GB virus C: cellular interactions, HIV inhibition and natural history

Emma Louise Mohr

University of Iowa

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GB VIRUS C: CELLULAR INTERACTIONS,
HIV INHIBITION AND NATURAL HISTORY

by
Emma Louise Mohr

An Abstract
Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Molecular and Cellular Biology
in the Graduate College of
The University of Iowa

May 2012

Thesis Supervisor: Professor Jack T. Stapleton
ABSTRACT

GB virus C (GBV-C) is a nonpathogenic lymphotropic virus that replicates in B and T lymphocytes. Infection with GBV-C is documented worldwide and is common: between 1% and 5% of healthy blood donors are viremic at the time of donation. Antibodies to GBV-C proteins are not usually detected during viremia, and antibodies to the GBV-C envelope glycoprotein E2 develop following the clearance of viremia. Although GBV-C viremia may persist for decades, viremia usually clears within 2 years following infection in the majority of individuals infected by blood transfusion. A chimpanzee variant of GBV-C, designated GBV-C<sub>cpz</sub>, is found in captive and noncaptive chimpanzees and its prevalence and natural history are uncharacterized.

HIV-infected individuals who are co-infected with GBV-C survive longer than those without GBV-C. GBV-C infection of PBMCs inhibits the replication of HIV isolates and one of the mechanisms for this is the downregulation of HIV coreceptors and secretion of the coreceptor ligands. Additional mechanisms of HIV inhibition by GBV-C are examined in this dissertation.

The GBV-C envelope glycoprotein E2 contributes directly to the inhibition of HIV infection. Incubation of recombinant E2 with PBMCs at 4°C prior to HIV infection results in a decrease in HIV replication, and only HIV enveloped pseudoparticle transduction, not VSV-G enveloped pseudoparticle transduction, is inhibited by GBV-C E2. This suggests that GBV-C E2 inhibits HIV infection at an entry step when the HIV envelope proteins interact with cellular receptors and membranes. How GBV-C E2 interacts with cellular surfaces and which cellular proteins are utilized for GBV-C binding and entry are unknown. Here, we characterize GBV-C E2 binding to human PBMCs, murine cells, and multiple transformed cell lines to identify the PBMC subset to which E2 binds and to identify candidate cellular receptors involved in GBV-C binding and entry. Understanding how GBV-C E2 interacts with cellular surfaces is critical to determining how it inhibits HIV entry.
Anti-GBV-C E2 antibodies are also associated with improved survival in HIV-infected individuals. Recent studies demonstrated that anti-E2 antibodies neutralize HIV infection *in vitro* and immunoprecipitate HIV virions. In these studies, we describe how anti-E2 antibodies immunoprecipitate retroviral particles regardless of the specific viral envelope protein on the surface, but only neutralize particles bearing the HIV envelope protein. We also found that the cellular antigen recognized by anti-E2 antibodies is accessible only in permeabilized cells and not on the cell surface. These studies provide insight into the HIV-inhibitory mechanisms of anti-E2 antibodies, which should aid in the development of GBV-C E2 as an immunogen in an HIV vaccine.

Finally, no animal models exist for studying GBV-C infection or GBV-C vaccines as HIV therapeutics *in vivo*. We examined the natural history of GBV-C \textit{cpz} in a captive chimpanzee population, and found that the prevalence of GBV-C \textit{cpz} viremia and anti-E2 antibodies, as well as the length of persistent infection, were similar to those found in healthy human blood donors. The GBV-C \textit{cpz} 5’ntr and RdRp sequences from chimpanzee subspecies \textit{troglodytes} and \textit{verus} shared a high level of sequence identity and indicate that the chimpanzee variant should be designated GBV-C \textit{cpz} rather than the currently used GBV-C \textit{trog}. These findings demonstrate that GBV-C \textit{cpz} viremia and E2 antibody status should be tested in animals involved in clinical research trials because affected animals may have altered responses to HIV vaccines, and that the chimpanzee would be a good animal model in which to study GBV-C infection.

Abstract Approved: ____________________________________________

Thesis Supervisor

__________________________________

Title and Department

__________________________________

Date
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HIV INHIBITION AND NATURAL HISTORY

by

Emma Louise Mohr

A thesis submitted in partial fulfillment
of the requirements for the Doctor of
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May 2012

Thesis Supervisor:  Professor Jack T. Stapleton
CERTIFICATE OF APPROVAL

_______________________

PH.D. THESIS

This is to certify that the Ph.D. thesis of

Emma Louise Mohr

has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Molecular and Cellular Biology at the May 2012 graduation.

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Jack T. Stapleton, Thesis Supervisor

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Wendy Maury

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Aloysius J. Klingelhutz

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Jeffery L. Meier

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Christopher Stipp
To my husband and family, who have inspired me to do what I love and do it well
Education is not the filling of a pail, but the lighting of a fire.

William Butler Yeats, Poet
ACKNOWLEDGMENTS

We thank Dr. Wendy Maury from the University of Iowa for providing defective VSV particles and the MLV envelope protein plasmid.

We thank Drs. Georg Hess and Alfred Engel (Roche Diagnostics and Laboratories, Germany) for providing GBV-C E2 ELISA kits and the M5 and M6 antibodies.

We thank Dr. Inara Souza and Donna Klinzman from the University of Iowa for technical assistance.

We thank Dr. Kristin Barnhart and Rebekah Jones from the University of Texas MD Anderson Cancer Center, Department of Veterinary Sciences for providing chimpanzee serum samples. We thank La Shayla Morrow at SFBR for providing assistance with the samples.

We thank Dr. Fayyaz Sutterwala and Tyler Ulland from the University of Iowa for providing primary mouse cells and assistance.

We thank Jim McLinden and Jinhua Xiang for technical assistance, helpful discussions and critical comments.
ABSTRACT

GB virus C (GBV-C) is a nonpathogenic lymphotropic virus that replicates in B and T lymphocytes. Infection with GBV-C is documented worldwide and is common: between 1% and 5% of healthy blood donors are viremic at the time of donation. Antibodies to GBV-C proteins are not usually detected during viremia, and antibodies to the GBV-C envelope glycoprotein E2 develop following the clearance of viremia. Although GBV-C viremia may persist for decades, viremia usually clears within 2 years following infection in the majority of individuals infected by blood transfusion. A chimpanzee variant of GBV-C, designated GBV-C_{cpz}, is found in captive and noncaptive chimpanzees and its prevalence and natural history are uncharacterized.

