cells can accumulate to up to 10% of total B cells during primary infection (6). The concurrent development of EBV specific CD8+ T cells can eliminate many of these cells. The infected B cells that persist enter a latency state where no viral genes are expressed and thus can escape T cell recognition. The average persistently infected individual has a viral load of approximately 1 infected cell per 1,000,000 circulating B cells (7). Occasionally, latently infected B cells are
thought to traffic back to the oral mucosa and undergo lytic replication. The biologic signal that switches B cells from latent to lytic infection is currently unknown. Virus then infects epithelial cells, replicates, and is shed into the saliva, completing the cycle.

**EBV-associated diseases**

EBV is a human pathogen that has been associated with a diverse set of diseases (Figure 1.1) (6, 8, 9). EBV-associated diseases can be categorized by at least three different characteristics. First, disease can be associated with either acute or persistent EBV infection. Diseases associated with acute, or primary, infection include Infectious Mononucleosis (IM) and certain cases of post transplant lymphoproliferative disease (PTLD). IM occurs within the first 4 to 6 weeks after initial exposure to EBV (10). PTLD can be a complication that arises within the first few months to a year after solid organ transplantation and young children who are EBV naïve but receive a donor organ from an EBV infected individual are particularly at risk (11). In these patients, primary EBV infection is occurring in the setting of severe immunosuppressive drugs that inhibit the development of the EBV specific T cells necessary to control the virus. Reducing the amount of immunosuppressive drugs can combat PTLD but can also increase the risk of organ rejection (12). The majority of EBV-associated diseases are diseases of persistent infection. These include Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma (NPC), and gastric carcinoma. EBV encodes oncogenes, such as the latent infection protein 1 (LMP-1), but not all EBV infected cells will develop into cancers, primarily because latently infected B cells in healthy individuals do not actively express the EBV latency genes (13). As with most cancers, these EBV-associated cancers likely rely on a “two-hit” model in order to become malignancies. First, the cells must be infected by EBV, and second, they must
stochastically accumulate additional cellular mutations that can drive the expression of the EBV oncogenes in order to become fully transformed. Since cellular mutations do not rapidly accumulate, these malignancies develop after many years of carrying the virus.

Second, EBV-associated malignancies can occur in both immunocompetent and immunosuppressed individuals. IM, Burkitt lymphoma, Hodgkin lymphoma, NPC, and gastric carcinoma are all diseases of immunocompetent individuals. While PTLD, X-linked lymphoproliferative disorders and oral hairy leukoplakia are diseases of immunocompromised individuals. Immunosupression can occur from genetic insufficiencies, therapeutic drugs, or progression to acquired immunodeficiency syndrome (AIDS).

Finally, EBV-associated diseases can arise directly from EBV infected B cells or epithelial cells or from alternative mechanisms such as the nonspecific activation of T cells and overproduction of cytokines. Since EBV infects and latently persists in B cells, it is not surprising that many EBV-associated malignancies arise from B cells. These include Burkitt lymphoma, Hodgkin lymphoma, and PTLD. However, EBV is thought to only transiently infect epithelial cells and has not been shown to establish a long term, latent infection in epithelial cells. Thus it is unclear exactly how EBV-associated epithelial cell malignancies arise. Interestingly, symptoms of IM are thought to result from the rapid expansion and activation of T cells, and not directly from any EBV infected cell (14). Again, it is unclear exactly how EBV causes IM but one model suggests that viral loads correlate with disease severity, thus the kinetics of viral infection and the number of infected cells in the blood might be a contributing factor (15).
**Figure 1.1 Epstein Barr virus associated diseases**

EBV-associated diseases are grouped according to whether they occur during primary or persistent EBV infection and whether they occur in an immunocompetent or immunocompromised host. Diseases that arise from EBV infected B cells are highlighted in blue and diseases that arise from EBV infected epithelial cells are highlighted in purple. Infectious mononucleosis, which likely results from hyperproliferation of non-infected T cells, is listed in black.
It is estimated that 200,000 new cases of EBV-associated cancers occur each year (16, 17). Given that EBV is also the most common cause of IM and PTLD, EBV infection is a public health problem and development of an EBV vaccine would provide significant benefit. With such a complex array of EBV-associated disease, it is currently unknown if a single vaccine can prevent multiple types of diseases. Such a “universal” vaccine would have to provide sterilizing protection and prevent infection, not just prevent disease. This is a lofty goal for any viral vaccine, and particularly far reaching for viruses as prevalent and persistent as herpesviruses. A commercially available herpesvirus vaccine targeting VZV is sufficient to prevent chickenpox and shingles, but does not prevent VZV infection (18). A similar goal of preventing an EBV-associated disease, such as IM, but not preventing infection may be more attainable. It is possible that an IM vaccine would lower viral setpoints and thus lower the risk of developing certain EBV-associated cancers.

Currently, EBV vaccine efforts have focused on either a prophylactic vaccine for preventing IM or a therapeutic vaccine for treating cancers such as NPC (19). Most prophylactic vaccine candidates were designed to induce neutralizing antibodies against the major membrane glycoprotein, gp350 (20-23). Gp350 specific neutralizing antibodies have been shown to prevent EBV infection of B cells in vitro and presumably would limit B cell infection in vivo, thus lowering acute viral loads during primary infection to protect against IM (24, 25). Therapeutic vaccine candidates, however, were designed to induce or boost CD8+ T-cell responses against the viral latent proteins latent membrane protein 2 (LMP2) and EBV nuclear antigen-1 (EBNA-1), as many EBV-associated cancers only express EBNA-1 or a combination of EBNA-1, LMP-1, and LMP-2 (26, 27). Proof-of-principle studies have shown that infusion of EBV specific
cytotoxic T lymphocytes can extend the median survival of NPC patients (28, 29). Thus, CD8+ T cells specific for EBNA-1 and LMP-2 induced by vaccination could reduce or eliminate tumor burden by killing the EBV infected tumor cells.

To date, only one EBV vaccine candidate has been tested in a phase II clinical trial (22). A recombinant, soluble gp350 vaccine was tested for the ability to prevent IM in EBV negative college students. Ninety individuals were vaccinated with a three dose regimen of 50ug recombinant gp350 in an aluminum salt based adjuvant AS04 administered at months 0, 1, and 5 and 91 individuals were similarly vaccinated with AS04 alone as a control. After completion of the 19 month study, 86 gp350 vaccinated individuals and 90 control vaccinated individuals had finished the trial in accordance with the original study design. In this population, 11 gp350 vaccinated individuals developed asymptomatic EBV infection and 2 developed IM while 9 control vaccinated individuals developed asymptomatic EBV infection and 8 developed IM. The difference in IM incidence and asymptomatic infection were not significantly different between the two populations. The one control vaccinated individual who did not complete the trail was suspected of developing IM and if this individual is included in the analysis, the difference in IM incidence between the gp350 vaccinated and placebo groups becomes significant. From this trial, it is clear that gp350 vaccination did not prevent asymptomatic EBV infection but it is much less clear whether this vaccine was effective at preventing IM. This uncertainty about protective efficacy may have contributed to the pharmaceutical industry choosing to stop development of this vaccine candidate.

Although not statistically significant, the reduction in IM incidence from 8 cases in the control group to 2 cases in the vaccine group would suggest that gp350 is an important component of an IM vaccine, but that recombinant gp350 alone may not be sufficient. As
previously mentioned, the induction of neutralizing antibodies is assumed to be the mechanism by which a prophylactic EBV vaccine would be effective. In the phase II trial, all gp350 vaccinated individuals developed gp350 specific antibodies but the levels of neutralizing antibodies were not specifically reported (22). One might hypothesize that a gp350 vaccine that induced high titers of neutralizing antibodies would be more effective, but gp350 neutralizing antibodies have not been tested against orally acquired EBV and it is unknown if they would be sufficient to control infection and prevent disease. During primary infection, epithelial cells within the oral mucosa are thought to be infected and contribute to acute virus amplification and spread. Thus, preventing epithelial cell infection by targeting the EBV glycoproteins involved in epithelial cell entry may be just as important as preventing B cell infection in order to control primary EBV infection.

**EBV infection of B cells and epithelial cells: entry, replication, and latency**

**EBV Entry**

EBV has a tropism for both B cells and epithelial cells. The entry pathways for each cell type have been characterized in vitro and use an overlapping, but distinct, set of viral glycoproteins (Figure 1.2). Infection of B cells begins at the plasma membrane but fusion occurs in the endosome. EBV infection of B cells is initiated by binding of the major membrane glycoprotein gp350 to the EBV receptor on B cells, CD21 (30, 31). Subsequently gp42, in complex with gH/gL, binds to the EBV coreceptor MHCII (32). This triggers a conformational change in gp42 that allows gH/gL to act as the fusion trigger (33, 34). Fusion of the viral and cellular membranes is completed by gB.
In contrast to B cell entry, EBV entry into most epithelial cell lines occurs at the plasma membrane and is much less well defined (Figure 1.2). In a polarized epithelial cell infection model, entry is initiated by BMRF2 binding to β1 containing integrins to attach virus (35, 36). In other epithelial cell infection models, heterodimeric complexes of gH/gL can bind to integrins αvβ5, αvβ6, and αvβ8 to attach virus, although use of gH/gL as the sole attachment factor results in only low levels of viral entry (37-39). This might suggest: 1) epithelial cell entry, like B cell entry, requires separate attachment and coreceptor binding stages, 2) the gH/gL:integrin binding functions similar to the gp42:MHCII coreceptor binding step and 3) the epithelial cell attachment factors have not yet been properly identified. Nevertheless, gH/gL binding to integrins is sufficient to allow gH/gL to act as the fusion trigger for gB, allowing gB to fuse the viral and cellular membranes. The function of gH/gL as the fusion trigger and gB as the fusion proteins is common to both epithelial cell and B cell entry.
Figure 1.2 EBV entry into epithelial cells and B cells

Schematic for the pathways of entry into B cells (left) or epithelial cells (right). Entry into B cells can be divided into three stages (attachment, coreceptor binding, and fusion) as listed in the middle. B cell attachment is mediated by the viral glycoprotein gp350 binding to the cellular receptor CD21. Coreceptor binding occurs between the viral glycoprotein gp42, in complex with gH/gL, binding to MHCII on the B cell. Finally, gH/gL functions as the fusion trigger to facilitate viral and cellular membrane fusion through the viral fusion protein gB. The precise events that occur during epithelial cell entry are still an area of active investigation and are less well defined. gH/gL binding to cellular integrins is important for epithelial cell entry and can either facilitate viral attachment or function in between attachment and fusion, presumably as a coreceptor binding step. The BMRF2/BDLF2 complex binding to cellular integrins has been described as an additional epithelial cell attachment event but may be cell type dependent. Following gH/gL:integrin binding, gH/gL can again trigger gB to promote fusion.
Thus, heterotrimeric gH/gL/gp42 complexes are necessary for B cell entry and heterodimeric gH/gL complexes are necessary for epithelial cell entry. The binding site of gp42 on gH/gL overlaps with the KGD integrin binding motif and a gH/gL complex that is bound to one cannot simultaneously bind the other (40). Interestingly, gp42 expression on the virion differs depending on whether virus is produced from B cells or epithelial cells (41). During replication in B cells, newly synthesized gp42 can bind MHCII in or on the cell and be sequestered (42). Thus gp42 is not efficiently incorporated into the virion, and virions produced in B cells have a higher proportion of heterodimeric gH/gL complexes that bind integrins but not MHCII. These virions preferentially infect epithelial cells over B cells. Epithelial cells do not express MHCII and in these cells gp42 can be efficiently incorporated into the viral envelope. Thus, virions produced from epithelial cells are high in gp42 and have a greater proportion of heterotrimeric gH/gL/gp42 complexes that will bind MHCII but not integrins. These virions preferentially infect B cells over epithelial cells. For this reason, gp42 has been described as a tropism switch (41).

*Events following entry: lytic replication or establishment of latency*

Once EBV has entered a B cell or epithelial cell, the viral capsid must move from the cytoplasm to the nuclear periphery to allow the viral genome to be deposited into the nucleus. From here, the virus can begin expressing genes to either establish a latent infection, as is the default for most B cell infections, or undergo lytic replication to produce more virus, as is thought to be common in epithelial cells (43, 44). Latent infection begins with the expression of EBNA-2 and EBNA-LP, two viral proteins that function with cellular transcription factors to induce viral and cellular genes important for B cell immortalization. These include viral genes
such as LMP-1, LMP-2A/B, EBNA-1, and EBNA-3A/B/C and cellular genes such as c-myc. Binding of gp350 to CD21 during viral entry can induce intracellular signaling cascades such as NF-κB and TNF that aide in initial transcription of EBNA-2 and EBNA-LP (45, 46). Lytic replication begins with expression of the immediate early proteins BZLF1 and BRLF1. These proteins act as transcription factors to induce the expression of early proteins. Most early proteins encode the DNA replication machinery and are necessary to replicate the viral genome for subsequent packaging. Following DNA replication, late genes, including the EBV glycoproteins, are translated and are necessary for virion packaging and viral egress.

**EBV glycoproteins and glycoprotein specific antibodies**

EBV encodes at least 11 virion-associated glycoproteins: gp350, gp42, gH, gL, gB, BMRF2, BDLF2, gM, gN, gp150, and gp78 (Figure 1.3). All glycoproteins are expressed on the plasma membrane of infected cells, are incorporated into the viral envelope and can be found in purified viral particles (47, 48). gH, gL, gB, gM, and gN are conserved among all three herpesvirus families (alpha, beta, and gamma) and have similar, but not necessarily identical, functions in viral entry and exit (9). BMRF2 and BDLF2 are conserved between the gamma-1 and gamma-2 herpesviruses (49). Gp350, gp42, gp150, and gp78 have no known herpesviral homologs and are unique to the lymphocryptoviruses. EBV glycoproteins are named based on one of three criteria: 1) the EBV gene that encodes the glycoprotein (ex. BMRF2) 2) apparent molecular weight of the glycoprotein (ex. gp350) or 3) based on homology to glycoproteins of other herpesviruses (ex. gH).
<table>
<thead>
<tr>
<th>Name</th>
<th>Gene</th>
<th>Cellular binding partner</th>
<th>Function</th>
<th>Phenotype of KO virus</th>
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<tr>
<td>gp350</td>
<td>BLLF1</td>
<td>CD21/CD35</td>
<td>B cell attachment</td>
<td>greatly reduced B cell infection</td>
</tr>
<tr>
<td>gp42</td>
<td>BZLF2</td>
<td>MHCII</td>
<td>B cell coreceptor binding</td>
<td>defective for B cell infection</td>
</tr>
<tr>
<td>gH/gL</td>
<td>BXLF2/BKRF2</td>
<td>ανβ6, ανβ8, ανβ5</td>
<td>epithelial cell attachment fusion trigger</td>
<td>defective for B cell and epithelial cell infection**</td>
</tr>
<tr>
<td>gB</td>
<td>BALF4</td>
<td>Neuropilin I*</td>
<td>fusion</td>
<td>defective for B cell and epithelial cell infection</td>
</tr>
<tr>
<td>BMRF2/BDLF2</td>
<td>BMRF2/BDLF2</td>
<td>α5β1</td>
<td>epithelial cell attachment</td>
<td>reduced infection of polarized epithelial cells***</td>
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<tr>
<td>gM/gN</td>
<td>BBRF3/BLRF1</td>
<td></td>
<td>viral egress</td>
<td>virus retained in nucleus or released with incomplete envelope****</td>
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<tr>
<td>gp150</td>
<td>BDLF3</td>
<td></td>
<td></td>
<td>enhanced epithelial cell infection</td>
</tr>
<tr>
<td>gp78</td>
<td>BILF2</td>
<td></td>
<td></td>
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* Neuropilin I has only been described to interact with gB on NPC cells
** Virus contained resistance gene in BXLF2 (gH) open reading frame
*** Virus was deleted for BMRF2 only
**** Virus contained resistance gene in BLRF1 (gN) open reading frame

**Figure 1.3 EBV glycoproteins and their functions**
Listed are the 11 EBV glycoproteins, the genes that encode them, their known cellular binding partners, their general function during the viral life cycle and the phenotypes of knockout (KO) viruses.
**Gp350**

Gp350 is a 907 amino acid protein encoded by the BLLF1 gene and is responsible for viral attachment by binding to the EBV receptor on B cells, CD21 (CR2) (30, 31, 50). Recently, CD35 has been reported as an additional receptor for gp350 (51). Exogenous expression of CD35 and MHCII, but not MHCII alone, on a CD21 negative pre-B cell line was sufficient to allow EBV entry. However, CD21 and CD35 are coexpressed on B cells, and the importance of CD35 initiated entry is still unclear. Binding of gp350 to CD21 initiates receptor capping and endocytosis of the virus to initiate entry (30). Additionally, gp350 binding to CD21 has been reported to activate NFκ-B, which may be important for expression of the latent genes following B cell entry (45). The first 470 amino acids of gp350 are sufficient for receptor binding, and a soluble 1-470 fragment of gp350 will block virus binding to B cells and decrease infectivity (52).