GBV-C research was initially performed by viral hepatitis research groups because it was predicted to cause hepatitis. The realization that GBV-C did not cause hepatitis resulted in a marked reduction in research activity. Because Hepatitis C virus co-infection worsens the clinical course of HIV-infected patients, researchers hypothesized that the related virus, GBV-C, may impact HIV disease. In 1998, researchers found that HIV-infected individuals who were co-infected with GBV-C survived longer than those without GBV-C. These findings provide the rationale for examining the relationship of GBV-C and HIV and the development of GBV-C as a novel therapeutic for HIV. GBV-C infection of PBMCs inhibits the replication of HIV isolates and one of the mechanisms for this is the downregulation of HIV coreceptors and the induction of the release of soluble ligands for HIV coreceptors (RANTES, macrophage inflammatory proteins (MIP)-1\(\alpha\) and MIP-1\(\beta\) and SDF-1).

The GBV-C envelope glycoprotein E2 contributes directly to the inhibition of HIV infection. Incubation of recombinant E2 with PBMCs at 4°C prior to HIV infection results in a decrease in HIV replication, and only HIV enveloped pseudoparticle transduction, not VSV-G enveloped pseudoparticle transduction, is inhibited by GBV-C E2. This suggests that GBV-C E2 inhibits HIV infection at an entry step when the HIV
envelope proteins interact with cellular receptors and membranes. How GBV-C E2 interacts with cellular surfaces and which cellular proteins are utilized for GBV-C binding and entry are unknown. Here, we characterize GBV-C E2 binding to human PBMCs, murine cells, and multiple transformed cell lines to identify the PBMC subset to which E2 binds and to identify candidate cellular receptors involved in GBV-C binding and entry. Understanding how GBV-C E2 interacts with cellular surfaces is critical to determining how it inhibits HIV entry.

Anti-GBV-C E2 antibodies are also associated with improved survival in HIV-infected individuals. Recent studies demonstrated that anti-E2 antibodies neutralize HIV infection in vitro and immunoprecipitate HIV virions. In these studies, we describe how anti-E2 antibodies immunoprecipitate retroviral particles regardless of the specific viral envelope protein on the surface, but only neutralize particles bearing the HIV envelope protein. We also found that the cellular antigen recognized by anti-E2 antibodies is accessible only in permeabilized cells and not on the cell surface. These studies provide insight into the HIV-inhibitory mechanisms of anti-E2 antibodies, which should aid in the development of GBV-C E2 as an immunogen in an HIV vaccine.

Finally, no animal models exist for studying GBV-C infection or GBV-C vaccines as HIV therapeutics in vivo. We examined the natural history of GBV-C_{cpz} in a captive chimpanzee population, and found that the prevalence of GBV-C_{cpz} viremia and anti-E2 antibodies, as well as the length of persistent infection, were similar to those found in healthy human blood donors. The GBV-C_{cpz} 5’ntr and RdRp sequences from chimpanzee subspecies troglodytes and verus shared a high level of sequence identity and indicate that the chimpanzee variant should be designated GBV-C_{cpz} rather than the currently used GBV-C_{trog}. These findings demonstrate that GBV-C_{cpz} viremia and E2 antibody status should be tested in animals involved in clinical research trials because affected animals may have altered responses to HIV vaccines, and that the chimpanzee would be a good animal model in which to study GBV-C infection.
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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>5’ntr:</td>
<td>5’ nontranslated region</td>
</tr>
<tr>
<td>AIDS:</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ART:</td>
<td>anti-retroviral therapy</td>
</tr>
<tr>
<td>ATCC:</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>bp:</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA:</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BVDV:</td>
<td>Bovine viral diarrhea virus</td>
</tr>
<tr>
<td>CHO:</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>CLDN1:</td>
<td>claudin-1</td>
</tr>
<tr>
<td>CMV:</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CTLA:</td>
<td>cytotoxic T-lymphocyte-associated</td>
</tr>
<tr>
<td>DNA:</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOB:</td>
<td>date of birth</td>
</tr>
<tr>
<td>DTP:</td>
<td>Developmental Therapeutics Program</td>
</tr>
<tr>
<td>DV:</td>
<td>Dengue virus</td>
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<tr>
<td>EBV:</td>
<td>Epstein Barr virus</td>
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<tr>
<td>ELISA:</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER:</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>F:</td>
<td>female</td>
</tr>
<tr>
<td>Fc:</td>
<td>fragment, crystallizable</td>
</tr>
<tr>
<td>GAG:</td>
<td>glycosaminoglycans</td>
</tr>
<tr>
<td>GBV-A:</td>
<td>GB Virus A</td>
</tr>
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<td>GBV-B:</td>
<td>GB Virus B</td>
</tr>
<tr>
<td>GBV-C:</td>
<td>GB Virus C</td>
</tr>
<tr>
<td>GBV-C_{cpz}:</td>
<td>GB virus C, chimpanzee variant</td>
</tr>
<tr>
<td>GBV-C_{trog}:</td>
<td>GB virus C, troglodyte variant</td>
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</table>
GBVpp: GBV-C E1/E2 enveloped retrovirus pseudoparticles
GE: genome equivalents
GFP: green fluorescent protein
gp: glycoprotein
HCA: hydrophobic cluster analysis
HCV: Hepatitis C virus
HGV: Hepatitis G virus
HIV: Human immunodeficiency virus
HIVpp: HIV enveloped retrovirus pseudoparticles
HOS: human osteosarcoma cell line
IC\(_{50}\): half maximal inhibitory concentration
IFN: interferon
Ig: immunoglobulin
IL: interleukin
IRES: internal ribosomal entry site
IP: immune precipitation
JEV: Japanese encephalitis virus
LDLR: low density lipoprotein receptor
LTR: long terminal repeat
M: male
mAb: monoclonal antibody
µM: micromolar
MIP: macrophage inflammatory protein
ml: milliliter
MLV: Murine leukemia virus
NHLBI: National Heart, Lung, and Blood Institute
NCI: National Cancer Institute
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>NIH:</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NS:</td>
<td>nonstructural protein</td>
</tr>
<tr>
<td>nt:</td>
<td>nucleotide</td>
</tr>
<tr>
<td>NMR:</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NS5A:</td>
<td>GBV-C nonstructural protein 5A</td>
</tr>
<tr>
<td>NSBB:</td>
<td>GBV-C nonstructural protein 5B</td>
</tr>
<tr>
<td>ORF:</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PBMC:</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS:</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBSA:</td>
<td>phosphate buffered saline with BSA</td>
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<tr>
<td>PE:</td>
<td>phycoerythrin</td>
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<tr>
<td>PHA:</td>
<td>phytohemagglutinin</td>
</tr>
<tr>
<td>PKR:</td>
<td>protein kinase R</td>
</tr>
<tr>
<td>Pr:</td>
<td>probe sequence</td>
</tr>
<tr>
<td>R5:</td>
<td>CCR5 tropic HIV-1 isolates</td>
</tr>
<tr>
<td>RANTES:</td>
<td>Regulated upon Activation, Normal T-cell Expressed, and Secreted</td>
</tr>
<tr>
<td>RBC:</td>
<td>red blood cells</td>
</tr>
<tr>
<td>RdRp:</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>RLU:</td>
<td>relative light units</td>
</tr>
<tr>
<td>RNA:</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR:</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDF-1:</td>
<td>stromal-derived-factor-1</td>
</tr>
<tr>
<td>SFBR:</td>
<td>Southwest Foundation for Biomedical Research</td>
</tr>
<tr>
<td>SHIV:</td>
<td>Simian human immunodeficiency virus</td>
</tr>
<tr>
<td>SR-BI:</td>
<td>scavenger receptor class B type I</td>
</tr>
<tr>
<td>TBEV:</td>
<td>Tick-borne encephalitis virus</td>
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</table>
TM: transmembrane
VSV: Vesicular Stomatitis Virus
VSV-G: VSV envelope glycoprotein
VSVΔG: VSV particle with genome lacking VSV-G envelope glycoprotein
X4: CXCR4 tropic HIV-1 isolates
YFV: Yellow Fever virus
CHAPTER I. INTRODUCTION