Gp350 is the most abundant viral glycoprotein on the surface of the EBV virion (47). It is heavily glycosylated, with both N-linked and O-linked sugars, and over half of the apparent molecular mass of the protein is sugar (53). The crystal structure of the first 443 amino acids revealed 14 glycosylated arginines (54). These sugars coat the majority of the protein, except for a glycan-free patch. Mutations in amino acids 21, 22, 28, 29, 151, 155, 158-160, 162-164, 208, 210, or 296, all of which map to this glycan-free patch, lose CD21 binding (52, 54, 55). Thus, CD21 binds gp350 within the glycan-free patch.

Gp350 binding to CD21 is important, but not strictly necessary for viral entry into B cells. Fusion to B cells can occur in the absence of gp350, and in vitro fusion assays with the EBV glycoproteins rarely include gp350 (56). A virus deleted for gp350 has a roughly 10-fold reduction in B cell infectivity, but can clearly still enter cells (57). Interestingly, however, gp350-specific neutralizing antibodies can completely block EBV infection of B cells (24, 25, 58). One
might hypothesize that with the abundance of gp350, the addition of antibody could create a
shield around the virion that prevents other glycoproteins from initiating entry, but
centrations of antibody that completely neutralize B cell infection can enhance epithelial cell
infection (58). As previously mentioned, B cell entry and epithelial cell entry use a subset of the
same glycoproteins. Thus, the other glycoproteins that may initiate B cell entry are exposed on
the virion, since they can initiate epithelial cell entry, but B cell entry is still prevented in the
presence of gp350-specific neutralizing antibodies.

BLLF1 transcripts can additionally be spliced to generate a second isoform of gp350,
called gp220 (59, 60). Gp350 and gp220 share identical N and C terminal amino acids, including
the receptor binding and transmembrane domains, but gp220 lacks amino acids 500-750.
Different EBV positive cell lines make various ratios of gp350/gp220 (61). For example, B958
almost exclusively makes gp350 while P3HR1 almost exclusively makes gp220. It is currently
unknown if these two different isoforms of gp350 function differently.

**Gp42**

Gp42 is 223 amino acid protein encoded by the BZLF2 gene and it is essential to viral
entry into B cells through binding to the EBV coreceptor, MHCII (32, 62). Interaction between
gp42 and MHCII involves a glutamic acid at residue 46 of the MHC beta chain (63). This
 glutamic acid is not conserved in all MHCII alleles, and as such, gp42 only interacts with the
HLA−DR and HLA−DP alleles but not all HLA−DQ alleles (64). Gp42 can be cleaved during
processing to generate a soluble form of the protein and it is expressed and retained on the
surface of the virion by binding to gH/gL (42, 65). A gp42 deleted virus cannot infect B cells and
soluble gp42 can rescue this phenotype, suggesting that gp42 can associate with gH/gL even
after gH/gL has been expressed on the virion envelope (62, 66). The N-terminal portion of gp42, residues 36-81, binds gH/gL while the C-terminal residues, 94-221, are involved in MHCII binding (67-69). Binding to MHCII induces a conformational change in gp42, widening a hydrophobic pocket within the protein (67, 70). It has been suggested that this conformational change in gp42 is necessary for gH/gL to act as the fusion trigger. A soluble peptide derived from amino acids 36-81 of gp42 is sufficient to block B cell infection, supporting an active role for the interaction of gp42 and MHCII during entry (68). Interestingly, although gp42 is not involved in epithelial cell infection, this gp42 peptide can also block epithelial cell infection. As described above, gp42 binding to gH/gL can prevent the association of gH/gL with other binding partners, acting as a tropism switch between B cells and epithelial cells, and this gp42 peptide is sufficient to block the interaction of gH/gL with its epithelial cell receptors (40, 41).

**gH/gL**

gH/gL is a heterodimeric complex that requires expression and interaction of both proteins for proper processing and function (71). gH/gL can also associate with gp42 to form a heterotrimeric complex. gH is a 706 amino acid protein encoded by the BXLF2 gene and gL is a 137 amino acid protein encoded by the BKRF2 gene. gH/gL is anchored in the membrane by the C-terminal transmembrane domain of gH and the C-terminus of gL interacts at the N-terminal region of gH to hold the complex together (72). The gH/gL complex has two distinct functions. First, it acts as the fusion trigger for both B cell and epithelial cell entry and is necessary for the function of the fusion protein, gB (73). Second, it can bind to integrins on the surface of epithelial cells and function as the receptor binding protein for epithelial cell entry (73). It is currently unknown exactly how gH/gL triggers gB, but the two proteins appear to directly
interact (74). Residues 54 and 94 of gL are important for interaction with gB. Amino acid 594 in the membrane proximal region of gH is also necessary for both B cell and epithelial cell entry but it is unknown exactly how this region of the protein contributes to entry (75). Interestingly, mutation of amino acid 595 increases B cell fusion but decreases epithelial cell fusion, suggesting this region is also involved in cell type specific entry functions. gH/gL has been described to bind three cellular integrins to attach virus to epithelial cells: αvβ5, αvβ6 and αvβ8 (37, 38). There is a KGD motif at residues 188-190 that binds these integrins and the location of this motif overlaps with the binding site for gp42 (40).

gB

gB is an 857 amino acid protein encoded by the BALF4 gene and is the fusion protein for EBV entry into both B cells and epithelial cells. The crystal structure of EBV gB revealed structural homology to post-fusion forms of class III fusion proteins such as VSV G and baculovirus gp64 (76-78). gB functions as a homotrimer and each monomer contains two putative fusion loops located at the base of the post fusion structure. The prefusion structure has not yet been solved. Interestingly, gB is predominantly expressed in the perinuclear region of the cell during replication, with a minimal amount of protein on the plasma membrane (79). gB is dependent on gH/gL for fusion and residues 456-807 are important for the interaction between gB and gH/gL (74). Mutations within the 104 amino acid cytoplasmic tail of EBV gB can result in gH/gL independent fusion (80). Truncation of the cytoplasmic tail can also result in enhanced trafficking of gB to the plasma membrane but does not enhance the ability of gB to mediate fusion (81). In fact, mutants with enhanced plasma membrane expression showed a decreased fusion for both B cells and epithelial cells.
**BMRF2/BDLF2**

Similar to gH/gL, BMRF2/BDLF2 is a heterodimeric protein complex and expression of both proteins is required for proper processing and function of the complex (48). BMRF2 is a 357 amino acid protein encoded by the BMRF2 gene, and BDLF2 is a 421 amino acid protein encoded by the BDLF2 gene. BMRF2 contains an RGD motif that is critical for binding to the integrin α5β1 on the basolateral surface of polarized epithelial cells (35, 82). BMRF2 has been described as the attachment factor for EBV entry in polarized epithelial cells but does not appear necessary for entry into monolayer epithelial cells (56). BMRF2 and BDLF2 have homology to the murine herpesvirus 68 (MHV-68) ORF58 and ORF27, respectively (49). ORF27 is important for MHV-68 cell to cell spread, and it has been suggested that the BMRF2/BDLF2 complex interacts with the cellular cytoskeleton to facilitate cell to cell spread of EBV (49, 83).

**gM/gN**

The gM/gN heterodimeric complex again requires coexpression of both proteins for proper stability, processing, and function (84). gM is a 405 amino acid protein encoded by the BBRF3 gene, and gN is a 102 amino acid protein encoded by the BLFR1 gene. This glycoprotein complex is most important for exit of encapsidated virus from the nucleus of replicating cells but may have an additional role in post entry movement of capsid to the nucleus. A recombinant virus that does not express gN also loses expression of gM and has two distinct defects (85). Most strikingly, virus is not readily released from the nucleus into the cytoplasm, and encapsidated DNA that does leave the cell does not contain a proper envelope. Second, of the rare properly enveloped viral particles, infection of new cells is inhibited at some step post
fusion. Thus, the gM/gN complex may associate with tegument proteins to properly assemble enveloped virus, both during nuclear egress and exit from the cell, and these associations may also be necessary for post fusion movement of the capsid to the nucleus. gM/gN do not appear to have any function during viral attachment and fusion.

**gp150 and gp78**

gp150 is a 234 amino acid protein encoded by the BDLF3 gene. A specific function for this protein has not yet been elucidated. A virus that does not express gp150 infects B cells similar to wild type but showed increased infection of epithelial cells (86).

gp78 is a 248 amino acid protein encoded by the BILF2 gene (87). It is the least well studied of the EBV glycoproteins and currently has no known function.

**EBV neutralizing antibodies**

There are a number of murine monoclonal antibodies that can prevent EBV entry into B cells or epithelial cells. These antibodies target gp350, gp42, and gH/gL. There are currently no EBV neutralizing monoclonal antibodies that target gB.

The gp350 specific neutralizing antibodies that prevent B cell infection include: 72A1, C1, F30-3C2, F34-4G8, F34-4E3, F29-167, F34-1F2, F34-5D3, F34-6B5, F34-6B1, and F29-3C6 (24, 25, 88). The latter 9 antibodies were a subset of 18 gp350 specific antibodies identified from a screen of 3500 hybridomas generated following immunization of mice with EBV positive cells induced for lytic replication (88). All 18 antibodies were classified into seven competition groups, group I-VII, based on the ability of one antibody to prevent binding of another antibody to gp350. Presumably antibodies within a single competition group bind the same or very similar
epitopes on gp350. Thus, gp350 has at least 7 distinct antibody binding epitopes. Antibodies from groups I and IV, but not any of the other groups, were able to neutralize EBV infection of B cells. 72A1, which was independently isolated from mice immunized with purified EBV, is also a group I antibody (25, 88, 89). A proposed epitope for group I antibodies can be identified from mutational studies of gp350 that result in a loss of 72A1 binding. Residues 151, 155, 158-160, 162-164, 208, and 210 are all necessary for 72A1 binding (30, 54, 55). These residues map to the non-glycosylated face of gp350 and overlap with the residues important for CD21 binding. Thus, 72A1 and the other group I antibodies inhibit infection by preventing the interaction of gp350 and CD21. It is much less clear how group IV antibodies neutralize infection. Their binding site maps to the membrane proximal 200 amino acids of gp350 (90). Given that gp350 has no known function besides CD21 binding, the mechanism of these antibodies is still a mystery. Interestingly, recombinant virus expressing a CD21 binding antibody in place of gp350 could not infect B cells as efficiently as wild type virus and suggests that gp350 has a secondary function in addition to CD21 binding (91). Group IV antibodies may inhibit this unknown function. 72A1 has become the prototypic EBV neutralizing antibody and is now used almost exclusively.

There are fewer neutralizing antibodies that target other glycoproteins. B cell infection can also be inhibited by the gp42 specific antibody F-2-1 (92). This antibody prevents the binding of gp42 and MHCII. Presumably this prevents gH/gL from being able to trigger gB to allow fusion. Additionally, there are three neutralizing antibodies that inhibit gH/gL: E1D1, CL40, and CL59 (38, 73). Interestingly, these antibodies differentially inhibit infection of B cells and epithelial cells. The E1D1 monoclonal antibody most specifically neutralizes epithelial cell infection, and does not inhibit B cells very well, if at all. E1D1 binds gH/gL near the KGD integrin binding motif and inhibits the interaction of gH/gL with integrins on the surface of
epithelial cells. The CL40 monoclonal antibody inhibits B cell infection the best of the three 
gH/gL monoclonals and also neutralizes epithelial cells. Its binding site on gH/gL is unknown, 
but likely neutralizes infection by blocking the ability of gH/gL to trigger fusion. Neutralization 
with the CL59 monoclonal antibody falls in the middle; it is not as good as CL40 at neutralizing 
infection of B cells but it is better than E1D1 and can neutralize epithelial cell infection. CL59 
binds gH alone, close to the C-terminus, and therefore the membrane and again, likely prevents 
gH/gL from functioning as the fusion trigger.

**Antibodies to EBV glycoproteins in infected humans**

The antibody response to EBV glycoproteins has not yet been extensively characterized, 
but various reports have shown that a subset of the glycoproteins induce antibodies during 
natural infection. Gp350, gp42, gB, BMRF2, and gp78 are all antibody targets (87, 93-96). 
Gp350 is a target for human neutralizing antibodies that can prevent B cell infection and BMRF2 
is a target for human neutralizing antibodies that can prevent infection of polarized epithelial 
cells (93, 97). How prevalent these neutralizing antibodies are in the majority of EBV infection 
humans and whether other glycoproteins are also targeted has yet to be determined.

**EBV animal models**

EBV is a human specific virus, and the hallmarks of EBV infection in humans, namely 
transmission through the oral mucosa and establishment of a life-long, persistent infection, do 
not occur with EBV infection of any other animal. High doses of EBV injected into cotton top 
tamarins or humanized mice can result in B cell lymphomas (98, 99). Low doses of EBV injected 
in humanized mice can result in long-lived, persistent infection and the development of EBV
specific T cells. Both of these models, however, bypass the traditional route of infection through the oral mucosa and thus bypass any potential involvement of epithelial cells.

Infection of rhesus macaques with their own, naturally occurring lymphocryptovirus, rhLCV, is the most accurate animal model for recapitulating the biology and immunology of natural EBV infection. Similar to humans, almost all macaques naturally become infected with rhLCV by the time they are 12 months old, and animals used for experimental infections must be bred specifically to be LCV negative (100). Naïve animals can be infected by non traumatic oral inoculation, and virus must pass through the oral mucosa to infect B cells, similar to how EBV naturally infects humans (101, 102). Virus is detected in circulating B cells as early as one week post inoculation and all animals become persistently infected with viral loads that are stable over time. rhLCV can be sporadically detected in the oral secretions of infected animals, reproducing the natural route of transmission as well (101).

Macaques naturally and experimentally infected with rhLCV develop cellular and humoral immune responses targeting a similar repertoire of viral proteins to that seen in EBV infected humans (102-105). CD8+ T cells target a range of viral proteins, including latent proteins such as EBNA-1 and EBNA-3 and all classes of lytic antigens (immediate early, early, and late). Interestingly, gp350 and gB specific T cells can develop in both LCV infected macaques and EBV infected humans (104). Antibody responses to all classes of lytic antigens are also common and almost all infected macaques develop antibodies to gp350 and gB (103).

Finally, rhLCV infection can result in sporadic diseases that are similar to those seen in humans. During primary infection, some, but not all, macaques develop symptoms such as atypical lymphocytosis, lymphadenophathy, and splenomegaly (101). These are characteristic symptoms of infectious mononucleosis. Immunosuppression of rhLCV infected macaques can
also result in B cell lymphomas or oral hairy leukoplakia (106, 107). The development of an epithelial cell disease like oral hairy leukoplakia is particularly important because it demonstrates that macaque epithelial cells can be infected.

*RhLCV and rhLCV glycoproteins*

The rhLCV genome has been sequenced and a reverse genetics system using a bacterial artificial chromosome (BAC) has been established (108, 109). The rhLCV genome encodes a repertoire of viral proteins identical to EBV. All EBV genes have a rhLCV homolog and visa versa. The amino acid similarity of EBV and rhLCV proteins range from 29% to 97%. The most homologous genes are those that are well conserved across the herpesviral families, such as genes involved in DNA replication. Surprisingly, the least well conserved genes are the latent genes, such as LMP-1/2 and EBNA-2. Despite the low sequence homology, rhLCV latent proteins are able to substitute for EBV latent proteins in some in vitro assays, demonstrating that they function through conserved molecular pathways (110, 111).

Most, but not all, of the glycoproteins share a high sequence similarity between EBV and rhLCV. Gp42 is 78% similar, gH is 85%, gL is 82%, gB is 86%, BMRF2 is 86%, BDLF2 is 67%, gM is 90%, gN is 75%, and gp78 is 75%. However some of these highly conserved glycoproteins cannot substitute for one another to facilitate viral fusion. This can be attributed to the fact that the interaction between gL and gB appears to be species specific, i.e EBV gB and rhLCV gL cannot interact with one another (74, 112). The most divergent glycoproteins are gp150, which shares 47% amino acid identity between EBV and rhLCV, and gp350, which shares only 49% amino acid identity between the two viruses. Given that macaque B cells express CD21, it is unclear why gp350 is one of the most divergent glycoproteins.
**EBV cannot infect macaques**

Despite the strong genetic and biologic similarities between EBV and rhLCV and macaques and humans, EBV does not appear to infect rhesus macaques. Most studies attempting to infect rhesus macaques with EBV were performed from 1940-1980 and revealed little or no evidence for infection (113-115). After many years of additional EBV research, this outcome could have been predicted in some, but not all, of the studies. The largest of these studies attempted to infect macaques with a virus that is unable to transform B cells because of a deletion that eliminates a crucial latent gene (113, 116).

The strongest argument for successful EBV infection of macaques came from serologic responses in some inoculated animals (113). However, it was not yet well recognized that macaques are naturally infected with rhLCV, and inoculation of EBV in these animals may have boosted the natural immune responses, some of which could have been cross-reactive with the highly homologous EBV proteins. Alternatively, new antibody responses may have developed in response to the antigenic stimulation of the viral inoculum without significant viral infection. Detection of true experimental infection would have required sensitive and specific molecular techniques to detect viral nucleic acids, which were not available at the time, in order to clearly distinguish experimental EBV infection from natural persistent rhLCV infection.