Discovery of Human and Chimpanzee GBV-C

Following the discovery of Hepatitis C virus (HCV) in 1989, virus discovery groups searched for novel etiological agents responsible for non-A, non-B, non-C hepatitis because it became clear that HCV was not detected in 10% to 20% of individuals with non-A, non-B hepatitis (Alter et al., 1989; Choo et al., 1989; Kuo et al., 1989; Simons et al., 1995a). In the process, human and primate viruses related to HCV were identified. Virus discovery groups at Genelabs, Inc. and Abbott Laboratories independently reported the identification of a human virus sharing many features with hepatitis C virus (HCV) in subjects with non-A, non-B, non-C hepatitis in 1995 and 1996 (Linnen et al., 1996; Simons et al., 1995a). Because a putative hepatitis F virus had been described (Deka et al., 1994), Genelabs named the virus they identified in a non-A, non-B, non-C hepatitis patient “Hepatitis G virus” (HGV) (Linnen et al., 1996), although there were limited epidemiological data to support an association with hepatitis. Abbott Laboratories had previously found two viruses in marmosets that had been inoculated with serum from a surgeon with non-A, non-B hepatitis whose initials were G.B. (Schaluder et al., 1995; Simons et al., 1995b). These viruses were closely related to HCV and were called GB virus A and B (GBV-A, GBV-B) (Schaluder et al., 1995; Simons et al., 1995b). GBV-A and GBV-B were not found in humans, but using degenerate primers based on GBV-A and B, Abbott Laboratories subsequently identified a closely related virus in humans which they called GBV-C (Simons et al., 1995a). Sequence analysis revealed that HGV and GBV-C were two isolates of the same virus, and the proper taxonomic name is HGV/GBV-C (Kim & Fry, 1997; Leary et al., 1996). Based on phylogenetic relationships, the GB viruses and HCV are classified as members of the Flaviviridae.

Abbott laboratories continued the search for hepatitis-like viruses and identified a variant of GBV-C (GBV-Ctrog) in an HCV-infected chimpanzee with resolving hepatitis.
Birkenmeyer et al., 1998). They reported a near-complete genome sequence (GenBank accession number AF070476) (Birkenmeyer et al., 1998), which remains the only near-complete GBV-C\textsubscript{trog} sequence published to date. GBV-C\textsubscript{trog} infection was not found in human or macaque monkey blood samples (Birkenmeyer et al., 1998). Adams et al. also identified GBV-C RNA in 3 noncaptive chimpanzees (subspecies troglodytes and verus) that they called GBV-C\textsubscript{cpz} (Adams et al., 1998). For the remainder of the thesis, the chimpanzee variant of GBV-C will be noted with the designation GBV-C\textsubscript{cpz} rather than GBV-C\textsubscript{trog}. The GBV-C\textsubscript{cpz} polyprotein shares 83.6% amino acid identity with GBV-C, while human GBV-C isolates are >95% identical (Mohr & Stapleton, 2009; Muerhoff et al., 2005; Pavesi, 2001). Based on limited phylogenetic analysis of sequences from the 5’ nontranslated region (5’ ntr), helicase and RNA dependent, RNA polymerase (RdRp) regions, all of the GBV-C\textsubscript{cpz} sequences are monophyletic within a group of GBV-C viruses from humans and chimpanzees (Adams et al., 1998). Thus, GBV-C\textsubscript{cpz} is considered a chimpanzee variant of GBV-C rather than a separate genotype. Sequence analyses of all available chimpanzee GBV-C sequences from Abbott Laboratories (named GBV-C\textsubscript{trog}) and Adams et al. (named GBV-C\textsubscript{cpz}) demonstrated that these viruses were different isolates of the GBV-C\textsubscript{cpz} variant (Adams et al., 1998; Birkenmeyer et al., 1998).

**GBV-C Epidemiology and Disease Association**

Numerous studies designed to determine if HGV/GBV-C represented an etiological agent for acute or chronic hepatitis were reported between 1996 and 1998, and prospective and well-controlled retrospective studies did not observe an epidemiological association between this new virus and acute or chronic hepatitis (Alter, 1997; Stapleton, 2003; Theodore & Lemon, 1997). Consequently, this virus is by definition not a “hepatitis” virus and the term HGV is misleading. Similarly, no evidence exists to suggest that the surgeon G.B. was infected with GBV-C (Alter, 1997). Consequently, neither HGV nor GBV-C accurately describes this virus (Simons et al., 1995a). Since the
virus does not cause hepatitis, it will be referred to as GBV-C. No convincing association between the virus and any disease entity has been identified since the discovery of GBV-C (Alter, 1997; Stapleton, 2003), and thus it appears to be a nonpathogenic human virus.

Like HCV, human GBV-C infection is found worldwide and is capable of persistent infection (Barnes et al., 2007; Lefrère et al., 1997). GBV-C infection is common, and between 1% and 5% of healthy blood donors in developed countries are viremic at the time of donation. The prevalence is higher in blood donors from developing countries (Fig. 1.1), and the prevalence of GBV-C is significantly higher in individuals with coexistent bloodborne or sexually transmitted infections (Alter, 1997; Stapleton, 2003). For example, the prevalence of GBV-C viremia in HCV- and HIV-infected individuals is approximately 20% and 30%, respectively (Alter, 1997; Stapleton, 2003). Unlike HCV, antibodies to GBV-C proteins are not usually detected during viremia, and antibodies to the GBV-C envelope glycoprotein E2 develop following the clearance of viremia (Pilot-Matias et al., 1996; Surowy et al., 1997; Tacke et al., 1997a; Tacke et al., 1997b; Tanaka et al., 1998; Thomas et al., 1998). Anti-E2 antibodies appear to be somewhat protective against re-infection; thus, E2 antibodies are thought to contain neutralizing activity (Hassoba et al., 1998; Tillmann et al., 1998). Active viremia is determined by the detection of viral RNA in sera or plasma using RT-PCR methods, while prior infection is inferred by the detection of GBV-C E2 antibodies (Alter, 1997; Stapleton, 2003; Stapleton et al., 2004). Because E2 antibodies may disappear during longitudinal follow up, prevalence studies probably underestimate the rate of prior exposure (Schwarze-Zander et al., 2006; Stark et al., 1996).

Although GBV-C viremia may persist for decades, viremia usually clears within 2 years following infection in the majority of individuals infected by blood transfusion (Alter, 1997; Theodore & Lemon, 1997). By comparing the ratio of E2 antibody positive blood donors to donors with GBV-C viremia, it appears that approximately 75% of GBV-
C infections are spontaneously cleared by the infected host, at least in immune competent individuals (Alter, 1997; Linnen et al., 1996; Simons et al., 1995a; Stapleton, 2003; Theodore & Lemon, 1997). Specifically, the prevalence of E2 antibody to GBV-C viremia in blood donors is approximately 6:1, while the ratio of individuals with HCV antibody compared to those with viremia is approximately to 1:4; thus, HCV infection is more likely to persist than GBV-C (Haaskjold et al., 1992). Among individuals with HIV infection, the ratio of E2 antibody prevalence to viremia is generally less than 2:1, suggesting that GBV-C viral clearance is reduced in individuals with impaired cellular immunity (Rey et al., 2000; Tillmann et al., 2001; Tillmann & Manns, 2001; Williams et al., 2004).

Unlike human GBV-C, the natural history of GBV-C<sub>cpz</sub> has not been well characterized. Because of its high sequence identity with human GBV-C, GBV-C<sub>cpz</sub> is predicted to be a nonpathogenic lymphotropic virus and to be transmitted by the same routes as human GBV-C (sexually, parenterally, vertically). GBV-C<sub>cpz</sub> viremia has only been detected in chimpanzees, both captive and noncaptive animals, and not in humans or macaques (Adams et al., 1998; Birkenmeyer et al., 1998). The single near-complete GBV-C<sub>cpz</sub> genome sequence was generated from an HCV-infected chimpanzee with resolving hepatitis (Birkenmeyer et al., 1998). It is unknown whether GBV-C<sub>cpz</sub> elicits persistent infection as does human GBV-C. The single population-based study of GBV-C<sub>cpz</sub> was performed by Adams et al. in 1998 (Adams et al., 1998). Here, they studied a population of noncaptive chimpanzees and detected GBV-C<sub>cpz</sub> viremia in 3 of 39 noncaptive chimpanzees (Adams et al., 1998). Partial sequences for one chimpanzee with samples 24 months apart were generated (Adams et al., 1998). The prevalence and natural history of GBV-C<sub>cpz</sub> has not been otherwise examined (Adams et al., 1998; Birkenmeyer et al., 1998). At the time GBV-C<sub>cpz</sub> was identified, serological reagents to detect GBV-C E2 antibodies were not available, so there are no data published on the presence of E2 antibodies in chimpanzees.
Genome Organization

Overview

Based on nucleotide sequence and genome organization, GBV-C, GBV-C<sub>cpz</sub> and HCV are classified as members of the *Flaviviridae*, which has three known genera (flavi, pesti, and hepaci). The GB viruses are unassigned, although GBV-B has been proposed to be within the hepaciviruses genera. The phylogenetic relationships between GBV-A, GBV-B, GBV-C, HCV and representative members of the pesti and flavi genera are shown in Figure 1.2. GBV-C shares considerable sequence identity with GBV-A (48%) and to a lesser extent with GBV-B and HCV (~30% for both) (Leary *et al.*, 1996). GBV-A infection in primates is not associated with disease; GBV-B infection in primates is associate with hepatitis (Adams *et al.*, 1998; Schaluder *et al.*, 1995). The GBV-C<sub>cpz</sub> polyprotein shares 83.6% amino acid identity with GBV-C (Birkenmeyer *et al.*, 1998; Mohr & Stapleton, 2009).

GBV-C, and presumably GBV-C<sub>cpz</sub>, contain a 9.4 kb single-stranded, positive sense RNA genome that is organized similarly to HCV (Fig. 1.3). HCV and GBV-C genomes contain 5’ and 3’ nontranslated regions (ntrs) and contain a long open reading frame (ORF) encoding approximately 3000 amino acids that are post-translationally cleaved into structural and nonstructural proteins. The predicted 5’ntr of GBV-C (555bp) is longer than the HCV 5’ntr (341 nt) and both contain an internal ribosome entry site (IRES) that directs translation of the mRNA (Simons *et al.*, 1996). The coding region for HCV and GBV-C structural proteins is found in the N-terminal one third of the ORF (Penin *et al.*, 2004). The C-terminal portion of the ORF encodes the nonstructural proteins (NS), NS2, 3, 4, 5A and 5B. The GBV-C 3’ntr (~300bp) is different from the HCV 3’ntr in that it does not contain poly-(A) or poly-(U) tracts, though it shares predicted structural elements (Xiang *et al.*, 2000).
Translation initiation

The GBV-C 5’ntr shares very little identity with the HCV 5’ntr, and consistent with these differences, the activity of the GBV-C 5’ntr IRES is considerably lower than that of the HCV 5’ntr IRES (Simons et al., 1996). The location of the GBV-C translation initiation site cannot be easily deduced by comparisons with HCV because of these sequence differences as well. The GBV-C 5’ntr has up to four AUG codons that are in-frame and could initiate translation, depending on the isolate (Simons et al., 1996; Xiang et al., 2000). In vitro transcription translation studies with the GBV-C 5’ntr directing translation of a chloramphenicol acetyltransferase reporter demonstrated that the AUG codon starting at nt 555 (based on Genbank accession no. AF121950) is the site of translation initiation, although the first in-frame AUG codon (nt 462) is highly conserved as well (Simons et al., 1996). There are no data to confirm if the translation initiator AUG codon identified in vitro is utilized in vivo.

Protein processing

Although HCV translation and protein processing were experimentally demonstrated, GBV-C processing is largely based on sequence comparisons with HCV. A cellular signal peptidase is predicted to cleave GBV-C E1-E2, E2-P7 and P7-NS2, based on consensus eukaryotic signal sequence cleavage sites (Leary et al., 1996). The NS2 viral serine protease mediates cleavage at the NS2/NS3 (Belyaev et al., 1998) as in HCV (Fig. 1.3). The NS3 protease mediates NS3/NS4 and NS5A/NS5B cleavage and, together with NS4A, mediates cleavage of the NS4B/NS5A junction (Belyaev et al., 1998). The protease responsible for mediating the cleavage of NS4A/NS4B was undetermined in these studies, but the NS3 protease is predicted to mediate this cleavage, as with HCV (Belyaev et al., 1998).