The more recent observation that EBV cannot immortalize rhesus macaque B cells in tissue culture suggests that even if EBV could enter some cells in monkeys, it would be functionally unable to transform the rhesus B cells and establish persistent B cell infection in a macaque (117).
**Goals of this dissertation**

The fundamental question of whether B cells or epithelial cells are the first cell type infected by EBV as it crosses the oral mucosa has not yet been answered. Understanding how viral acquisition occurs would provide valuable insight into how infection could be altered or prevented by a vaccine or other therapeutics. We approached this question in a therapeutically relevant way by asking whether neutralizing antibodies that prevent infection of B cells or epithelial cells are able to alter viral infection when acquired though the oral mucosa using the rhesus macaque animal model for EBV infection. However, as described in the following chapters, testing neutralizing antibodies in the macaque first required adaptation of both rhLCV and the neutralizing antibodies to create 1) a virus that was neutralized by the prototypic EBV neutralizing antibodies yet would still infect macaques and 2) antibodies that would have minimal immunogenicity, and thus maximal tolerance, when infused into macaques. The rhLCV-hugp350 virus and rhesusized 72A1 and E1D1 antibodies developed in this dissertation have met those goals and can now be used to test the effects of preventing B cell infection or epithelial cell infection on viral infection in the macaque animal model.

The testing of B cell and epithelial cell neutralizing antibodies in the rhesus macaque animal model could have direct implications for future vaccine design, as future vaccine candidates might need to induce the types of neutralizing antibodies that are most effective at preventing primary infection. For example, if B cell neutralizing antibodies are sufficient to control infection, then immunogens that induce B cell neutralizing antibodies would be necessary. As a secondary goal for this project, We began characterizing the neutralizing antibody response in EBV infected humans to define the EBV glycoproteins that are immunogenic and could be considered as immunogens for future vaccines.
Chapter Two

Replacement of Rhesus Lymphocryptovirus gp350 with EBV gp350 Supports Infection of Macaque B cells in vitro and of Rhesus Macaques Following Oral Inoculation

Acknowledgements: Portions of this chapter are taken from a manuscript that is currently in preparation: *Rhesus Lymphocryptovirus Can Use EBV gp350 to Confer Sensitivity to an EBV Neutralizing Antibody and Infect Macaques*. Authors include Marissa Herrman, Janine Mühe, Carol Quink, and Fred Wang. JM performed all the rhesus EBER assays and CQ constructed the rhLCV-hugp350 BAC.
Abstract

Epstein-Barr virus infects most humans by adulthood, and primary EBV infection is the most common cause of Infectious Mononucleosis (IM). Developing a vaccine that prevents IM is challenging because our understanding of how EBV infects humans and causes disease is poorly understood. EBV infection begins at the oral mucosa, potentially infecting both B cells and epithelial cells, and a fundamental question is whether serum antibodies that neutralize EBV infection of B cells can be effective against EBV infection initiated at the oral mucosa. The recently developed rhesus macaque animal model can stringently test this question. However, the only available neutralizing antibodies target EBV, and EBV cannot be used because an unknown block prevents successful infection of rhesus macaques. Therefore, we created a system where the EBV-related monkey virus is humanized with the envelope protein of EBV. These experiments show that humanization makes the monkey virus susceptible to neutralization with an EBV-specific monoclonal antibody while still preserving the ability to infect simian cells and monkeys. Thus, viral attachment is not the reason why EBV cannot infect monkeys, and this chimeric virus can be used as an important tool for understanding how neutralizing antibodies that prevent B cell infection alter EBV infection.
**Introduction**

Infectious Mononucleosis (IM) occurs with primary EBV infection and causes significant morbidity and loss of productivity, i.e. prolonged absence from school. EBV is also associated with various cancers like Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma, and gastric carcinoma, which arise as a result of persistent infection, years or decades after primary infection. A vaccine that prevents EBV-associated disease would provide significant public health benefits. It is unknown whether a single EBV vaccine could be effective against both IM and EBV-associated malignancies.

A candidate EBV vaccine based on the major membrane glycoprotein, gp350 has been tested in phase I and II human clinical trials for safety and prevention of IM (21, 22). Vaccination of EBV-naïve college students lowered IM incidence from 9 cases among 91 controls to 2 cases among 90 vaccinated individuals (22). This result achieved statistical significance using an intent-to-treat analysis, but lost statistical significance on a more stringent according-to-protocol basis since one IM case in the control population was not confirmed. Vaccination did not prevent infection since there was no significant difference in the total number of seroconversions between the two groups. Human clinical testing of an EBV vaccine is a major accomplishment, but the lack of convincing efficacy is problematic for deciding whether to move this particular vaccine forward into larger, more definitive, and more expensive clinical trials.

Gp350 was initially considered an attractive vaccine candidate because it is important for viral attachment to B cells by binding to the EBV receptor, CD21 (30, 31). Antibodies against gp350 can neutralize EBV infection of B cells in tissue culture, and gp350 is believed to be the major target for serum neutralizing antibodies in EBV-infected humans (24, 25, 97). Preclinical
studies in a New World monkey model showed gp350 vaccination could protect animals from EBV-induced B cell lymphomas (118-121). This was the best available animal model for EBV at the time, but this model fails to accurately reproduce EBV infection of humans. In these animals, virus has to be injected intramuscularly or intraperitoneally and causes B cell lymphomas within 2-3 weeks (118). In humans, EBV is transmitted via saliva and must first penetrate the oral mucosa, where it may infect epithelial cells and B cells. Over the next 6-8 weeks, virus is amplified by lytic replication and/or proliferation of latently infected B cells and, in patients who develop IM, a vigorous immune response leads to characteristic symptoms of IM, such as fever, atypical lymphocytosis, lymphadenopathy, and splenomegaly (14). Thus, gp350-specific antibodies can neutralize EBV infection of B cells in tissue culture, and gp350 vaccination can be protective in animals that model direct virus infection and B cell tumorigenesis, but it is less clear whether gp350-specific neutralizing antibodies would be the most effective strategy to disrupt EBV infection through the oral mucosa and to prevent triggering the immune response that leads to IM. Indeed, targeting EBV infection of epithelial cells may be more effective than, or synergistic with, targeting B cell infection.

Infection of rhesus macaques with their own EBV-related lymphocryptovirus (LCV) provides an accurate animal model for EBV infection where the role of gp350 specific neutralizing antibodies against orally acquired virus could be stringently tested (101). The biology of rhLCV infection in rhesus macaques mirrors EBV infection of humans. The rhLCV genome encodes an identical repertoire of viral genes as EBV, which function through the same molecular pathways as their EBV orthologues (108). Importantly, LCV-naive rhesus macaques can be experimentally infected with rhLCV by oral inoculation, recapitulating the natural route of infection and allowing the potential involvement of epithelial cells within the oral mucosa.
There is an acute viremia followed by a life-long, persistent infection controlled by an immune response with virus-specific humoral and cellular responses that closely parallel the human immune responses to EBV infection (103-105).

However, there are no neutralizing monoclonal antibodies (mab) against rhLCV gp350 that can be used for investigation. Only a few EBV gp350 specific neutralizing mabs have been described, and it was not known whether they would cross-react and neutralize rhLCV since rhLCVgp350 is the most poorly conserved LCV glycoprotein sharing only 49% amino acid identity with EBV gp350 (108). Therefore, we tested the prototypic EBV gp350-specific neutralizing mab 72A1 and found that it did not cross-neutralize rhLCV. Since EBV cannot be used to infect rhesus macaques and the mechanism for this restriction is unknown, we asked whether replacing the major membrane glycoprotein of rhLCV with EBV gp350 could confer sensitivity to neutralization and still retain rhesus macaque infection in vitro and in vivo.

**Results**

**72A1 does not neutralize rhLCV infection of macaque B cells**

We compared the ability of an EBV neutralizing antibody, 72A1, to neutralize EBV infection of human peripheral blood mononuclear cells (PBMC) and rhLCV infection of rhesus PBMC. To measure EBV/rhLCV infection, we used virus driven B cell proliferation as a surrogate marker. EBV/rhLCV infection of PBMC drives B cell proliferation that can be detected by monitoring loss of the cytoplasmic dye, carboxyfluorescein succinimidyl ester (CFSE). Recombinant 72A1 inhibited EBV infection of human B cells, as expected, with a >75% reduction in B cell proliferation when virus was preincubated with 100, 50 or 25 ng/ml of antibody (Figure 2.1). The same concentrations of recombinant 72A1 had no effect on rhLCV
infection of rhesus B cells.
Figure 2.1 EBV but Not Wild Type rhLCV is Sensitive to 72A1 Neutralization

Graphical representation of CFSE low, proliferated B cells from A) EBV infection of human PBMC with or without preincubation of recombinant 72A1 and B) rhLCV infection of rhesus macaque PBMC with or without preincubation of recombinant 72A1. Concentrations of recombinant 72A1 ranged from 100-25 ng/ml and were identical for both assays.
Generation of a chimeric rhLCV expressing EBV gp350

Since rhLCV was not neutralized by the prototypic EBV neutralizing antibody, we tested whether creating a chimeric rhLCV that expressed EBV gp350 would confer sensitivity to 72A1 neutralization while preserving the ability to infect rhesus macaques. We replaced the native rhLCV gp350 gene (rhgp350) with the EBV gp350 gene (hugp350) in the rhLCV BAC (Figure 2.2A). Proper insertion of the hugp350 sequence was confirmed by BamHI restriction digest and Southern blotting. Probes specific for rhgp350 and hugp350 hybridized to the expected BamHI DNA fragments of the wild type and rhLCV-hugp350 BACs respectively (Figure 2.2B). Integrity of the hugp350 coding sequence was confirmed by sequencing. Virus derived from the rhLCV-hugp350 BAC was recovered by immortalization of rhesus B cells and generation of a hygromycin-resistant, immortalized lymphoblastoid cell line (LCL). PCR analysis confirmed that the viral genome in the LCL was chimeric, i.e. was positive for hugp350 and rhEBNA2, but negative for other EBV genes and rhgp350 (Figure 2.2C).

Expression of the hugp350 protein in the rhLCV-hugp350 LCL was detected by immunoprecipitation with 72A1, followed by immunoblotting with EBV-positive human sera. After induction of lytic replication, a high molecular weight protein, consistent with hugp350, was readily precipitated from the rhLCV-hugp350 LCL, but not the WT-rhLCV LCL (Figure 2.2D). Precipitation by 72A1 demonstrates that the gp350 in the chimeric virus can be bound by this neutralizing antibody and subsequent work (shown in Chapter 3) demonstrates that this virus is indeed neutralized by 72A1. Thus, a recombinant rhLCV deleted for the native rhgp350 and expressing the EBV major membrane glycoprotein has been successfully recovered.
Figure 2.2 Generation of the Chimeric rhLCV-hugp350

A) Schematic representation of rhLCV BAC (top line) zoomed in to the 10kb that contain the gp350 open reading frame (the native rhgp350 of the wild type-rhLCV BAC in the middle or the introduced hugp350 of the rhLCV-hugp350 BAC on the bottom) and surrounding genes. BamH1 restriction sites are denoted by a ”B”. The distance between BamH1 restriction sites is noted. Southern probes for rhgp350 or hugp350 are denoted as an open or filled upside down triangle, respectively. B) BamH1 digest of wild type rhLCV and chimeric rhLCV-hugp350 BACs ran on an agarose gel and stained with ethidium bromide is shown on the left. On right are the Southern blots of the BamH1 digests transferred to nylon and probed with gene specific probes. C) PCR from DNA of EBV, wild type rhLCV, and chimeric rhLCV-hugp350 LCLs. Specific genes amplified are listed on the right. D) 72A1 immunoprecipitation of wild type rhLCV and chimeric rhLCV-hugp350 cell lysates following TPA/butyrate induction for lytic gene expression. EBV positive human sera was used for immunoblotting.
**rhLCV-hugp350 infects macaque B cells comparable to wild-type rhLCV**

To determine whether use of hugp350 altered the ability of rhLCV to enter macaque B cells, we assessed the in vitro transformation efficiency of rhLCV-hugp350 compared to the parental, BAC derived wild type rhLCV. Any differences in the entry properties of the two gp350s should be detected as a difference in transforming activity since the chimeric, BAC derived rhLCV-hugp350 and WT-rhLCV are otherwise identical, i.e. have identical latent proteins (EBNAs and LMPs) necessary for B cell immortalization.

First, multiple lots of cell-free virus were generated from WT-rhLCV and rhLCV-hugp350 LCLs, and the relative amount of virus present in each lot was determined by real time PCR (Figure 2.3). All viral lots were assayed simultaneously to allow direct comparison between the wild type and chimeric virus and values were expressed as arbitrary DNA units. A wide range of viral DNA was expected since each lot was independently derived from a varying number of cells where the induction of lytic replication and virus production may differ. Next, the number of transforming units/ml for each viral lot was determined by infecting multiple replicates of rhesus PBMC with serial dilutions of virus to derive the titer required to induce LCL outgrowth in 50% of the wells. The transformation efficiency was represented by the number of arbitrary DNA units required for each transforming unit (DNA/TU). The transformation efficiency for rhLCV-hugp350 was 12.5 DNA units per transforming unit and was not significantly different from the 10 DNA/TU for WT-rhLCV (Figure 2.3). Thus, rhLCV infection of macaque B cells in vitro is comparable whether the EBV or rhLCV gp350 is used for entry, i.e. EBV gp350 does not restrict rhLCV infection in vitro.
Figure 2.3 rhLCV-hugp350 Immortalizes Rhesus B cells Similar to Wild Type

The transformation efficiency of WT-rhLCV and rhLCV-hugp350 was compared by calculating the DNA/TU. Multiple large scale virus preparations (Lot 1, 2, etc.) were assayed for B cell transforming activity, and transforming units (TU)/ml were calculated as the dilution necessary for 50% outgrowth. Relative DNA content was assayed by real time PCR, and all samples were amplified in the same PCR run to allow relative comparison of arbitrary DNA units. Relative DNA units were normalized so that the transforming activity of WT-rhLCV was 10 DNA units/TU.

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<th>B Cell Transforming Units/ml (10^7)</th>
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rhLCV-hugp350 infects and persists in rhesus macaques

To test whether the chimeric rhLCV-hugp350 could successfully infect a primate host, two rhLCV-naïve rhesus macaques were orally inoculated with $10^6$ transforming units of virus. To look for virus penetration of the oral mucosa and infection of peripheral blood B cells during acute infection, we assayed for the presence of the hyperabundant small RNA rhEBER in multiple aliquots of 5 million PBMC as a semi-quantitative measurement of acute and persistent viral load.

Virus-infected B cells were first detected in at least one PBMC aliquot starting at week 1 post inoculation for both animals and remained detectable throughout the time course (Figure 2.4A). Overall, 18/48 (38%) and 31/48 (65%) of the aliquots were rhEBERs positive during the acute phase (0-16 weeks) for Mm218-05 and Mm143-97 respectively. Virus-infected B cells were also detectable at all time points assayed during the persistent phase, with 21/24 (88%) and 7/18 (39%) aliquots scoring rhEBERs positive (Figure 2.4A).

As a more quantitative measure of viral infection, we analyzed the frequency of infected B cells in the blood at various time points during persistent infection by limiting dilution analysis. The frequency of LCV infected B cells in the peripheral blood at 6 different time points between weeks 19 and 35 post inoculation ranged from 1 infected cell in 707,276 B cells to 1 infected cell in 127,735 B cells for Mm218-05 (Figure 2.4B). Mm143-97 had a range of 1 infected cell in 1,002,143 B cells to 1 infected cell in 1,668,387 B cells when assayed at three different time points from weeks 24-32 (Figure 2.4B). Together these results showed that rhLCV-hugp350 can successfully cross the oral mucosa and establish a stable, persistent infection in macaques. Thus, EBV gp350 can substitute for the native rhLCV major membrane glycoprotein to confer susceptibility to 72A1 neutralization and still support acute and persistent
infection of the natural host.
Figure 2.4 The Chimeric rhLCV-hugp350 Infects Rhesus Macaques Following Oral Inoculation

A) Detection of rhEBERs in aliquots of 5x10^6 PBMC for two macaques (Mm218-05 and Mm143-97) after experimental infection with rhLCV-hugp350. RhEBERs positive aliquots are represented by filled circles and negative aliquots are represented by unfilled circles. B) Frequency of rhLCV-hugp350 infected B cells as calculated by limiting dilution analysis over time during persistent infection (>16 weeks). Mm218-05 is represented by triangles and Mm143-97 is represented by circles.
rhLCV-hugp350 infected macaques can develop an EBV gp350-specific antibody response

To test whether the differences in sequence between EBV gp350 and rhLCV gp350 resulted in unique antibody responses, the EBV gp350 specific serologic responses of the rhLCV-hugp350 infected macaques were analyzed and compared to macaques naturally infected with rhLCV. EBV gp350 reactive antibodies were first detected in both rhLCV-hugp350 infected animals at week 2 post inoculation, with an initial endpoint titer of 733 for Mm143-97 and 38 for Mm218-05 (Figure 2.5A). Mm143-97 continued to develop higher titers of gp350 antibodies, ending at week 32 with a titer of 19,414. EBV gp350 reactive antibodies peaked in Mm218-05 at week 10 with a titer of 2,082 and ended at week 32 with a titer of 1,285. Most naturally infected macaques (96%) develop antibodies against rhgp350, but it was not known if serum antibodies to rhgp350 would cross-react with EBV gp350. Interestingly, 12 of 13 naturally infected macaques had only low titers of cross-reactive antibodies. For these 12 animals, the average endpoint titer was 170 with a standard deviation of 128 (Figure 2.5B). Only 1 naturally infected macaque had a titer of cross-reactive antibodies within the range seen during persistent infection of Mm143-97, indicating that the development of high titer serum antibodies reactive with EBV gp350 is rare in macaques infected with wild type rhLCV. Thus, expression of EBV gp350 on the chimeric virus results in the development of a unique immune response that is rarely seen during rhLCV infection.