Structural proteins

Although the HCV ORF encodes a core protein upstream of E1 and GBV-C particles have similar biophysical characteristics with HCV, the coding region for a
GBV-C core protein has not been definitively identified (Xiang et al., 1998). The processing sites of the structural proteins and the translation initiation AUG codon utilized in vivo have not been examined experimentally and the characteristics of a core protein are unclear. Biophysical studies demonstrate that GBV-C particles are similar to HCV particles, supporting the existence of a nucleocapsid (Xiang et al., 1998). If the amino terminus of E1 is processed at a predicted signal peptide site using the predicted initiator AUG codon at nt 555, a 23 amino acid long, 2.2 kD core protein with a pI of 5.4 would result. This would result in the smallest flavivirus core to date. Together with its low pI, this is not consistent with a flavivirus core protein. However, if the translation initiation site was located at nt 462, the core protein would be 5.4 kD and the pI would be 11.0, about the size and charge of other flaviviral core proteins. The lack of a definitive AUG initiator codon makes numbering the proteins and their processing sites difficult. In addition, there is an alternative ORF within the HCV core coding region (Walewski et al., 2001) which does not appear to exist in GBV-C.

The envelope glycoproteins (E1 and E2) appear to form a heterodimer and are thought to be inserted into the viral envelope. Based on similarities with the envelope glycoproteins of HCV, the ectodomains of GBV-C envelope glycoproteins were proposed to be targeted to the ER lumen and their transmembrane domains inserted into the ER membrane to become type I transmembrane proteins (Kaiser et al., 2004). Like HCV, the GBV-C E1/E2 heterodimer is predicted to be retained in the ER because of the presence of N-linked oligosaccharides on E2 (Pilot-Matias et al., 1996), although some HCV heterodimers are detected on cellular plasma membranes when the envelope glycoproteins are overexpressed (Dubuisson, 2007). HCV envelope proteins E1 and E2 contain multifunctional transmembrane regions (TM) in their C-terminal regions. The TM domains function as membrane anchors, ER retention signals and are involved in E1/E2 heterodimerization (Cocquerel et al., 2002). The same is predicted for GBV-C envelope proteins. GBV-C does not have hypervariable regions at the N-terminal end of
HCV E2 (Stapleton et al., 2004), which may impair the virus from developing neutralization escape variants and contribute to the increased rate of clearance observed in infected humans (Haaskjold et al., 1992).

Another aspect of the GBV-C structural proteins that has not been studied yet is a putative amphipathic protein at the C-terminus of E2 analogous to the HCV P7 protein. This protein is an ion channel in HCV and there are two putative signal peptidase sites surrounding an amino acid sequence that may encode a small (5.6 kD) amphipathic helical protein which may serve as an HCV P7 homolog.

**Nonstructural proteins**

The functions of the GBV-C nonstructural proteins have not been experimentally characterized with one exception, but are proposed to be similar to HCV. GBV-C NS2 and NS3 are predicted to function as viral proteases. The C-terminal region of NS3 is predicted to have NTPase and helicase functions, and NS5B is an RNA-dependent RNA polymerase (;Leary et al., 1996). NS4B and 5A are less well characterized, but are predicted to function as a membrane alteration inducer and multifunctional phosphoprotein interfering with numerous cellular pathways respectively (Dubuisson, 2007;Macdonald & Harris, 2004). A summary of the HCV and predicted GBV-C genome organization and processing is shown in Figure 1.3.

**Glycosylation**

Human and chimpanzee GBV-C E1 and E2 proteins contain fewer predicted N-linked glycosylation sites than HCV E1 and E2 proteins (Goffard & Dubuisson, 2003;Stapleton, 2003) based on the consensus sequence for N-linked glycosylation Asn-X-Ser/Thr, where X is any amino acid except Pro (Fig. 1.4). Treatment of secreted E2 lacking the C-terminal transmembrane domain with N-glycanase reduces the molecular weight of protein, indicating that N-linked oligosaccharides are present (Pilot-Matias et al., 1996).
Fusion peptides

The envelope proteins of flaviviruses are class II fusion proteins which are predominantly non-helical, are not cleaved during biosynthesis, and appear to have fusion peptides within internal loop structures far from the N-terminus (Larios et al., 2005a). Several studies characterized putative fusion peptides within GBV-C E1 and E2 in recent years. For example, interactions of two overlapping peptides from GBV-C E2 with model membranes were studied (E2 (267-284) LLGTEVSEALGGAGLTTGG and E2 (279-298) AGLTGGFYEPLVRRCSELAG) (Larios et al., 2005a; Larios et al., 2005b). Lipid mixing and rabbit red blood cell hemolysis studies identified the E2 (279-298) peptide as more able to disrupt lipid bilayers, suggesting that it is the best candidate internal fusion peptide (Larios et al., 2005a; Larios et al., 2005c). Further physiochemical property analyses with Langmuir phospholipid monolayers demonstrated that the E2 (279-298) peptide modifies the surface behavior of phospholipid monolayers, suggesting that this peptide interacts with lipids and should be a candidate in future studies of GBV-C fusion peptides (Larios et al., 2006). Of note, the E2(279-298) peptide is unstructured in aqueous solution, but forms an amphipathic helical structure in the presence of model membranes, further supporting a role in virus-cell fusion (Mazzini et al., 2007).

Recent studies examining peptide adsorption at the air/water interfaces and their interaction with phospholipid monolayers identified other potential fusion peptides within GBV-C E2: E2 (267-284) LLGTEVSEVLGGAGLTTGG (Casas et al., 2006) and E2 (347-363) VLLYMKLAEARLVPLI (Perez-Lopez et al., 2009a). Two E1 fusion peptides were also proposed as internal fusion peptides based on predicted lipid-interacting structures: E1 (53-66) AGLAVRPKSAAQL (Perez-Lopez et al., 2009b) and E1 (145-162) WKVPFDWVRGVISLTPLL (Sanchez-Martin et al., 2009). Further studies of the candidate peptides in the context of viral particles are needed to determine if these domains are truly involved in fusion events.
**GBV-C Genotypes**

Based on phylogenetic analysis of more than 30 full-length human GBV-C genome sequences, at least five, and possibly six genotypes of GBV-C have been identified (Muerhoff et al., 2006). The global distribution of different genotypes follows distinct patterns consistent with the migration patterns of humans out of Africa, suggesting that GBV-C has co-evolved with its human hosts (Pavesi, 2001; Smith et al., 2000). Despite this presumed ancient history, GBV-C has a surprising lack of genetic diversity between GBV-C variants (< 14%) compared with HCV (>30%) (Fig. 1.2) (Pavesi, 2001). The chimpanzee variant of GBV-C, GBV-C\(_{cpz}\), is significantly more diverse than human GBV-C isolates, further supporting species co-evolution (Adams et al., 1998; Birkenmeyer et al., 1998). The GBV-C\(_{cpz}\) polyprotein shares 83.6% amino acid identity with GBV-C, while human GBV-C isolates are >95% identical (Mohr & Stapleton, 2009; Muerhoff et al., 2005; Pavesi, 2001). GBV-C\(_{cpz}\) is predicted to have the same genome organization and protein functions as human GBV-C and HCV (Birkenmeyer et al., 1998). The phylogenetic relationships between GBV-C genotypes and the human and chimpanzee variants are shown in Figure 1.2.

**Tropism**

Following translation and processing of the polyprotein, the GBV-C NS5B RNA dependent, RNA polymerase transcribes negative strand RNA from which positive strand RNA is subsequently transcribed. Thus, detection of negative strand RNA in cells is indicative of active viral RNA replication. Initial reports suggested that GBV-C negative strand RNA was present in liver tissue (Madejon et al., 1997; Saito et al., 1997). However, comparison of HCV and GBV-C RNA levels in liver and serum of co-infected individuals found that HCV RNA was consistently higher than GBV-C RNA levels in liver tissues, despite higher serum GBV-C RNA levels (Pessoa et al., 1998). Furthermore, the median liver/serum ratio of GBV-C RNA was < 1.0, consistent with serum contamination of liver tissue (Pessoa et al., 1998). Laskus et al. were unable to
demonstrate minus-strand GBV-C RNA in any of ten liver samples tested (Laskus et al., 1997). In a clinical study, GBV-C serum RNA levels did not significantly decrease following liver transplantation, although this routinely occurs with HCV RNA levels (Bert et al., 1999). These data support a non-hepatic source of GBV-C replication.

Like HCV, GBV-C virions are expected to bind to cellular receptors and enter the cell by receptor-mediated endocytosis. Multiple attachment and entry receptors have been identified for HCV, including the low density lipoprotein receptor (LDLR), glycosaminoglycans (GAG), scavenger receptor class B type I (SR-BI), the tetraspanin protein CD81 and claudin-1 (CLDN1) (Moradpour et al., 2007). Because of its high sequence identity with HCV, GBV-C is predicted to utilize multiple receptors as well. Although no studies on the GBV-C life cycle have been performed, the life cycle is predicted to continue with acidification of the endosomal vesicle, conformational change in the virion, and fusion of the viral and cellular membranes, as with HCV.

GBV-C RNA is found in, and is produced by T and B lymphocytes from infected individuals studied ex vivo (George et al., 2006; Mellor et al., 1998). Both CD4+ and CD8+ T cells contain GBV-C RNA, and the most widely-reported cell culture system for in vitro growth of GBV-C utilizes primary human peripheral blood mononuclear cells (PBMCs), suggesting that GBV-C is a lymphotropic virus (Fogeda et al., 1999; Fogeda et al., 2000; Jung et al., 2005; Xiang et al., 2000; Xiang et al., 2001) and explaining the lack of association with hepatitis (Alter, 1997; Stapleton, 2003). Nevertheless, questions remain about the primary site of GBV-C replication in humans. Negative strand GBV-C RNA is either very low or not detected in PBMCs of infected humans (Mellor et al., 1998), and negative sense RNA was found in 3 of 4 bone marrow samples and 2 of 2 spleen samples in one study (Laskus et al., 1998), suggesting that a hematopoietic progenitor cell may be the primary site of infection. Consistent with lymphotropism, GBV-C infection is transmitted by bloodborne, vertical, and sexual routes (Berzsenyi et al., 2005; Bjorkman et al., 2001; Bourlet et al., 1999; Fiordalisi et al., 1997; Frey et al.,
GBV-C – HIV Interactions

Clinical studies

GBV-C research was initially performed by viral hepatitis research groups, and the realization that GBV-C did not cause hepatitis resulted in a marked reduction in research activity. Because Hepatitis B virus and Hepatitis C virus co-infection worsens the clinical course and outcome of HIV-infected patients, researchers hypothesized that the related virus, GBV-C, may impact HIV disease. In 1998, two groups examined the seroprevalences of GBV-C viremia and anti-E2 antibodies, and correlated the results with clinical follow-up data (Heringlake et al., 1998; Toyoda et al., 1998). They reported that HIV-infected individuals who were co-infected with GBV-C survived longer than those without GBV-C (Heringlake et al., 1998; Toyoda et al., 1998), and these results were confirmed in several, though not all, subsequent studies (Bjorkman et al., 2004; Lefrère et al., 1999; Tillmann et al., 2001; Van der Bij et al., 2005; Williams et al., 2004; Xiang et al., 2001; Yeo et al., 2000). It became clear that persistent viremia with GBV-C was important for this association, as some individuals who clear viremia during follow up have a worse prognosis compared to those who had never had GBV-C viremia or in whom E2 antibodies were detected (Bjorkman et al., 2004; Van der Bij et al., 2005; Williams et al., 2004). A meta-analysis of survival in studies of 1,294 HIV-infected individuals in the era prior to effective combination anti-HIV (antiretroviral; ART) therapy found that persistent GBV-C viremia is associated with a significant reduction in the risk of death (relative risk 0.41; 95% confidence interval 0.23 to 0.69) compared to those without GBV-C viremia (Zhang et al., 2006). Although persistent viremia with GBV-C is associated with the highest reduction in mortality, HIV-infected patients with E2 antibodies still have a survival advantage compared to HIV-infected patients with no history of GBV-C infection (Tillmann et al., 2001; Williams et al., 2004).
Studies conducted after widespread use of combination ART, which began in 1996, reported that the association between GBV-C and survival remains (Tillmann et al., 2001). HIV-infected patients treated with ART and coinfected with GBV-C still have a significantly better survival rate compared to GBV-C naïve patients (Tillmann et al., 2001). However, there are no longer significant survival differences between patients with E2 antibodies, who had a more modest survival advantage than GBV-C viremia even before widespread use of ART, and patients with no GBV-C infection (Tillmann et al., 2001).

GBV-C viremia is associated with improved clinical response to antiretroviral therapy (Rodriguez et al., 2003; Souza et al., 2006), and there is an inverse relationship between GBV-C and HIV plasma viral load (Bjorkman et al., 2007; Tillmann & Manns, 2001). Recent studies also found that GBV-C viremia is associated with a block in CD4+ T cell proliferation following IL-2 therapy, suggesting that GBV-C also influences CD4 proliferation in response to IL-2 (Stapleton et al., 2009). Taken together, these findings suggest that GBV-C modulates T cell homeostasis in ways that are beneficial for people infected with HIV.