We additionally measured the level of serum antibodies that could compete with the 72A1 monoclonal antibody for binding to EBV gp350. In Mm143-97, serum antibodies that compete with 72A1 could first be detected at week 8, with an initial 50% inhibitory concentration (IC50) of 15. The level of competing antibodies gradually increased over time, reaching a peak IC50 of 51 at 6 months (Figure 2.5C). No 72A1 competing antibodies were
detected in Mm218-05. In rhesus macaques naturally infected with rhLCV, 21 of 22 animals had no detectable 72A1 competing antibodies, indicating that serum antibodies competing with 72A1 binding are rare in rhLCV positive macaques (Figure 2.5D). The one animal with detectable 72A1 competing antibodies was the same animal to generate high titer cross-reactive antibodies to EBV gp350. Thus, the rising titer of serum antibodies competing with 72A1 in 1 of 2 animals experimentally infected with rhLCV-hugp350 is suggestive of a specific immune response to EBV gp350 and the chimeric rhLCV.
The gp350 endpoint titer was determined for A) the rhLCV-hugp350 infected macaques and B) macaques naturally infected with rhLCV by elisa. The C) rhLCV-hugp350 infected macaques and D) macaques naturally infected with rhLCV were assayed for 72A1 competing antibodies by a competition elisa. IC50 was calculated as the dilution where 50% of 72A1 binding was inhibited and all sera that failed to inhibit 72A1 binding by 50% at the starting dilution of 1:10 were assigned an IC50 of 0. Mm143-97 is represented by circles, Mm218-05 is represented by triangles, and macaques naturally infected with rhLCV are represented by diamonds.
Mm218-05 loses EBV specific antibodies over time

Given the low level of EBV gp350 specific antibodies detected in Mm218-05, we tested additional rhLCV specific antibody responses to ensure that this animal was not somehow immunocompromised. Surprisingly, antibodies to both the small viral capsid antigen (VCA) and EBNA2 peaked during the acute phase and became virtually undetectable by week 25 (Figure 2.6A and B). This was in contrast to Mm143-97 where antibodies to the VCA and EBNA2 were readily detectable from week 5 on, generally increasing over time as would be expected (Figure 2.6A and B). Mm218-05 is clearly infected during the late time points (Figure 2.4), thus the drop in antibody responses cannot be explained by clearance or loss of the virus. To test whether this macaque had developed hypogammaglobulinemia, a rare immunodeficiency that occurs following primary EBV infection and IM, we looked at total serum immunoglobulin (Ig) levels and antibody titers to measles virus from vaccination prior to infection with rhLCV-hugp350. Given that the IgG isotype predominates in serum, one would have expected total Ig levels to have decreased significantly if this animal had developed hypogammaglobulinemia. There was no noticeable decrease in total Ig in Mm218-05 between weeks 1, 9, and 19 and these levels were comparable to Mm143-97 at similar time points (Figure 2.6C). Additionally, serum antibodies to measles virus were still readily detected across all time points tested (Figure 2.6D). Thus it appears that there is a specific, currently unexplainable decrease in EBV-specific antibodies over time in Mm218-05.
Figure 2.6 EBV antibodies decrease over time in Mm 218-05
A) Development of serum antibodies that bind the small viral capsid antigen (rhBFRF3) in Mm143-97 (circles) and mm218-05 (triangles). Values are expressed as signal/3x background and the limit of detection is indicated with a grey dashed line. B) Serum antibodies from various time points post infection were assayed for EBNA2 binding antibodies by immunoprecipitation of EBNA2 from a rhLCV+ LCL. The EBNA2 specific antibody PE2 was used as positive control. PE2 was additional used for immunoblotting. C) Total serum protein levels for various time points post infection were visualized by SDS page electrophoresis followed by Coomassie staining. D) Measles titers induced by prior vaccination were measured by elisa at various time points post infection with rhLCV-hugp350 in Mm218-05.
Discussion

Why can’t EBV infect macaques?

Almost every primate species is naturally infected by their own EBV-related herpesviruses, or lymphocryptoviruses (LCV) (1). Unlike all other herpesvirus genera, LCV have not been found in any non-primate species. This would suggest that the LCV genus evolved more recently than other herpesviruses and that these viruses have only had to evolve in response to pressures from closely related primate hosts. The identical repertoire of viral genes in EBV and rhLCV and their manipulation of the same molecular pathways underscores the close evolutionary relationship of these viruses.

Despite the strong genetic and biologic similarities between primate hosts and their LCVs, EBV does not appear to successfully infect rhesus macaques, i.e. EBV does not establish life-long persistent infection in macaques as in humans. Most studies attempting to infect rhesus macaques with EBV revealed little or no evidence for infection (113-115). The strongest argument for successful EBV infection of macaques came from serologic responses in some inoculated animals (113). However, pre-existing natural infection of rhesus macaques with their own native LCV was not well-recognized at the time, so antibody responses to EBV antigens in this setting are not necessarily indicative of successful experimental infection. Apparent seroconversion may have been due to stimulation of memory responses to native rhLCV antigens which can be cross-reactive to the related EBV antigens. Alternatively, new antibody responses may have developed in response to the antigenic stimulation of the viral inoculum without significant viral infection. Detection of true experimental infection would have required sensitive and specific molecular techniques to detect viral nucleic acids, which were not available at the time, in order to clearly distinguish experimental EBV infection from natural persistent rhLCV
infection. The more recent observation that EBV cannot immortalize rhesus macaque B cells in tissue culture suggests that even if EBV could enter some cells in monkeys and express viral proteins, it would be functionally unable to transform growth of rhesus B cells and establish persistent B cell infection in a macaque (117).

Why EBV cannot immortalize rhesus macaque B cells is unknown. The most divergent viral genes between EBV and rhLCV are gp350 and the latent infection proteins, like EBNA-2 and LMP1 (29-49% amino acid similarity) (108). Gp350 is important for viral entry and the latent infection proteins are important for B cell transformation (6). A defect in either pathway could feasibly result in a loss of immortalization. The experiments presented here demonstrate that EBV gp350 can replace rhgp350 for infection of rhesus macaque B cells in vitro and that rhLCV can use EBV gp350 to penetrate the oral mucosa and establish persistent infection in rhesus macaques. Thus, gp350 is not responsible for the restriction of EBV infection in macaque cells. Given the high homology of the other glycoproteins important for viral entry (gp42, gH, gL, and gB; 78, 85, 82 and 86% amino acid similarity respectively), the mechanism for the restriction of EBV is likely downstream of viral entry (108).

We would hypothesize that EBV cannot immortalize macaque B cells because of functional differences in the latent infection proteins EBNA-2 and/or the EBNA-3s. These viral proteins bind to a range of host transcription factors and regulate global gene expression during transformation and latency (9). If the EBV EBNA-2 or the EBNA-3s cannot properly bind to macaque host proteins, a large number of virus and host genes would fail to be activated and transformation could not be initiated or maintained.

Once the proteins that are responsible for preventing EBV immortalization of macaque cells are identified, the rhLCV variants of these proteins could be introduced into EBV to create
a mostly human virus that is infectious in macaques. A “humanized” virus would be particularly advantageous for certain therapeutic studies, as discussed below.

“Humanizing” the rhesus macaque animal model

Infection of rhesus macaques with rhLCV is an invaluable model for studying the basic biology of EBV, but may be limited in translational applications. The EBV proteins that have been most extensively studied for vaccines and other therapies are gp350 and the latent infection proteins (19). As previously mentioned, these are the most divergent proteins between EBV and rhLCV. Given that therapy is critically dependent on sequence, i.e. induction of antibodies or T cells against particular epitopes or binding of a drug to specific pockets, the divergence of these proteins may prove problematic. The finding that rhLCV is not neutralized by 72A1, the monoclonal antibody that binds EBV gp350, illustrates that EBV specific reagents do not always cross react with rhLCV. Similarly, one might imagine that small molecule inhibitors developed against the EBV variants of these divergent proteins may not be testable in the current rhLCV infection model because they would not bind the homologous protein in rhLCV. A “humanized” virus that expresses the minimal number of rhLCV proteins necessary for persistence in macaque cells and all other proteins expressed are from EBV could expand the therapeutic potential of the macaque model. The chimeric rhLCV-hugp350 virus presented here is a step towards “humanization” and will allow the direct testing of EBV gp350 specific reagents, such as 72A1.

Infection of rhesus macaques with the chimeric rhLCV-hugp350 may also be a more accurate model for future gp350 vaccine studies. The EBV gp350 specific antibody responses that developed in Mm143-97 following oral inoculation with rhLCV-hugp350 closely mirrors the responses of EBV infected humans (see chapter 4) and is different from the vast majority of rhLCV infected macaques. rhLCV infected macaques almost always develop high titer rhLCV
gp350 specific antibodies but these antibodies do not cross-react with EBV gp350, suggesting there are very few conserved antibody binding epitopes between rhLCV gp350 and EBV gp350. The high titer antibodies induced following rhLCV-hugp350 infection show that macaques are capable of developing EBV gp350 specific antibodies when given the correct immunogen. Similarly, rhLCV gp350 does not have a 72A1 binding site and consequently natural infection with rhLCV does not readily induce 72A1 competing antibodies but 72A1 competing antibodies can be generated if the macaque immune system encounters EBV gp350. The antibody data presented here would suggest that the immune responses induced by EBV gp350 and rhLCV gp350 are fundamentally different. Thus, future vaccine studies in the macaque model using EBV gp350 as an immunogen followed by infection with rhLCV-hugp350 would result in a more human like response than if rhLCV gp350 and rhLCV were used.

*How does rhLCV-hugp350 infection compare to infection with wild-type rhLCV?*

This experiment was not designed to compare infectivity and persistent viral setpoints between wild type rhLCV (WT-rhLCV) and the chimeric rhLCV-hugp350, and thus, there was no control arm of macaques infected with WT-rhLCV for direct comparison. The goal of these experiments was to determine whether rhLCV-hugp350 could penetrate the oral mucosa to establish acute and persistent infection of a macaque. The small number of animals used in this study was sufficient to clearly show that use of EBV gp350 does not prevent the chimeric rhLCV-hugp350 virus from infecting macaques after oral inoculation, but does not allow stringent comparison to past infections.

Historical data of macaques infected with WT-rhLCV can be used, but the comparison is additionally limited by the small number of animals infected with BAC-derived WT-rhLCV (n=2) (102) and recent improvements to the rhEBERs assay. Current data from the rhEBERs
assay were expressed as frequency of infected B cells, while historic data were expressed as
frequency of infected PBMC (i.e. current viral setpoints incorporate the percentage of B cells in
the blood at any given timepoint).

The most appropriate approach might be to compare experimental infection to naturally
infected macaques where the same rhEBERs RT-PCR was used. Historically, macaques
naturally infected with rhLCV were found to have persistent viral setpoints roughly 6 fold higher
than macaques experimentally infected with BAC derived WT-rhLCV (1 infected cell per
116,000 PBMC compared to 1 infected cell per 724,000 PBMC) (102). In the current studies, a
separate cohort of naturally infected macaques had a roughly 17.5 fold higher setpoint compared
to experimental rhLCV-hugp350 infection (1 infected cell per 45,000 B cells vs 1 infected cell
per 780,000 B cells) (Janine Mühe, data not shown). This crude comparison suggests that there
may be a subtle difference in persistent viral setpoints after infection with rhLCV-hugp350
versus WT-rhLCV, but an experiment powered with many more macaques infected in parallel
with WT-rhLCV and rhLCV-hugp350 viruses and using the same rhEBERs assay for
determination of viral setpoints would be required for a more stringent analysis.

Given that the chimeric virus and the wild type virus infect comparably in tissue culture,
it would be surprising if the rhLCV-hugp350 virus does have an infection phenotype in vivo.
Such a phenotype would also suggest that infection of macaque B cells in tissue culture is not
that same as infection of a macaque. Small differences in the kinetics of receptor binding may
not impact infection in a static tissue culture dish but may result in less infection when exposed
to the biophysical pressures of tissues and body fluids. An in depth study of the binding kinetics
of EBV gp350 and rhLCV gp350 to the rhesus macaque CD21 may reveal unappreciated
differences. Alternatively, EBV gp350 is a slightly larger protein than rhLCV gp350 and
expression of EBV gp350 on the rhLCV virion may create greater steric hindrance for other viral
glycoproteins to interact with each other or their cellular receptors. If gH/gL cannot readily bind
to integrins on the epithelial cells of the oral mucosa, viral entry into the body may be limited
and would result in a lower viral set point. Comparing infection of epithelial cells with the
chimeric rhLCV-hugp350 and wild type rhLCV would reveal this potential hindrance.
Additionally, EBV gp350 could induce a more potent immune response that leads to rhLCV-
hugp350 being better neutralized or otherwise cleared. This would be much more difficult to
study but infecting immunocompromised macaques and testing viral setpoints in the absence of a
strong immune response may point to this as a possible mechanism.

Why are EBV gp350 and rhLCV gp350 so poorly conserved?

The studies presented here show that EBV gp350 can functionally replace rhLCV gp350
to allow infection of macaque B cells, even though the EBV gp350 and rhLCV gp350 proteins
are only 49% homologous. One might have hypothesized that as long as the CD21 binding site is
conserved, other residues on the protein could be free to mutate, perhaps as a way of escaping
gp350-specific antibodies or other immune pressures. Interestingly, however, even the CD21
binding site does not appear to be well conserved between these two proteins. Mutational studies
have identified 13 residues on CD21 and 13 residues on EBV gp350 that are important for the
interaction of these two proteins (Figure 2.7) (52, 54, 55, 122, 123). 11 of the 13 residues on
CD21 are conserved between humans and macaques whereas only 7 of the 13 residues on gp350
are shared between EBV and rhLCV. Thus, gp350 has accumulated more mutations within the
CD21 binding site than would have been expected from directed coevolution of gp350 and
CD21.

One could argue that many of the differences between rhLCV gp350 and EBV gp350
within the CD21 binding site have made EBV gp350 a better binding partner for CD21. The CD21 residues involved in binding to EBV gp350 are particularly lysine and arginine rich, resulting in a positively charged interface (Figure 2.7). Within the 13 defined binding residues on gp350, EBV has 7 negatively charged amino acids and no positively charged amino acids, whereas rhLCV has 4 negatively and 2 positively charged amino acids. Thus, EBV gp350 has a much more negatively charged binding interface. Based on this charge difference, one might have expected EBV gp350 to facilitate better binding to rhesus CD21 than rhLCV gp350. Additional amino acids throughout rhLCV gp350 may compensate for the charge difference or rhLCVgp350 may bind CD21 through a slightly different set of amino acids.
Figure 2.7 Sequence comparisons of gp350s and CD21s

Sequence alignment of

A)
the first 470 and 453 amino acids of EBV gp350 and rhLCV gp350, respectively

B)
the first 192 and 300 amino acids of human and macaque CD21s, respectively.

An * under each pair of amino acids indicates conservation of that residue between the two proteins. A : under each pair indicates amino acids with strong similarities and a . under each pair indicates amino acids with weak similarities. Residues that have been identified as important for gp350 and CD21 binding are highlighted in red.
In addition to the variations in sequence, EBV gp350 is about 14% longer than rhLCV gp350. rhLCV gp350 is only 783 amino acids long whereas EBV gp350 is 907 amino acids long. EBV gp350 can also be spliced into a smaller protein, gp220, which loses amino acids 500-750 (52). rhLCV gp350 is missing the splice donor and is very unlikely to be spliced. Differential roles for EBV gp350 and gp220 have not been described so it is unclear why the EBV protein has two different variants while the rhLCV protein does not. It is possible that gp350 has a function in addition to binding CD21, although this function is unlikely to be critical for viral entry.

Gp350 binding partners other than CD21 have not been extensively tested. It would be interesting to compare EBV gp350, EBV gp220 and rhLCV gp350 binding partners by mass spectrometry. Based on size, EBV gp220 and rhLCV gp350 may be more similar than EBV gp350 and rhLCV gp350. EBV gp350 may have gained a binding site that is not present on the other two proteins, or perhaps the extra amino acids disrupted a binding site that is still intact on the smaller two variants. Differences in binding partners may have contributed to the divergence of EBV gp350 and rhLCV gp350, but based on our current knowledge of gp350 there is no convincing explanation for the variance.

Conclusions

The rhLCV-hugp350 infection studies presented here provide both technical and conceptual advancements with important translational implications. We have created the first chimeric LCV where viral genes from different LCV species were exchanged, and we used this recombinant genetic approach to better understand the evolutionary differences between LCV species. The chimeric virus provides a novel modification to the rhesus macaque animal model by conferring sensitivity to a potent EBV neutralizing mab targeting gp350. This virus will also
be sensitive to cross-reactive neutralizing antibodies to gH/gL, allowing use of the rhesus macaque model to test whether serum neutralizing antibodies to B cell or epithelial cell infection are most effective in blocking viral amplification during acute infection after oral virus transmission. This “humanization” of the rhesus macaque animal model allows direct testing of EBV neutralizing mabs to provide important pre-clinical data for their potential therapeutic use in humans.

**Materials and Methods**

**Cell Culture**

All LCLs were grown in RPMI media supplemented with 10% fetal calf serum and 100 units/ml Penicillin and 100 ug/ml Streptomycin.

**CFSE Neutralization assay**

Viruses and antibody were preincubated in at total of 50ul for 90 minutes at 37°C prior to addition of 2x10^6 peripheral blood mononuclear cells (PBMC) labeled with carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies) according to the manufacturers protocol for an additional 90 min at 37°C in a total volume of 100ul. Following incubation, cells, virus, and antibody were moved to a 24 well plate and incubated for 5 days (EBV) or 10 days (rhLCV). Cells were collected, washed with FACS buffer (PBS+1%FCS and 0.1% sodium azide), stained with CD20-APC and analyzed by flow cytometry. All assays included no virus and virus alone samples as controls.

**BAC cloning and generation of lymphoblastoid cell line (LCL) carrying rhLCV-hugp350**

The gp350 open reading frame from the EBV B95-8 strain open was used to replace the rhgp350 in the molecular clone of wild type rhLCV (WTr) using a two step galactokinase
recombination approach, as described previously (124) (102). The galactokinase gene (GalK) was inserted in place of the rhLCV gp350 by lambda recombinase mediated homologous recombination, and recombinants were selected by growth on agar plates with galactose as the only carbon source. Correct insertion and general BAC integrity was confirmed by PCR, BamHI restriction digest, and sequencing analysis. Next, the GalK sequence was replaced with the PCR amplified EBV gp350 open reading frame using lambda recombinase mediated homologous recombination, and recombinants were selected for growth on 2-deoxy-galactose containing agar plates. Correct insertion of an intact EBV gp350 open reading frame and general BAC integrity was confirmed by PCR, BamHI restriction digest, and sequencing analysis.

The rhLCV-hugp350 BAC was transfected into the P3HR1 cell line and stable, BAC containing clones were selected by growth in hygromycin media (400ug/ml). Virus was induced by culturing cells in media containing 20ng/ml phorbol 12-myristate 13-acetate (TPA) and 3 mM butyrate. Cell supernatants were harvested after 7 days, filtered, and used to infect rhesus PBMC. Infected cells were cultured for 6 to 8 weeks in RPMI with 10% FCS, and 0.5 ug/ml cyclosporine and observed for outgrowth of a virus immortalized lymphoblastoid cell line (LCL). A rhLCV-hugp350 LCL clone was confirmed to be free of P3HR1 virus by PCR, and the integrity of the chimeric rhLCV was confirmed by restriction digest analysis and sequencing of BAC DNA recovered from the LCL clone in bacteria. The BAC vector sequence was removed by CRE expression in the LCL, as previously described.

**Immunoprecipitations**

For the gp350 immunoprecipitation, rhLCV-hugp350 LCLs and wild type rhLCV LCLs were induced by culturing cells in the presence of 20ng/ml TPA and 3mM butyrate for 3 days to stimulate lytic replication and expression of the major membrane glycoprotein. For the EBNA-2
immunoprecipitation, untreated wild type rhLCV LCLs were used. Cells were lysed in lysis buffer (50mM Hepes pH 7.4, 250mM NaCl, 2mM EDTA, 1mM PMSF, 0.5% NP-40, protease inhibitor) on ice. Precleared lysates were incubated with 50ul 72A1 hybridoma supernatant (gp350), 10ul macaque sera (EBNA2), or 1ug of purified PE2 (anti-EBNA2 antibody) overnight at 4°C. The next day samples were incubated with protein G sepharose beads for 2 hours at 4°C., Beads were washed three times by centrifugation and addition of fresh lysis buffer, and eluted proteins were analyzed by SDS-PAGE and western blot with EBV immune human sera (gp350) or PE2 (EBNA2) diluted 1:1000.

**Large scale production of virus**

Large numbers (>1x10^9) of wild type rhLCV or rhLCV-hugp350 LCLs were induced by culture with 20ng/ml TPA and 3mM butyrate for one day, and then cells were resuspended in fresh media at a concentration of 3x10^6 cells/ml. Cell free supernatants were collected 7 days after induction and concentrated by centrifugation at 13000g for 2 hours. Virus was resuspended in concentrations ranging from 1:300-1:550 of starting culture volume.

**RT-PCR for DNA content and B cells titering**

Virus lots were diluted 1:4000 in water and boiled for 10 minutes. 5ul of diluted virus lots were quantified using real time PCR with SYBR green (Life Technologies) detection (35 cycles of 95°C. for 15 seconds, 65°C for 30 seconds, and 72°C. for 40 seconds) using rhBALF4 specific primers (5’-tacaatactgacacggtggg-3’) and (5’-atgagacgcaggtactctgtgcttgctg-3’). All virus lots were tested by three independent dilutions with duplicate amplifications, and all virus lots were tested in the same run against a standard curve derived from a plasmid DNA clone containing a portion of the rhBALF4. The DNA units were arbitrarily assigned so 10 DNA units were required for each transforming unit of the wild type rhLCV viral lots and all relative values
were scaled accordingly.

Viral lots were titered for transforming units by making serial ten-fold dilutions of virus and the plating multiple replicates of each virus dilution into microwell plates with 200,000 rhesus lymphocytes/well with RPMI media, 10% fetal calf serum, and 0.5 ug/ml cyclosporine A. Wells were scored positive at 8 weeks post infection by visual confirmation of definite outgrowth of LCL. Transforming units were calculated as the dilution necessary for outgrowth in 50% of the wells by the Reed-Muench equation.

Animal Infection

rhLCV-naïve rhesus macaques from the extended-specific pathogen free colony at the New England Primate Research Center were assigned and confirmed to be rhLCV-naive shortly before infection. Animals were inoculated with 10⁶ transforming units of virus applied non-traumatically throughout the oral cavity. All animal experiments were approved by the Harvard Institutional Animal Care and Use Committee and performed in accordance with guidelines from the National Institutes of Health, US Department of Agriculture, and Harvard Medical School. Utmost care was taken by animal care workers and veterinary staff to ensure minimized stress and pain during all procedures.

Reverse transcriptase mediated PCR detection of rhLCV EBER in PBMC

PBMC were isolated from whole blood by centrifugation over a Ficoll gradient and were counted using Count Bright counting beads in flow cytometric analysis. For rhEBERs detection, total RNA was isolated from aliquots of 5x10⁶ PBMC and reverse transcribed by Mu-MLV reverse transcriptase using primers for rhEBER (173) and rhGAPDH (R) for 1 hour at 37°C (102). 90% of the cDNA was tested for rhEBER by PCR amplification (35 cycles 95°C, for 30 seconds, 60°C. for 30 seconds, 68°C for 30 seconds) and the remaining 10% of the cDNA was
tested for GAPDH using identical cycling parameters. PCR products were separated by agarose
gel electrophoresis, transferred to a nitrocellulose membrane by Southern blotting, and
hybridized with a dig-labeled rhEBERs probe.

**Frequency analysis for rhLCV infected cells by limiting dilution.**

In order to determine the frequency of rhLCV-infected cells in the peripheral blood, serial
two-fold dilutions of PBMC were prepared and then aliquoted into multiple replicates for each
dilution with the largest aliquots containing 2x10^6 cells. RNA was then isolated from each
replicate and each replicate was tested for rhEBERs by reverse transcriptase mediated PCR
amplification as described above. The frequency of infected cells was calculated by the number
of positive and negative replicates at all dilutions using Extreme Limiting Dilution Analysis
(125).

**Enzyme Linked Immunosorbant Assay (Elisa)**

**Coating antigens**

Recombinant EBV gp350, truncated directly before the transmembrane anchor sequence,
was isolated from stably transfected GH3 cells by size exclusion chromatography (126). Cells
were grown to 90% confluency and switched to serum free DMEM for 7 days. Spent
supernatants were precipitated in 60% ammonium sulfate for 1 hour at 4°C and then spun for 10
minutes at 12000 g. The precipitate was resuspended in a small volume of PBS and dialyzed
overnight against 4L of PBS. The dialyzed solution was then fractionated over a Sepharose 4B
(Sigma-Aldrich) column and fractions containing gp350 were identified by immunoblot with
EBV positive human sera. For each assay, gp350+ fractions were diluted 1:200 in bicarbonate
buffer (15 mM Na_2CO_3, 35 mM NaHCO_3 pH 9.6) and 96-well polystyrene plates were incubated
overnight at 4°C with 200ul/well. Small viral capsid antigen (sVCA) assays were coated with
approximately 50ng/well of a commercially manufactured rhBFRF3 peptide.

Assay

Plates were washed 3 times with PBS+0.1% Tween 20 and blocked for 2 hours with blocking solution (PBS+0.1% Tween 20 and 0.3% I-Block (Applied Biosystems)) at room temperature. Plates were washed 3 times with PBS+0.1% Tween 20. gp350 plates were incubated with a two fold serial dilutions of sera (starting at a dilution 1:20) and sVCA plates were incubated with a 1:100 dilution of sera for 1 hour at room temperature. Plates were again washed 3 times and incubated with horseradish peroxidase conjugated goat anti-human IgG (Jackson ImmunoResearch) diluted 1:1000 in blocking solution for 1 hour at room temperature. Plates were washed a final 3 times and peroxidase activity was detected using SigmaFast OPD (Sigma-Aldrich) according to the manufacturer’s protocol. All samples were tested in duplicate. Gp350 endpoint titer was calculated as the reciprocal of the last dilution to give a signal of three times the assay background. Measles titers were determined by a clinical elisa performed at Brigham and Women’s Hospital Clinical Labs.

72A1 Competition Assay

Microtiter plates were coated with gp350, as described above and blocked with PBS+0.1% Tween 20 and 0.3% I-Block for two hours at room temperature. Plates were washed 3 times with PBS+0.1% Tween 20 and incubated with four dilutions of human sera (1:10, 1:50, 1:100, and 1:200) for 1 hour at room temperature. Plates were washed 3 times and incubated with a 1:500 dilution of 72A1 from spent hybridoma supernatants. Plates were again washed 3 times and incubated with horseradish peroxidase conjugated goat anti-mouse IgG (Jackson ImmunoResearch) diluted 1:1000 in blocking solution for 1 hour at room temperature. Plates were washed a final 3 times and peroxidase activity was detected using SigmaFast OPD (Sigma-
Aldrich) according to the manufacturer’s protocol. All samples were run in duplicate and all plates included 72A1 reference wells where gp350 was not preincubated human sera.
Chapter Three

Development of recombinant antibodies targeting gp350 and gH/gL for use in rhesus macaques

Acknowledgements: Portions of this chapter are taken from a manuscript that is currently in preparation: *Rhesus Lymphocryptovirus Can Use EBV gp350 to Confer Sensitivity to an EBV Neutralizing Antibody and Infect Macaques*. Authors include Marissa Herrman, Janine Mühe, Carol Quink, and Fred Wang. The immunoglobulins from the 72A1 hybridoma were originally sequenced by a former graduate student, Nina Orlova. Nina also created the first 72A1 human chimeric antibody constructs. The 72A1-CDR grafted heavy chain sequences were analyzed in collaboration with Hayat Abdulkerim from Dr. Keith Reimann’s lab.
Abstract

Despite over 50 years of research on EBV, the series of events that allows EBV to infect its human host is still not completely understood. EBV is transmitted in oral secretions and the virus must cross the oral mucosa and infect B cells in order to establish life long, persistent infection. One model for crossing the mucosal barrier is direct infection of epithelial cells within the oropharynx and replication within these cells prior to B cell infection. This model has been particularly difficult to prove because primary EBV infection in humans is often asymptomatic. Well-described neutralizing monoclonal antibodies that selectively prevent infection of B cells or epithelial cells could be used to test the relative contributions of these two cell types during primary infection. The rhesus macaque animal model of EBV infection is the most accurate model for testing the effect of these neutralizing antibodies, but the use of murine monoclonal antibodies in macaques can be problematic because of their inherent immunogenicity. To generate recombinant, “rhesusized” monoclonal antibodies that would be minimally immunogenic in macaques, we first sequenced the immunoglobulin mRNAs from the 72A1 and E1D1 hybridomas that produce the antibodies that neutralize EBV infection of B cells and epithelial cells, respectively. Surprisingly, we found the 72A1 hybridoma produces multiple antibody molecules and determined the heavy and light chain pair that bound gp350 and neutralized EBV and rhLCV-hugp350 infection of B cells. The variable regions of the heavy and light chains of 72A1 and E1D1 were cloned upstream of the rhesus immunoglobulin constant regions, and here we show that these chimeric antibodies retain binding to their cognate antigens gp350 and gH/gL. These recombinant antibodies are valuable tools that can be used in combination with the chimeric rhLCV-hugp350 to test of the effects of blocking B cell and/or epithelial cell neutralization on infection in an animal host.
Introduction

During primary infection, EBV may infect both epithelial cells and B cells as the virus crosses the oral epithelium. It has been difficult to detect infection of epithelial cells during primary infection because acute infection is often asymptomatic. Even when acute infection causes disease, such as Infectious Mononucleosis, symptoms occur 6-8 weeks after initial virus exposure (10, 14). EBV has been shown to replicate in epithelial cells in vitro and it is thought that EBV is periodically replicated and shed from epithelial cells within the oral mucosa during persistent infection (127, 128). If EBV infects and replicates in epithelial cells during primary infection, then preventing epithelial cell infection may be just as important as preventing B cell infection to control virus.

Infection of rhesus macaques with the chimeric rhLCV-hup350 virus provides a novel modification to the rhesus macaque animal model that will allow testing of B cell and epithelial cell specific neutralizing antibodies during primary infection. RhLCV-hup350 expresses both EBV gp350 and rhLCV gH/gL and is therefore sensitive to neutralization by two well-characterized murine monoclonal antibodies that prevent infection of B cells or epithelial cells. 72A1 neutralizes infection of B cells by binding to the major membrane glycoprotein gp350 and preventing the association of gp350 and the EBV receptor CD21 (25). As shown in chapter 1, 72A1 is specific for EBV gp350 and does not neutralize rhLCV. E1D1 neutralizes infection of epithelial cells by binding to gH/gL and preventing the association of gH/gL with integrins (38). Unlike 72A1, E1D1 cross-reacts with rhLCV and is able to bind rhLCV gH/gL (129).

Direct use of murine monoclonal antibodies in primates, however, has generally been problematic. When murine antibodies are introduced into macaques, the macaque immune system recognizes the sequences as foreign and can mount an antibody response against the
infused murine antibody. The development of anti-antibody or anti-idiotype antibodies can negatively impact the activity of the infused antibody and is undesirable. A majority of the immunogenicity comes from the constant regions (Fc) and from the conserved framework sequences of the variable regions (FW1-4) of the murine antibody (Figure 3.1) (130). The complementarity determining domains (CDRs) within the variable region, which are responsible for antigen specificity, are not particularly immunogenic.
Figure 3.1 General antibody structure.
An antibody is composed of two identical heavy chains paired with two identical light chains. Each heavy and light chain can be divided into a constant region (purple), and a variable region (yellow). The variable regions can be further subdivided into framework regions (FW1-4) shown in yellow and complementarity determining regions (CDR1-3) shown in red.
Murine monoclonal antibodies are now routinely engineered through chimerism and CDR-grafting to produce an antibody molecule with reduced immunogenicity. A chimeric antibody is created by replacing the murine constant regions with constant regions from human or rhesus antibodies (131). CDR-grafting is achieved by replacing certain amino acids within the murine framework regions to create framework regions that are more similar to human or rhesus framework regions (132). Replacing the murine heavy and light chain constant regions with human or rhesus sequences can reduce the immunogenicity of a given antibody by 60-80% (133). Replacing the framework regions of the variable domain can further reduce immunogenicity by >10%. Thus an antibody that has been both chimerized and CDR-grafted has the lowest probability of inducing a detrimental anti-antibody response when infused into macaques.

The work presented here shows that recombinant, “rhesusized” 72A1 and E1D1 antibodies can still bind to their cognate antigens. These engineered antibodies can be used to test the impact of blocking B cell infection or epithelial cell infection following oral virus challenge in the rhesus macaque animal model.

Results

Cloning of an EBV neutralizing monoclonal antibody, 72A1

The murine monoclonal antibody 72A1 is the prototypic EBV neutralizing antibody that can prevent EBV infection of B cells. Surprisingly, flow cytometric analysis of the 72A1 hybridoma cell line revealed simultaneous expression of at least two different immunoglobulin molecules, one with a kappa light chain and one with a lambda light chain (Figure 3.2A). Both kappa and lambda containing immunoglobulin molecules could be detected by solid phase
antigen detection in all 72A1 hybridoma supernatants obtained from multiple sources (data not shown). Sequence analysis of the immunoglobulin mRNAs from the 72A1 hybridoma confirmed expression of two unique IgG1 heavy chains and two unique light chains, one kappa and one lambda (Supplemental Figure 6.1).