**Mechanisms of HIV inhibition**

The finding that GBV-C replicates in CD4+ T cells *in vitro* (Xiang et al., 2000) stimulated research on identifying potential interactions between GBV-C and HIV. GBV-C infection of PBMCs inhibits the replication of both CCR5-tropic and CXCR4-tropic HIV isolates in a co-infection model (Jung et al., 2005; Xiang et al., 2001; Xiang et al., 2004; Xiang et al., 2005b). HIV isolates representing all HIV Clades (A-H) and group O virus were inhibited by GBV-C (Jung et al., 2005). Inhibition of HIV did not depend on GBV-C replication, as transfection of either an infectious viral RNA or an RNA containing a deletion rendering GBV-C replication incompetent inhibited HIV (Jung et al., 2005; Xiang et al., 2000). Furthermore, incubation of virus-free supernatants from GBV-C infected PBMCs inhibited HIV *in vitro* (Jung et al., 2005).
The HIV coreceptors, CCR5 and CXCR4, are important players in GBV-C inhibition of HIV. Cell culture studies demonstrated that GBV-C infection alone induces the release of soluble ligands for HIV entry receptors (RANTES, macrophage inflammatory proteins (MIP)-1α and MIP-1β and SDF-1) (Jung et al., 2005; Nattermann et al., 2003; Xiang et al., 2004). HIV inhibition in GBV-C co-infected PBMCs is mediated at least in part by the induction of the chemokine ligands of the HIV entry receptors CCR5 and CXCR4 (Jung et al., 2005; Xiang et al., 2001; Xiang et al., 2004; Xiang et al., 2005b). These chemokine receptors are internalized upon ligand binding and CCR5 surface expression is decreased in GBV-C infected PBMCs (Jung et al., 2005; Nattermann et al., 2003; Xiang et al., 2004). Consistent with this, one clinical study found that HIV-infected patients with GBV-C viremia have significantly reduced CCR5 expression on their CD4+ T cells (Nattermann et al., 2003; Schwarze-Zander et al., 2010). Similarly, another study found that GBV-C co-infection inhibited the increase in CCR5 surface expression density normally associated with HIV immune deficiency and reduced CXCR4 surface density expression on CD4+ T cells in HIV-infected individuals whether or not they received HAART (Schwarze-Zander et al., 2010). Epidemiological studies also found that either decreased CCR5 surface expression or increased serum levels of CCR5 and CXCR4 chemokine ligands (RANTES, MIP-1α, MIP-1β and SDF-1) is associated with prolonged survival in HIV-infected individuals (Rowland-Jones, 1999). These finding indicate that GBV-C specifically alters the surface expression of HIV coreceptors and induces the secretion of soluble ligands for HIV coreceptors, and that this is responsible, at least in part, for HIV inhibition.

GBV-C infection has pleotropic effects on immune system activation: activation of the innate immune system and decreased activation of T cells. The level of interferon activation in dendritic cells was significantly higher in GBV-C – HIV coinfected individuals compared to interferon activation levels in HIV-monoinfected people (as
measured by endogenous levels of IFN-γ and RNA-dependent protein kinase (PKR) mRNA levels) (Lalle et al., 2008). In addition, interferon regulated gene expression correlated with IFN-γ expression, and dendritic cell activation (measured by CD80 expression) correlated with GBV-C viral load, supporting activation of innate immunity by GBV-C infection (Lalle et al., 2008). In contrast, GBV-C viremia reduces the level of T-cell activation in HIV infected individuals (Maidana Giret et al., 2009). Specifically, active GBV-C infection reduces the level of activation markers CCR5 and CD38 on CD8+ T cells and CD38 on CD4+ T cells (Maidana Giret et al., 2009). These multiple effects of GBV-C on immune cells provide a model in which GBV-C may inhibit HIV replication through the modulation of immune system activation, counteracting chronic immune activation which is characteristic of progressive HIV disease. The mechanisms of HIV inhibition described to date are summarized in Table 1.1.

NS5A inhibition of HIV

One of the GBV-C nonstructural proteins, NS5A, inhibits HIV replication in vitro. Expression of NS5A downregulates CXCR4 and induces the expression of SDF-1 (the CXCR4 ligand) in a CD4+ T cell line, rendering the cells nearly completely resistant to HIV infection (Chang et al., 2007; Xiang et al., 2006). Expression of a 16 amino acid domain within NS5A is sufficient to inhibit HIV replication, and the addition of a synthetic peptide containing this domain resulted in dose-dependent HIV inhibition (Xiang et al., 2008), suggesting therapeutic potential for this small molecule. During GBV-C infection, this nonstructural protein is only expressed in cells actively infected with GBV-C, and the protein is anchored in the endoplasmic reticulum; thus, any effect on neighboring cells would require the induction of soluble factors like chemokines to result in a widespread or potent effect (Xiang et al., 2000).
E2 inhibition of HIV

GBV-C E2 is one of the viral factors specifically involved in inhibition of HIV replication. Incubation of a recombinant E2 protein or a recombinant E2-Fc fusion protein with PBMCs prior to HIV infection results in a decrease in HIV replication when compared to untreated HIV-infected PBMCs (Jung et al., 2007). Transient transfection of T7-transcribed RNA encoding E1 and E2 in CD4+ T cells prior to HIV infection also results in HIV inhibition (Jung et al., 2007). Expression of the first part of E1 protein alone did not inhibit HIV, indicating E2 is sufficient to inhibit HIV replication (Jung et al., 2007).

Transduction of X4-enveloped HIV pseudoparticles is also inhibited in a CCR5 and CXCR4-expressing human osteosarcoma cell line with increasing E2 concentrations in a dose-dependent manner (Mohr et al., 2008). The recombinant E2-Fc fusion protein inhibits the single cycle infection of both X4- and R5-enveloped HIV pseudoparticles by 50% in PBMCs compared with Fc controls (Jung et al., 2007). Neutralization of the recombinant E2-Fc protein with anti-E2 antibodies abrogates HIV inhibition indicating specificity (Jung et al., 2007). In contrast, the E2-Fc fusion protein does not inhibit entry of VSV-G-pseudotyped particles, indicating that the E2 inhibition of HIV replication appears to target the entry steps requiring HIV envelope proteins (Jung et al., 2007).

During HIV replication, the env gene gp160 is translated as a polyprotein and is cleaved by a cellular protease into two proteins: gp120 attaches to cellular receptors and gp41 contains the fusion protein. Recent work localizes the step of HIV replication inhibition even further than an early entry step. Single cycle infections of T cell lines expressing E2 and cell-virus fusion experiments on HEK 293T cells indicate that GBV-C E2 inhibits HIV replication soon after the gp120/CD4 dependent entry and prior to reverse transcription (Hanel et al., 2009).
GBV-C E2-mediated HIV inhibition is relevant because the average titer of GBV-C particles in plasma is roughly 1000-fold higher than HIV titers, and GBV-C particles are predicted to contain multiple copies of E1-E2 heterodimers on their envelope. Because of the high titers of GBV-C in the serum, lymphocyte and HIV interactions with E2 are predicted to occur often. With the high titer of E2 in infected humans, GBV-C is likely to induce a chemokine milieu inhibitory to HIV infection and to bind to multiple uninfected cells (without entering) to inhibit HIV entry.