To define the pair that binds EBV gp350 and to confirm that this pair neutralizes EBV infection, we expressed the variable domains of the two heavy chains and two light chains on the constant regions of human IgG1 (H1, H2) and IgK (L1, L2) respectively. All four possible combinations of heavy and light chains were expressed in 293T cells and could be detected at equal levels by immunoblotting, confirming that each combination of heavy and light chains could potentially interact to produce four unique antibody molecules (data not shown). Of the four combinations, only one (H1L2) bound gp350 in an ELISA (Figure 3.2B).

All four combinations were additionally tested for the ability to neutralize EBV infection of B cells. EBV infection of peripheral blood mononuclear cells (PBMC) drives B cell proliferation that can be detected by monitoring loss of the cytoplasmic dye, carboxyfluorescein succinimidyl ester (CFSE). B cells maintained in media alone are detected as a single peak of CD20+/CFSE bright cells (Figure 3.2C; upper left panel), and exposure to EBV results in B cell proliferation detected as multiple peaks of CD20+/CFSE low cells (Figure 3.2C, upper right panel). Preincubation of EBV with H1L2 (Figure 2.2C, lower left panel), but not any of the other combinations (H2L2 shown as representative example, Figure 3.2C, lower right panel), neutralized EBV-driven B cell proliferation and reduced the percentage of proliferated B cells back to background levels. These data show that the same unique combination of heavy and light chains (H1L2) was responsible for both gp350 detection and EBV neutralization.
Figure 3.2 Identification of the Immunoglobulin sequences from the 72A1 Hybridoma that confer Binding to gp350 and Neutralization of EBV Infection.

A) Dual expression of murine kappa (IgK) and lambda (IgL) light chains on 72A1 hybridoma cells by flow cytometry. B) Testing recombinant antibodies for EBV gp350 binding by ELISA. 293 cells were transfected with the four different combinations of immunoglobulin expression plasmids (i.e. the unique heavy (H1, H2) and light (L1, L2) chain variable regions of immunoglobulin mRNAs expressed in the 72A1 hybridoma cloned upstream of human IgG1 constant region or human IgK constant region). Cell supernatants were harvested 3 days after transfection and reactivity to recombinant gp350 was measured by ELISA. Human sera from EBV positive (Pos. sera) or EBV negative (Neg. sera) donors were used as controls. C) EBV neutralization with recombinant H1L2 72A1. Human PBMC were stained with CFSE and exposed to media, virus, or virus preincubated with recombinant antibody supernatant from transfected 293 cells. Proliferation of human B cells was detected using flow cytometry. Live, CD20+ cells were gated and levels of CFSE intensity were measured. Percentages indicate CFSE low, proliferating B cells. Results from preincubation with H2L2 were similar to those with H1L1 and H2L1.
Figure 3.2 (Continued)
rhLCV-hugp350 is sensitive to 72A1 neutralization

To determine whether the recombinant 72A1 antibody can be used to neutralize chimeric rhLCV-hugp350, virus was incubated with recombinant 72A1 or control antibody before infection of CFSE labeled rhesus macaque PBMC. Infection of macaque B cells with rhLCV-hugp350 was readily neutralized by 72A1, and titration experiments showed that chimeric rhLCV-hugp350 infection of macaque B cells was as sensitive to 72A1 neutralization as EBV infection of human B cells (50% inhibitory concentration =14.52 ng/ml and 15.8 ng/ml respectively)(Figure 3.3).
Fig 3.3 rhLCV-hugp350 is Sensitive to 72A1 Neutralization.
CFSE neutralization assay results for A) EBV infection of human PBMC or B) rhLCV-hugp350 infection of rhesus PBMC when preincubated with two fold dilutions of recombinant 72A1. Percent neutralization was calculated as ((% CFSE_{low \ [virus \ alone]} - % CFSE_{low \ [virus+antibody]})/% CFSE_{low \ [virus \ alone]})
A chimeric 72A1 retains binding to gp350

To facilitate use of 72A1 in the rhesus macaque animal model, it was necessary to create a “rhesusized”, chimeric antibody. The variable regions of 72A1 were cloned and expressed upstream of the rhesus IgG1 and Igλ constant regions (Figure 3.4A). Antibody produced from transient transfection of 293T cells was still able to bind gp350 expressing cells, as measured by flow cytometry (Figure 3.4B). Thus, the chimeric, “rhesusized” 72A1 (rh72A1) was properly cloned and retained antigen specificity.
Figure 3.4 A recombinant, chimeric 72A1 retains gp350 binding

A) Schematic of the heavy and light chains of the parental murine 72A1 (m72A1) and the chimeric rhesusized 72A1 (rh72A1). Heavy chains are shown in blue (light blue=mouse and dark blue=rhesus) and light chains are shown in green (light green=mouse and dark green=rhesus). The variable regions of the heavy/light chains are shown in the lightest blue/green, respectively.

B) EBV gp350 expressing cells were stained with spent hybridoma supernatants containing m72A1 or supernatants from transiently transfected 293T cells containing rh72A1 and binding was detected by flow cytometry. Cells stained with secondary alone (anti-mouse FITC for m72A1 and anti-human FITC for rh72A1) are shown in grey and cells stained with recombinant antibody and secondary are shown in black.
A CDR grafted 72A1 heavy chain can be used to bind gp350

To generate an antibody with the least possible immunogenicity, we have designed and have begun testing a CDR grafted variant of 72A1. To identify sequences within the framework regions of the 72A1 heavy chain that could be mutated, the murine 72A1 variable region was BLASTed and the 10 human antibody variable regions with the highest amino acid homology were chosen for analysis. Ignoring the CDRs, since those are always variable between antibodies and cannot be mutated in order to retain antigen binding, 28 of the 73 amino acids analyzed differed in at least 9 of the 10 human heavy chain sequences as compared to the 72A1 heavy chain sequences (Supplemental Figure 6.2). Seventeen of the varied amino acids have been previously identified as important for the three dimensional structure of the murine variable region and were not considered for mutagenesis in an attempt to preserve correct positioning of the CDRs and proper antigen recognition. The remaining 11 amino acids were changed within the chimeric, rhesusized 72A1 from the murine variant to the amino acid that was most frequent among the 10 human heavy chain variants at each position (Figure 3.5A and Supplemental Figure 6.3).

When this grafted heavy chain was expressed with the rhesusized 72A1 light chain, the resulting antibody still bound gp350 (Figure 3.5B). Given that this antibody retains gp350 binding, it may be possible to introduce additional mutations and more extensively CDR-grafted the heavy chain. However, it will first be necessary to test this heavy chain in combination with a CDR-grafted light chain.
Figure 3.5 A CDR-grafted heavy chain 72A1 retains gp350 binding

A) Schematic of the heavy and light chains of the chimeric rhesusized 72A1 (rh72A1) and the CDR-grafted heavy chain containing 72A1 (rh72A1-heavy graft). Heavy chains are shown in blue (light blue=mouse and dark blue=rhesus) and light chains are shown in green (light green=mouse and dark green=rhesus). The variable regions of the heavy/light chains are shown in the lightest blue/green, respectively and the heavy chain variable region that contains the CDR-grafting mutations is denoted by vertical black stripes. B) EBV gp350 expressing cells were stained with supernatants from transiently transfected 293T cells containing rh72A1 or rh72A1-heavy graft and binding was detected by flow cytometry. Cells stained with secondary alone (anti-human FITC) are shown in grey and cells stained with recombinant antibody and secondary are shown in black.
A rhesusized E1D1 retains gH/gL binding

The murine monoclonal antibody E1D1 is the most well characterized EBV neutralizing antibody that can selectively inhibit EBV infection of epithelial cells. Generating a recombinant, rhesusized E1D1 was much more straightforward than 72A1. Sequencing of the immunoglobulin mRNAs from the E1D1 hybridoma revealed only one productive IgG heavy chain and one productive Igκ light chain (Supplemental Figure 6.4). Since we used an unbiased sequencing approach to amplify all possible immunoglobulin mRNAs, there were additional sequences from an unproductive kappa chain rearrangement and from a kappa pseudogene, but these were not further pursued. The variable regions of E1D1 were cloned and expressed upstream of the rhesus IgG1 and Igλ constant regions (Figure 3.6A). Antibody produced from transient transfection of 293T cells retained binding to both EBV gH/gL and rhLCV gH/gL (Figure 3.6B). Thus, the proper heavy and light chains were identified and the chimeric “rhesusized” E1D1 (rhE1D1) was appropriately cloned and retained antigen specificity.
Figure 3.6 A rhesusized E1D1 retains gH/gL binding
A) Schematic of the heavy and light chains of the parental murine E1D1 (mE1D1) and the chimeric rhesusized E1D1 (rhE1D1). Heavy chains are shown in blue (light blue=mouse and dark blue=rhesus) and light chains are shown in green (light green=mouse and dark green=rhesus). The variable regions of the heavy/light chains are shown in the lightest blue/green with white hatch marks, respectively. EBV gH/gL expressing cells (B) or rhLCV gH/gL expressing cells (C) were stained with spent hybridoma supernatants containing mE1D1 or supernatants from transiently transfected 293T cells containing rhE1D1 and binding was detected by flow cytometry. Cells stained with secondary alone (anti-mouse FITC for mE1D1 and anti-human FITC for rhE1D1) are shown in grey and cells stained with recombinant antibody and secondary are shown in black.
Figure 3.6 (Continued)
Discussion

The finding that the 72A1 hybridoma does not produce a single antibody was surprising since this antibody has been used in research for decades. It is not clear why the functional mRNA sequences we identified for 72A1 differ from those recently identified by another laboratory (134). This group did not describe the presence of two different heavy and light chain mRNAs, and their putative 72A1 sequences were identical to our H2L1 sequences which did not react with EBV gp350 in our studies. The H2L1 sequences are highly homologous to immunoglobulin molecules produced from the MOPC myeloma fusion partner originally used to create the 72A1 hybridoma, suggesting they are unlikely to encode EBV-specific antibodies.

Identifying and cloning the functional EBV neutralizing monoclonal antibody from the 72A1 hybridoma has translational implications. Passive transfer of recombinant 72A1 may provide effective prophylaxis of EBV infection by blocking virus infection of B cells and limiting viral spread during primary infection. Controlling acute viral loads is especially important in clinical situations where patients are at high risk for complications associated with primary EBV infection. This group includes pediatric transplant patients who are frequently EBV-negative prior to transplantation and who have an extremely high risk of post-transplant lymphoproliferative disorder (PTLD) if they acquire primary EBV infection while immunosuppressed. Additionally, boys with the congenital immunodeficiency X-linked lymphoproliferative syndrome (XLP) are uniquely sensitive to EBV infection with severe and often fatal outcomes during primary EBV infection. Antibody from the 72A1 hybridoma has been tested for safety in a limited number of pediatric liver transplant patients where it led to the development of a severe hypersensitivity reaction, a common problem associated with clinical use of a murine antibody (135). Thus, human studies with a pure, recombinant 72A1 mab that
has been humanized to reduce immunogenicity will be safer and more potent.

The experiments presented here describe three recombinant antibodies, rh72A1, rh72A1-heavy graft, and rhE1D1, that have been engineered to have reduced immunogenicity in macaques. These antibodies could be similarly engineered for humans by replacing the rhesus constant regions with human constant regions. The potential to develop these antibodies for human therapy is the main reason for CDR grafting 72A1 to have human-like framework regions as opposed to rhesus-like framework regions. Our experiments show that chimerizing and/or CDR-grafting 72A1 and E1D1 does not abolish antigen recognition. It is still unclear whether these changes to the antibody sequence have resulted in different binding kinetics relative to the parental murine antibodies. This could be tested using surface plasma resonance to measure binding kinetics between purified antibody and antigen. Additionally, each of these engineered antibodies should be tested for the ability to neutralize rhLCV-hugp350 infection of B cells and epithelial cells before being used in macaques. It would be expected that the chimeric antibodies retain identical antigen binding and virus neutralization but CDR-grafting may result in subtle differences in binding and neutralization. Characterizing the potency of the rhesusized antibodies will be important prior to the macaque studies, as only the most potent but least immunogenic antibodies should be chosen.

**Materials and Methods**

**Cell Culture**

The 72A1 and E1D1 hybridomas were grown in RPMI media supplemented with 10% fetal calf serum (FCS), 100 units/ml Penicillin, and 100 ug/ml Streptomycin. 293T cells were grown in DMEM with 10% FCS, 100 units/ml Penicillin, and 100 ug/ml Streptomycin.
GH3Δ19.5 cells used for gp350 antigen production were grown in DMEM with 10% FCS, 100 units/ml Penicillin, 100ug/ml Streptomycin and 200ug/ml G418.

**Sequencing of the 72A1 and E1D1 hybridomas and cloning of variable regions upstream of human/rhesus constant regions**

RNA was isolated using RNA-bee (Tel-Test, Inc) and immunoglobulin chain variable regions were amplified by Rapid Amplification of cDNA Ends (RACE) PCR using primers specific for the murine IgG1 constant region and IgK/IgL1-4 constant regions.

For 72A1, sequencing of the cloned immunoglobulin chains revealed two unique heavy chain variable regions (IgG-H1 and IgG-H2) and two unique light chain variable regions (IgK-L1 and IgL-L2). Each of the heavy chain variable regions was cloned upstream of the human IgG1 constant region of the pCIRN plasmid (kindly provided by Dr. Lisa Cavacini) to create pCIRN-H1 and pCIRN-H2. Each of the light chain variable regions was similarly cloned upstream of the human IgK constant region of the pEIG plasmid (kindly provided by Dr. Lisa Cavacini) to create pEIG-L1 and pEIG-L2. To create rhesusized variants, the variable region from pCIRN-H1 was PCR amplified and subcloned upstream of the rhesus IgG1 constant region in the pCIRN plasmid (kindly provided by Dr. Keith Reimann). The variable region from pEIG-L2 was also PCR amplified and subcloned upstream of the rhesus Igλ constant region in the pEIG plasmid (kindly provided by Dr. Keith Reimann). The variable region of the 72A1 heavy chain that was CDR-grafted was synthesized as a codon optimized DNA fragment with mutations in the codons for the 11 amino acids highlighted in supplemental figure 6.3 and subcloned upstream of the rhesus IgG constant region in the pCIRN plasmid.

For E1D1, sequencing of the cloned immunoglobulin chains revealed only one IgG1 heavy chain and one productive Igκ light chain. The variable region of the heavy chain was PCR
amplified and subcloned upstream of the rhesus IgG1 constant region in the pCIRN plasmid. The variable region of the light chain was also PCR amplified and subcloned upstream of the rhesus Igλ constant region in the pEIG plasmid.

Flow cytometry

The 72A1 hybridoma was subcloned and cells growing out from single clones were stained with fluorescein isothyocyanate (FITC) conjugated mouse anti-kappa (Southern Biotech) and phycoerythrin (PE) conjugated mouse anti-lambda (Southern Biotech) antibodies for 30 min at 4°C. Cells were washed twice with phosphate buffered saline (PBS) containing 1% FSC and 0.1% sodium azide to remove unbound antibody and analyzed on a FACScalibur cytometer (BD Biosciences).

Recombinant antibodies used for staining were produced by transfecting the appropriate pCIRN and pEIG plasmids into 293T cells using Effectene (Life Technologies) according to the manufacturer’s instructions. After 6-10 days, cell free supernants were collected. Gp350 or gH/gL expressing cells were stained with a 1:3 dilution of spent hybridoma supernatants or straight transfection supernatants for 30 min at 4°C. Cells were washed with PBS containing 1% FSC and 0.1% sodium azide to remove unbound antibody then incubated with a 1:100 dilution of FITC conjugated goat anti-mouse or goat anti-human (Jackson ImmunoResearch) for 30 min at 4°C. Cells were washed once more and analyzed on the flow cytometer.

EBV gp350 ELISA

Recombinant EBV gp350, truncated directly before the transmembrane anchor sequence, was isolated from stably transfected GH3 cells by size exclusion chromatography. Cells were grown to 90% confluency and switched to serum free DMEM for 7 days. Spent supernatants were precipitated in 60% ammonium sulfate for 1 hour at 4°C and then spun for 10 minutes at
12000 g. The precipitate was resuspended in a small volume of PBS and dialyzed overnight against 4L of PBS. The dialyzed solution was then fractionated over a Sepharose 4B (Sigma-Aldrich) column and fractions containing gp350 were identified by immunoblot with EBV-positive human sera. For each assay, gp350+ fractions were diluted 1:200 in bicarbonate buffer (15 mM Na$_2$CO$_3$, 35 mM NaHCO$_3$ pH 9.6) and 96-well polystyrene plates were incubated overnight at 4°C with 200ul/well. Plates were washed 3 times with PBS+0.1% Tween 20 and blocked for 2 hours with blocking solution (PBS+0.1% Tween 20 and 0.3% I-Block (Applied Biosystems)) at room temperature. Plates were washed 3 times and supernatants from 293 cells transiently transfected with pCIRN-H1/H2 and pEIG-L1/L2 using Effectene (Life Technologies) according to the manufacturer’s protocol were collected 48 hours post transfection, diluted 1:2 in blocking solution and incubated for 1 hour at room temperature. Sera from an EBV-immune or EBV-naive donor were diluted 1:50 and used as controls. Plates were again washed 3 times and incubated with horseradish peroxidase conjugated goat anti-human IgG (Jackson ImmunoResearch) diluted 1:1000 in blocking solution for 1 hour at room temperature. Plates were washed a final 3 times and peroxidase activity was detected using SigmaFast OPD (Sigma-Aldrich) according to the manufacturer’s protocol. All samples were tested in duplicate.

**CFSE Neutralization assay**

Virus and antibody were combined in RMPI for a total volume of 50ul for and incubated for 90 minutes at 37°C. 2x10$^6$ peripheral blood mononuclear cells (PBMC) labeled with carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies) according to the manufacturers protocol were resuspended in 50ul RPMI and added for an additional 90 min at 37°C. Cells were then moved to a 24 well plate and cultured in RPMI for 5 (human PBMC) or 10 (rhesus PBMC) days. Cells were harvested, washed with FACS buffer (PBS+1%FCS and
0.1% sodium azide), stained with CD20-APC (BD Biosciences) for 30 minutes at 4°C, washed again with FACS buffer and analyzed by flow cytometry. All assays included no virus and virus with no antibody pretreatment as controls.
Chapter Four

The EBV neutralizing antibody response extends beyond 72A1-competing antibodies and likely targets multiple EBV glycoproteins
Abstract

EBV infection is known to induce neutralizing antibodies in humans, but the glycoprotein targets of these antibodies are largely unknown. Early studies have suggested that the major membrane glycoprotein gp350 is the primary, if not the only, target for neutralizing antibodies in human sera. Additional studies have defined antibodies that bind to a single epitope on gp350 as a correlate for total neutralizing activity. These epitope binding antibodies, termed 72A1-competing antibodies, are occasionally used as a surrogate marker for all neutralizing antibodies. Such a focused neutralizing antibody response has not been described for any other herpesvirus. Additionally, four of the five EBV glycoproteins involved in entry have been shown to induce neutralizing antibodies in mice. We hypothesized that the neutralizing antibody response in humans was in fact more complex than what has been reported in the literature. Surprisingly, we found that only half of EBV infected humans make 72A1-competing antibodies, even though they all neutralize EBV infection. Thus, 72A1-competing antibodies are clearly not the only type of neutralizing antibody in human sera. Additional data presented here would suggest that the repertoire of glycoproteins targeted by neutralizing antibodies extends beyond gp350, and a possible additional target is the gH/gL complex. Understanding the antibody response to EBV glycoproteins induced during natural infection can be used as a surrogate for the proteins that are naturally immunogenic and may be prime candidates for EBV vaccine immunogens.
**Introduction**

EBV entry into cells involves the step-wise coordination of up to five different viral glycoproteins. Murine monoclonal antibodies can block many of these entry steps, including viral attachment, coreceptor engagement and triggering of the fusion protein, by binding to the viral glycoproteins gp350, gp42, and gH/gL, respectively (25, 38). Gp350 is currently the most well defined target for neutralizing antibodies induced during EBV infection in humans.

Gp350 was recognized early on as a target for neutralizing antibodies in humans (97). Depletion of gp350 specific antibodies from the sera of 5 EBV positive individuals resulted in a decrease or total loss of EBV neutralizing activity compared to a control depletion. This cohort included 2 EBV positive, non-diseased individuals, 2 individuals with Burkitt lymphoma, and 1 individual with nasopharyngeal carcinoma. Given the small sample size and the fact that only two sera came from non-diseased individuals, it is still unclear whether gp350 neutralizing antibodies are present and abundant in the majority of EBV infected humans.

Additional studies have defined specific gp350 binding antibodies, termed 72A1-competing antibodies, as a correlate for total neutralizing activity in human sera (89). The murine monoclonal antibody 72A1 binds to gp350 and prevents the association of gp350 and the EBV receptor on B cells, CD21. The exact epitope that 72A1 binds has not been defined, but mutational studies have identified specific amino acids on gp350 that are required for 72A1 binding (Figure 4.1B) (52, 54, 55). Perhaps unsurprisingly, these same residues, in addition to others, have been defined as important for gp350 binding to CD21 (Figure 4.1A). Antibodies in human sera that bind gp350 within this region and subsequently block the binding of 72A1 are defined as 72A1-competing antibodies. The levels of 72A1 competing antibodies were shown to correlate with total neutralizing activity in EBV infected humans, i.e. the more 72A1-competing
antibodies, the better a given sera neutralized (89). Many of these 72A1 competing antibodies are likely to be neutralizing antibodies since they bind within or very close to the CD21 binding site, but this study does not rule out that other types of neutralizing antibodies are induced during EBV infection. Surprisingly, the depletion study and this correlation study have lead to the dogma that gp350 is the major, if not the only, target for neutralizing antibodies in human sera and the antibody responses to other viral glycoprotein have been largely ignored.
Figure 4.1 Residues important for CD21 or 72A1 binding to EBV gp350
Snapshot of the crystal structure of EBV gp350 showing approximately the first 300 amino acids (aa). Gp350 domain A (aa4-153) is shown in orange and domain B (165-305) is shown in blue. The amino acids important for A) CD21 or B) 72A1 have been highlighted in white: A) aa 21, 22, 151, 155, 158-160, 162-164, 208, 210, and 296. B) aa 151, 155, 158-160, 162-164, 208, and 210. PBD:2H6O.
Comparatively, the antibody responses to the glycoproteins of alpha- and beta-herpesviruses, like HSV-1, VZV and HCMV, have been much more extensively characterized (136-140). Humans infected with these other herpesviruses make a broad range of neutralizing antibodies that target multiple different viral glycoproteins. Some neutralizing antibodies target glycoproteins that are unique to each virus, such as HSV-1 gD or VZV gE. More interestingly, however, antibodies targeting the glycoproteins gH/gL and gB, which are conserved across all families of herpesviruses, are always present. Often, the majority of the neutralizing antibody response is directed at the fusion protein, gB. Thus, it would be surprising if the EBV neutralizing antibody response really was as narrow as reported.

Results

72A1 competing antibodies are not the only neutralizing antibodies in human sera

To begin examining the neutralizing antibody response in EBV infected humans, we assembled a cohort of sera from EBV negative individuals, EBV positive individuals, and individuals with NPC. NPC patients are known to have a dysregulated antibody response, with a general increase in the levels of many antibodies, and we were interested in comparing this population to otherwise healthy, EBV positive population. Sera were first tested for EBV gp350 reactive antibodies to rule out the possibility that there were individuals without gp350 specific antibodies that could still neutralize EBV infection, as this would automatically suggest the presence of non-gp350 neutralizing antibody targets. As expected, however, all 23 EBV positive samples and 20 NPC samples did have gp350 specific antibodies, as measured by Elisa (Figure 4.2A). For each serum, we calculated the endpoint titer (i.e. the reciprocal of the last dilution of
serum to give a positive signal). The EBV negative sera did not give a positive signal at any
dilution and did not have a titer. The average gp350 antibody titer in EBV positive sera was 2071
with a range from 721 to 8428 and a standard deviation of 1617. The average gp350 antibody
titer for NPC sera was 5207 with a range from 38 to 45861 and a standard deviation of 10447.
The greater range of antibody levels in NPC sera may result from disease progression and/or
clinical disease treatment, as samples were randomly collected from patients. Although the
average titer of gp350 antibodies in NPC sera was 2.5 times higher than in healthy individuals,
the difference between the two cohorts was not significant. Thus, all individuals tested had
gp350 specific antibodies.
Figure 4.2 Antibodies to EBV gp350 in human sera
A) EBV positive (circles) and NPC (squares) sera were tested for EBV gp350 binding antibodies by Elisa. Gp350 was incubated with serial dilutions of sera and the endpoint titer (reciprocal of the last serum dilution to give a signal of three times the assay background) was determined. B) EBV negative (triangles), EBV positive (circles) and NPC (squares) sera were tested for 72A1 competing antibodies. Dilutions of sera were used to block 72A1 binding to gp350 and the 50% inhibitory concentration (IC50) was determined. The IC50 represents the reciprocal of the serum dilution where the 72A1 binding signal was decreased by 50% relative to a no serum control. Sera were scored as negative (below cutoff) if the initial serum dilution of 1:10 did not inhibit 72A1 binding by 50%.
Next, we measured the levels of 72A1-competing antibodies. Surprisingly, in our cohort, only 14/25 (56%) healthy and 10/19 (52%) NPC sera had 72A1 competing antibodies (Figure 4.2B). For each of the competing sera we calculated the IC50, i.e. the dilution of serum that blocked 50% of 72A1 binding. EBV positive sera had an average IC50 of 111 with a range from 12 to 582 and NPC sera had an average of 415 with a range from 17 to 2986. Similar to the gp350 titers, although the average IC50 was almost 4 times higher in NPC than in healthy individuals, the difference between the two cohorts was not significant.

Since 72A1-competing antibodies have been defined as a correlate for total neutralizing activity and only half of the sera we tested had 72A1-competing antibodies, we measured the neutralizing activity of sera from 3 EBV negative individuals, 17 EBV positive individuals, and 21 NPC patients at a single dilution of 1:5. As in previous assays, neutralization was scored as a loss of virus driven B cell proliferation and measured by a decrease in the cytoplasmic dye CFSE. As expected, all the EBV positive and NPC sera were able to neutralize EBV infection of B cells, while none of the sera from EBV negative donors could (Figure 4.3A and B). In both populations there was a range of neutralization, from 18% to upwards of 95%. Interestingly, a greater proportion of NPC sera could neutralize EBV infection by more than 75% (86% of NPC sera vs 59% of EBV positive sera), suggesting that NPC sera have more neutralizing antibodies than the sera of EBV positive individuals. When the neutralizing activity of these sera was compared with their ability to compete with 72A1, it was clear that human sera can neutralize EBV infection without 72A1-competing antibodies (Figure 4.3A and B). Thus, 72A1-competing antibodies are not the only type of neutralizing antibodies in human sera.
Figure 4.3 Neutralizing activity of human sera that do or do not have antibodies that compete with 72A1

EBV negative (A), EBV positive (A), and NPC (B) sera were tested for the ability to neutralize EBV infection. Sera were diluted 1:5 and incubated with EBV prior to infection of CFSE labeled PBMC. B cell proliferation in the presence or absence of sera was measured five days post infection. Percent neutralization was calculated as ((% CFSE$_{\text{low}}$ [virus alone] - % CFSE$_{\text{low}}$ [virus + antibody])/% CFSE$_{\text{low}}$ [virus alone]). 72A1-competition status was previously determined (Figure 3.2B). The neutralization of sera with 72A1-competing antibodies are shown by black bars and the neutralization of sera with no 72A1-competing antibodies are shown by grey bars.
RhLCV infected macaques have neutralizing antibodies that inhibit EBV infection

72A1-competing antibodies are clearly not the only neutralizing antibodies induced during EBV infection of humans, but there is still an open question of whether the vast majority of neutralizing antibodies are directed at gp350 or whether the neutralizing antibody response targets other glycoproteins as well. In chapter 2, we found that rhesus macaques naturally infected with rhLCV do not readily generate antibodies that cross-react with EBV gp350 (Figure 2.5). If macaques also generate neutralizing antibodies that target multiple viral glycoproteins, as might occur in humans, we hypothesized that some of these antibodies will cross-react with well conserved glycoproteins, such as gH/gL or gB. Thus, we tested sera from rhLCV positive and negative macaques for the ability to neutralize EBV infection of human B cells. Sera from 8 rhLCV positive macaques could all neutralize EBV infection (Figure 4.4). The average neutralizing activity was 59%, with a range from 32% to 85%. These data show that rhLCV infection induces neutralizing antibodies that can clearly cross-neutralize EBV infection. Given that these animals have a very low titer of gp350 cross-reactive antibodies, these data would suggest that this neutralizing activity comes from cross-reactive antibodies to other, more well conserved, glycoproteins.
Figure 4.4 Macaque sera neutralize EBV infection of human B cells
rhLCV positive and rhLCV negative macaque sera were tested for the ability to neutralize EBV infection. Sera were diluted 1:5 and incubated with EBV prior to infection of CFSE labeled PBMC. B cell proliferation in the presence or absence of sera was measured five days post infection. Percent neutralization was calculated as ((% CFSE_{low} [virus alone] - % CFSE_{low} [virus+antibody])/% CFSE_{low} [virus alone]).
Most EBV infected humans generate gH/gL antibodies

The glycoproteins gH/gL and gB, which are conserved in all herpesviruses and have been shown to induce neutralize antibodies during HSV-1, VZV, and HCMV infection, are likely to induce neutralizing antibodies during EBV infection. To test if EBV infected humans generate gH/gL specific antibodies, we measured the ability of human sera to detect gH/gL expressed on the surface of 293T cells by flow cytometry. None of the five EBV negative sera tested could detect gH/gL, while 9/10 EBV positive and 8/8 NPC sera were able to detect surface expressed gH/gL (Figure 4.5). All of the sera tested here did not bind untransfected 293T cells above background levels. The average mean fluorescence intensity (MFI) for cells incubated with EBV negative sera was 7.3 and was similar to unstained cells. The average MFI for cells stained with EBV positive sera was 27 with a range of 9-42. The average MFI for cells stained with NPC sera was 42 with a range of 11 to 90. For comparison, saturating amounts of a gH/gL specific monoclonal antibody resulted in an MFI of 173. Thus, it would appear that the levels of gH/gL binding antibodies were not saturating at this dilution of sera. Similar to the gp350 antibody response, it appears that NPC sera have more gH/gL specific antibodies, but given the quantitative limitations of this particular assay, it is unclear if that difference is meaningful. This shows, for the first time, that gH/gL is immunogenic during natural infection and the majority of EBV infected humans generate antibodies to the gH/gL heterodimer. Thus, it is still possible that a portion of EBV neutralizing antibodies target gH/gL.
**Figure 4.5 EBV infected humans make gH/gL binding antibodies**

Summary of gH/gL detection by antibodies in human sera. 293T cells transfected with gH/gL were stained with EBV negative (circles), EBV positive (squares), or NPC (triangles) sera and analyzed by flow cytometry. Staining with the gH/gL specific monoclonal antibody, E1D1, was used as a positive control. The mean fluorescence intensity of all live cells is plotted.
Discussion

The neutralizing antibody response that is generated during EBV infection has not been well studied. The current dogma in the field that gp350 is the major, if not the only, glycoprotein targeted by neutralizing antibodies comes from only two studies (89, 141). Neither of these studies conclusively demonstrates that finding, as the first tested only two EBV positive individuals and the other was a statistical correlation. An additional glycoprotein, BMRF2, has been reported to induce neutralizing antibodies in EBV infected humans (93). These antibodies, however, have only been shown to be important in a polarized epithelial cell infection system. BMRF2 has not been described to have a role in B cell infection and does not seem to be important for entry in other epithelial cell models. Thus, the current characterization of the EBV neutralizing antibody response in humans is incredibly limited and likely incomplete.

It was surprising that only half of our EBV positive and NPC sera had 72A1-competing antibodies, given that the levels of 72A1 competing antibodies have been shown to correlate with total neutralizing activity in human sera. In the original study defining the correlation between 72A1-competing antibodies and total neutralization, there was evidence that some of the EBV positive human sera did not compete with 72A1 (89). It was not well documented, however, if those sera were then included when the correlation to neutralizing activity was made. If they were included, it is not at all clear how the amount of 72A1-competing antibodies were assigned. In the cohort presented here, there were sera without 72A1-competing antibodies that could neutralize better than sera with 72A1-competing antibodies, suggesting that 72A1 competition is not the most accurate correlate for neutralizing activity. This could become problematic, as 72A1 competition was used as a surrogate assay for neutralization in the phase II clinical trial.
evaluating a gp350 vaccine for IM (22). The results from this assay were not specifically reported during the trial, and one could speculate that these data were not reported because not all individuals developed a 72A1-competing antibody response. The 72A1 competition assay can provide important serological information, as antibodies that compete with 72A1 are likely binding within the CD21 binding site and thus are neutralizing, but this assay should not be used as a stand alone surrogate for neutralization.

*Is gp350 the only target for neutralizing antibodies?*

Since it has been shown that depleting gp350-specific antibodies from a small number of human sera also depletes the neutralizing activity, the next question to answer would be: how much of the neutralizing activity in human sera is directed at gp350? It is possible that there are human antibody binding epitopes on gp350, in addition to the epitope(s) bound by 72A1-competing antibodies, that could be neutralizing. A study describing 18 gp350-specific murine monoclonal antibodies showed that they grouped into 7 distinct competition groups, two of which, I and IV, were found to be neutralizing (88). 72A1 is a group I antibody, therefore this group must bind within the CD21 binding site, and group IV antibodies bind within the C-terminal 200 amino acids of gp350 (90). It is currently unknown how this antibody neutralizes, since the only described function for gp350 is binding to CD21. Human sera have not yet been tested for antibodies that compete with group IV antibodies. If there are human antibodies that bind to the epitopes defined by these monoclonals, they will likely be neutralizing. Thus, the sera that do not have 72A1-competing antibodies could still be neutralizing EBV infection by binding to gp350.

Determining the proportion of neutralizing antibodies that bind gp350 could be experimentally tested in at least two ways. First, gp350 antibodies could be depleted from human
sera and the neutralizing activity of depleted and non-depleted sera from the same donor could be compared. This approach is technically challenging and would require proper optimization to ensure that all gp350 specific antibodies, including any that bind the membrane proximal region, are depleted while all other antibodies remain unchanged. This approach is also limited in the amount of information that can be gained. The relative amount of gp350-specific neutralizing antibodies will be clear, but the exact repertoire of antibodies will remain unknown without a number of additional studies.

Alternatively, the relative abundance of gp350 specific antibodies could be determined by generating a panel of human monoclonal antibodies, screening them for neutralization, and then identifying the viral protein target that they bind. This approach would be labor intensive and would require the development of a more sensitive, rapid neutralization assay but would result in neutralizing antibodies that could be extensively characterized and would rapidly reveal the different viral glycoproteins targeted by neutralizing antibodies. This approach also has its limitations and would be very difficult to perform on a large panel of individuals. Additionally, there is no way to ensure the monoclonals that are generated accurately represent the entire repertoire.

The outcome of either of these experiments cannot be accurately predicted based on our current knowledge, but it would be expected that the gp350-specific neutralizing antibody response varies from individual to individual. Gp350-specific antibodies are likely to be the majority of the neutralizing activity in some individuals and a smaller proportion of the neutralizing activity in others. It would also be expected that the gp350-specific neutralizing antibodies are more abundant in individuals who have 72A1-competing antibodies relative to those that do not have 72A1-competing antibodies. Since the macaque neutralization
experiments presented here suggest that lymphocryptovirus infection can induce neutralizing antibodies that target multiple viral glycoproteins, it would not be expected for all the neutralizing activity in all EBV infected humans to be directed against gp350.

**What other EBV glycoproteins may be targeted by neutralizing antibodies?**

EBV has at least four other glycoproteins, gp42, gH/gL, and gB that may be targeted by neutralizing antibodies. gp42 and gB were previously shown to induce antibodies in the majority of EBV infected humans (94, 142). Here we show that gH/gL also induce antibodies in the majority of EBV infected humans. gH/gL and gB commonly induce neutralizing antibodies during infection with other herpesviruses. Gp42 is a glycoprotein unique to EBV but has been compared to the HSV-1 specific glycoprotein gD. Neutralizing antibodies to gD are commonly induced in HSV-1 infection (138). Thus, any or all of these glycoproteins could be inducing neutralizing antibodies in EBV infected humans.

Neutralizing antibodies targeting EBV gH/gL would be particularly interesting, given the dual role of gH/gL during B cell and epithelial cell entry. Three murine monoclonal antibodies specific for gH/gL can inhibit epithelial cell infection but differentially inhibit B cell infection (38). The E1D1 monoclonal antibody is the most specific for epithelial cell neutralization, i.e. does not inhibit B cells very well. E1D1 binds gH/gL near the KGD integrin binding motif and inhibits the interaction of gH/gL with integrins on the surface of epithelial cells. The CL40 monoclonal antibody is the best at inhibiting both cell types. Its binding site on gH/gL is unknown, but likely neutralizes infection by blocking the ability of gH/gL to trigger fusion since this would prevent infection of both cell types equally well. Neutralization with the CL59 monoclonal antibody falls in the middle; it is not as good as CL40 at neutralizing infection of B cells but it is better than E1D1. CL59 binds gH alone, close to the C-terminus, and therefore the
membrane. Again, it is most likely to neutralize by inhibiting gH/gL from triggering fusion.

Similar to the 72A1-competition assay, each of these three antibodies could be used to measure E1D1, CL40, or CL59 –competing antibodies in human sera. If most humans develop antibodies that compete with all three monoclonals, this would suggest that a portion of the neutralizing antibody response targets gH/gL. If there are E1D1-competing antibodies, but not CL40 or CL59 –competing antibodies, this would suggest that gH/gL specific neutralizing antibodies may be more likely to neutralize epithelial cell infection than B cell infection. Human sera could then be depleted of gH/gL specific antibodies and the neutralizing activity of depleted versus undepleted sera from the same donor could be compared. Here, both B cell neutralization and epithelial cell neutralization should be assayed. Based on the neutralizing antibody repertoire induced following most herpesvirus infections, it would be expected that humans generate gH/gL specific neutralizing antibodies and that they can neutralize both B cell infection and epithelial cell infection.

Accurately defining the neutralizing antibody response induced during EBV infection has implications for future EBV vaccine development. Neutralizing antibodies are important immune effectors for many vaccines and the EBV glycoproteins that can naturally induce neutralizing antibodies may be the best immunogens for an IM vaccine.

**Materials and Methods**

**EBV gp350 ELISA**

Recombinant EBV gp350, truncated directly before the transmembrane anchor sequence, was isolated from stably transfected GH3 cells by size exclusion chromatography. Cells were grown to 90% confluency and switched to serum free DMEM for 7 days. Spent supernatants
were precipitated in 60% ammonium sulfate for 1 hour at 4°C and then spun for 10 minutes at 12,000 g. The precipitate was resuspended in a small volume of PBS and dialyzed overnight against 4L of PBS. The dialyzed solution was then fractionated over a Sepharose 4B (Sigma-Aldrich) column and fractions containing gp350 were identified by immunoblot with EBV positive human sera. For each assay, gp350+ fractions were diluted 1:200 in bicarbonate buffer (15 mM Na$_2$CO$_3$, 35 mM NaHCO$_3$ pH 9.6) and 96-well polystyrene plates were incubated overnight at 4°C with 200ul/well. Plates were washed 3 times with PBS+0.1% Tween 20 and blocked for 2 hours with blocking solution (PBS+0.1% Tween 20 and 0.3% I-Block (Applied Biosystems)) at room temperature. Plates were washed 3 times and two fold serial dilutions of sera (starting at a dilution 1:20) were incubated for 1 hour at room temperature. Plates were again washed 3 times and incubated with horseradish peroxidase conjugated goat anti-human IgG (Jackson ImmunoResearch) diluted 1:1000 in blocking solution for 1 hour at room temperature. Plates were washed a final 3 times and peroxidase activity was detected using SigmaFast OPD (Sigma-Aldrich) according to the manufacturer’s protocol. All samples were tested in duplicate. The endpoint titer was calculated as the reciprocal of the last dilution to give a signal of three times the assay background.

72A1 Competition Assay

Microtiter plates were coated with gp350, as described above and blocked with PBS+0.1% Tween 20 and 0.3% I-Block for two hours at room temperature. Plates were washed 3 times with PBS+0.1% Tween 20 and incubated with four dilutions of human sera (1:10, 1:50, 1:100, and 1:200) for 1 hour at room temperature. Plates were washed 3 times and incubated with a 1:500 dilution of 72A1 from spent hybridoma supernatants. Plates were again washed 3 times and incubated with horseradish peroxidase conjugated goat anti-mouse IgG (Jackson
ImmunoResearch) diluted 1:1000 in blocking solution for 1 hour at room temperature. Plates were washed a final 3 times and peroxidase activity was detected using SigmaFast OPD (Sigma-Aldrich) according to the manufacturer’s protocol. All samples were run in duplicate and all plates included 72A1 reference wells where gp350 was not preincubated human sera.

**CFSE Neutralization assay**

Virus and 10 ul of sera were combined in RMPI for a total volume of 50ul for and incubated for 90 minutes at 37°C. 2x10^6 human peripheral blood mononuclear cells (PBMC) labeled with carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies) according to the manufacturers protocol were resuspended in 50ul RPMI and added for an additional 90 min at 37°C. Cells were then moved to a 24 well plate and cultured in RPMI for 5 days. Cells were harvested, washed with FACS buffer (PBS+1%FCS and 0.1% sodium azide), stained with CD20-APC (BD Biosciences) for 30 minutes at 4°C, washed again with FACS buffer and analyzed by flow cytometry. All assays included no virus and virus with no sera pretreatment as controls.

**Flow Cytometry**

293T cells were transfected with pCAGGS-gH and pCAGGS-gL expression plasmids (kindly provided by Lindsey Hutt-Fletcher) using Effectene (Life Technologies) according to the manufacturer’s protocol. Three days post transfection, cells were trypsinized, washed in FACS buffer (PBS+1%FCS and 0.1% sodium azide) and incubated with a 1:100 dilution of sera for 30 minutes at 4°C. Cells were washed and incubated with a 1:100 dilution of fluorescein isothiocyanate (FITC) conjugated goat anti-human antibody (Jackson ImmunoResearch) for 30 minutes at 4°C. Cells were washed again with FACS buffer to remove unbound antibody and analyzed on a FACScalibur cytometer (BD Biosciences).
Chapter Five

General Discussion and Conclusions
Testing neutralizing antibodies in the rhesus macaque animal model

One major goal of the work presented in this dissertation was to adapt the rhesus macaque animal model to allow studying EBV neutralizing antibodies in vivo. Neutralizing antibodies are assumed to be an important immune effector for an effective EBV vaccine, but this has never been tested in a model that recapitulates the natural route of infection, i.e. infection through the oral mucosa. The rhLCV-hugp350 virus created in chapter 2 and the rhesusized monoclonal antibodies created in chapter 3 will now allow the testing of neutralizing antibodies in macaques. Thus, the original goal has been accomplished and the macaque model has been appropriately adapted.

Neutralizing antibodies may only be effective during the first 6-8 weeks of infection as viral loads are still increasing and the virus is presumably replicating. After this point, the EBV specific immune response begins to control infection and viral loads decrease to a steady state level of latently infected cells where viral replication may be limited. We would expect the half life of the rhesusized 72A1 and rhesusized E1D1 to be around two weeks and should be able to achieve sufficient antibody levels during the first 6-8 weeks with one infusion directly before virus challenge and a second infusion after 3 weeks. Given that these antibodies will only be tested for a short amount of time, the attempts to CDR-graft 72A1 may be excessive. However, if these antibodies can significantly reduce viral loads during primary infection, then they may be useful as a therapy for transplant patients at high risk for post transplant lymphoproliferative disease. In this setting, patients would likely need multiple infusions over many months and a CDR-grafted antibody would be necessary to limit long term immunogenicity. Testing CDR-grafted antibodies in our proposed macaque experiments would provide preclinical data for such a therapy. If future attempts at CDR-grafting negatively impact antigen binding, macaque
experiments could still be conducted with the chimeric constructs that have been generated.

There are a number of different models for how EBV crosses the oral mucosa to initiate infection of a naïve host and the ability of neutralizing antibodies to limit viral spread depends on which model is biologically correct. We hypothesize that epithelial cells are the first cell type infected as the virus encounters the oral mucosa. Here virus is amplified by lytic replication and free virus is released. The free virus will then infect B cells and infected B cells will be driven to proliferate. Each replicated B cell will carry the EBV episome and thus be infected. In this model, the combination of both epithelial cell infection and B cell infection determine viral loads during primary infection. Alternative models postulate that EBV gains direct access to B cells though cracks in the epithelial cell barrier of the oral mucosa. Thus, epithelial cells are not infected and do not contribute to acute viral loads. If our model is correct, one would expect both epithelial cell neutralizing antibodies and B cell neutralizing antibodies to alter viral infection.

Future experiments to test the impact of neutralizing antibodies on rhLCV-hugp350 infection should include 4 groups of macaques: 1) preinfused with rhesusized 72A1 to neutralize B cell infection 2) preinfused with rhesusized E1D1 to neutralize epithelial cell infection 3) preinfused with both 72A1 and E1D1 to neutralize infection of both cell types and 4) mock infused as a control. If our model of virus infection is correct, all three groups of animals that receive 72A1 and/or E1D1 should have a significant reduction in both acute and persistent viral loads relative to the control group. Both antibodies should be equally effective at controlling infection and all three groups that receive 72A1 and/or E1D1 should be equally protected. If the alternative model of direct B cell infection is correct, then viral loads should be reduced only in the two groups that receive 72A1.

Each of these models could be a vast oversimplification of what occurs during primary...
infection and all three groups may behave differently. Incoming virus may be able to infect either cell type, i.e. both models occur simultaneously. In this case, the combination of 72A1 and E1D1 will be more effective than E1D1 alone at lowering viral loads. One might expect for 72A1 alone to be as effective as the combination of both antibodies, given that the virus must enter B cells in order to persistently infect its host. Infection of B cells, however, may occur through alternative mechanisms that are not sensitive to antibody neutralization. For example, virus could be transferred from epithelial cells directly to B cells through cell-to-cell spread or through the formation of an infection synapse. Thus, simultaneously blocking infection of both cell types would have the greatest effect on limiting infection and would be more beneficial than blocking infection of either cell type alone.

**Designing future EBV vaccines**

Ultimately, the results of the experiment proposed above will be informative for future vaccine design. If neutralizing antibodies against both B cell and epithelial cell infection can limit viral spread, then the goal would be to design a vaccine that induces both B cell and epithelial cell neutralizing antibodies. One option would be to add soluble gH/gL to the current gp350 subunit vaccine. If neutralizing antibodies against B cell infection are sufficient for reducing acute and persistent viral loads, then it would be better to focus on a vaccine that induces high levels of B cell neutralizing antibodies. Several of the gp350 vaccine formulations that have been tested do not produce a strong neutralizing antibody response. In the phase I human trials of the recombinant gp350 vaccine, only 50% of individuals developed neutralizing antibodies (21). An alternative approach using measles vectored gp350 could not induce any gp350 specific antibodies in macaques (143). Additionally, gp350 expressed in a New Castle disease viral vector was only slightly more immunogenic than soluble gp350 (144). Thus, it may
be more beneficial to incorporate other glycoproteins that could induce B cell neutralizing antibodies, such as gp42 or gH/gL.

Whether the fusion glycoprotein gB should be included in future EBV vaccine candidates is still unclear. gB is one of the most well conserved proteins across all three families of herpesviruses, and subunit vaccines for other herpesviruses have shown that gB is a strong inducer of neutralizing antibodies (9). EBV gB, however, has some unique properties compared to other herpesviruses and may not be as immunogenic or as sensitive to antibody neutralization. There are currently no gB-specific murine monoclonal antibodies that are neutralizing, suggesting that EBV gB does not readily induce neutralizing antibodies. For all other herpesviruses, gB has well described cellular binding partners that are necessary for fusion (34). Inhibition of binding between gB and its cellular binding partner is a common mechanism for gB neutralizing monoclonal antibodies (145). EBV gB may have a binding partner on NPC cells, but it has not been described to bind cellular proteins during fusion to B cells or other epithelial cells (146). If EBV gB does not directly interact with other proteins, neutralizing antibodies would have to bind to gB and prevent the conformational changes that are necessary for fusion. Thus, EBV gB may be a much less effective immunogen than other herpesvirus gBs and may not be an important component of an EBV vaccine.

Accurately defining the glycoproteins targeted by neutralizing antibodies during EBV infection would be beneficial for deciding which glycoproteins would be promising immunogens and which would not. The data presented in chapter 4 suggest that gp350 may not be the only glycoprotein to induce neutralizing antibodies in humans. If gp350, gp42, gH/gL, and gB can all induce neutralizing antibodies, then the most promising vaccine candidate may be a virus-like particle (VLP) that contains all EBV glycoproteins instead of a subunit vaccine that contains
only one or two glycoprotein(s).
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Supplemental Figures
Supplemental Figure 6.1 Four unique immunoglobulin variable regions cloned from the 72A1 hybridoma
Sequences of the two unique heavy chains (A and B) and two unique light chains (C and D) cloned from the 72A1 hybridoma. The cleaved signal sequences for each immunoglobulin chain are underlined in green.
Supplemental Figure 6.2 Alignment of 72A1 heavy chain variable region and the 10 most homologous human heavy chain sequences
The first 95 amino acids of the 72A1 heavy chain were aligned with the top 10 human heavy chain hits following a Blast search of the 72A1 heavy chain sequence (ms72A1-H vs Human Version 1-10). The CDR sequences, highlighted in light blue, were automatically changed to the 72A1 CDR sequences since they must be maintained. The amino acids of the human heavy chains that differed at residues predicted to be important for variable region secondary structure, highlighted in green, were also automatically changed to the amino acids naturally found in 72A1, as they must be maintained as well. The remaining amino acids that differed between the human heavy chains and 72A1 are highlighted in red and were the positions considered for CDR-grafting.
Supplemental Figure 6.3 Sequence of the 72A1-heavy graft variable region

Sequence of the original (A) 72A1 heavy chain as compared to the sequence of the 72A1 CDR-grafted heavy chain (B). The original murine signal sequence is underline in green and the albumin signal sequence that was introduced during grafting is underlined in blue. The amino acids that have been changed in the 72A1 grafted heavy chain are highlighted in red.
Supplemental Figure 6.4 Sequence of the heavy and light chain variable regions from the E1D1 hybridoma

Sequences of the heavy (A) and light (B) chain variable regions cloned from the E1D1 hybridoma. The cleaved signal sequences are underlined in green.