**E2 peptide inhibition of HIV**

Studies of the interaction of putative GBV-C fusion peptides with HIV-1 gp41 suggest that a candidate E2 fusion peptide (269-286 GTEVSEALGGAGLTGGFY) inhibits HIV fusion (Herrera et al., 2009). Vesicle leakage assays conducted with synthetic overlapping peptides found that the 269-286 E2 peptide inhibited membrane leakage induced by the HIV-1 gp41 fusion peptide by at least 55% compared to peptides synthesized from adjacent E2 sequences (Herrera et al., 2009). The inhibition was specific for the HIV-1 gp41 fusion peptide, as the E2 peptide does not inhibit the membrane lytic activity of melittin, a commonly used control membrane fusion protein. This E2 peptide also inhibits HIV-1 gp41 fusion peptide-induced lipid mixing and binds to the HIV-1 fusion peptide in a 1:1 ratio in an energetically favorable manner (Herrera et al., 2009). When both peptides are mixed together in a membrane-mimicking environment, their conformation changes from $\alpha$-helices to $\beta$-turns and random structures indicating that their interaction leads to conformational changes of both peptides (Herrera et al., 2009). NMR spectra of the mixed peptides indicate that they interact and form high-molecular-weight aggregates. The authors suggest that the binding of the E2 peptide may prevent oligomerization of the HIV-1 fusion peptide upon membrane binding. This would inhibit the membrane destabilization effect necessary for HIV-1 membrane fusion.
In addition to putative E2 fusion peptide inhibition of HIV fusion, multiple non-fusion peptides may have some HIV-inhibitory capability as well (Herrera et al., 2010). The E2 (133-150) SDRDTVVELSEWGVPCAT peptide (named P45) inhibits gp41-induced fusion of HeLa cells expressing the HIV env and TZM-bl cells expressing the HIV receptor and coreceptors (Herrera et al., 2010). In addition, the P11, P19, P20, P21, P34, P45, P46, P47, P109 and P124 E2 peptides inhibited HIV pseudoparticle (R5-tropic BaL envelope) transduction of TZM-bl cells, with IC\textsubscript{50} values (concentration (µM) of a peptide causing 50% inhibition of infection) ranging from 94.7 µM to 529.1 µM (Herrera et al., 2010). These IC\textsubscript{50} values are much higher than the IC\textsubscript{50} value of 0.021 µM for T20, a well-characterized HIV-1 fusion inhibitor and human HIV antiviral drug known as Fuzeon (Herrera et al., 2010). The fact that multiple peptides spanning almost the entire region of E2 inhibit HIV fusion and infection to some degree raises the question of how these peptides are acting, and whether there is specificity to this inhibition. One could argue that these \textit{in vitro} studies with high concentrations of purified E2 peptides may not completely reflect the main method of HIV replication inhibition \textit{in vivo} because E2 fusion peptides (and other domains of E2 represented by some, but not all, of the E2 peptides) are not predicted to be exposed when in the conformation of the E1/E2 heterodimer on the surface of GBV-C particles outside of an endosome. Although the studies of E2 peptides have only been performed in a manipulated system, the inhibition of HIV by multiple E2 domains is interesting and requires further study in more relevant systems.

\textbf{Overview of Thesis Chapters}

\textit{Chapter II: GBV-C E2 Inhibition of HIV and Cellular Interactions}

Expression of the one of the GBV-C structural proteins, E2, in CD4\textsuperscript{+} T cells or the addition of recombinant E2 protein to cells results in the inhibition of HIV replication (Jung et al., 2007; Mohr et al., 2008; Mohr & Stapleton, 2009; Nattermann et al., 2003). Incubation of PBMCs with recombinant E2 decreases the entry of HIV-enveloped
retroviral particles and not VSV-G enveloped retroviral particles, suggesting that E2 inhibits HIV infection at the step of HIV entry because the HIV envelope proteins are involved (Jung et al., 2007). Since E2 is present on virions, and GBV-C titers are typically between $10^7$ and $10^8$ genome equivalents per ml in plasma in infected individuals (Bert et al., 1999; Tillmann et al., 2001), the GBV-C E2 protein has ample access to HIV particles and infected cells. Furthermore, E2 is thought to mediate GBV-C binding to permissive cells, and thus it may be an important determinant of viral tropism (Kaufman et al., 2007; McLinden et al., 2006; Nattermann et al., 2003).

Because GBV-C E2 inhibition of HIV entry is dependent on the presence of HIV envelope proteins, which interact with surface receptors and membranes to initiate entry, it is predicted that GBV-C E2 inhibition of HIV entry may occur on the surface of the cell adjacent to cellular proteins and membranes. Recent studies reported that multiple peptides from within E2 interact with HIV gp41 and interfere with its interaction with vesicles, which results in the inhibition HIV infection in CEM-174 and TZM-bl cells (Herrera et al., 2010). The combination of epidemiological studies in HIV-infected individuals and in vitro studies indicate that GBV-C E2 is an HIV-inhibitory protein and its interaction with cellular surfaces must be studied to elucidate its role in HIV inhibition. These studies will be described in further detail in Chapter II.

Chapter III: GBV-C Anti-E2 Antibody Inhibition of HIV

The development of an HIV vaccine has proved to be a difficult task for many reasons. Some of these reasons include the extensive number of viral subtypes and sequence diversity; hidden conserved antibody targets on the outer envelope protein; and viral escape from humoral immune responses (Kim et al., 2010). In addition to eliciting an immune response which includes T cells that recognize and inactivate diverse HIV strains, successful HIV vaccines will also require a humoral response which includes broadly neutralizing antibodies. One of the largest stumbling blocks in the field of HIV
vaccine development is the failure to develop broadly neutralizing antibodies following immunization with HIV envelope proteins (Kim et al., 2010).

Although the presence of E2 antibodies is protective in HIV-infected patients, no groups have examined how E2 antibodies may inhibit HIV. In fact, it was hypothesized that the survival advantage of the GBV-C E2 antibody-positive patients may be due to the previous GBV-C infection, which increases CD4+ cell counts (Tillmann et al., 2001). However, recent findings from J. Xiang support the hypothesis that GBV-C E2 antibodies inhibit HIV infection directly in vitro (Mohr et al., 2010). GBV-C E2 antibody positive healthy blood donor sera and purified IgG neutralize both CCR5- and CXCR4-tropic HIV isolates. In addition, HIV neutralizing antibodies were elicited by immunization of mice and rabbits with recombinant GBV-C E2 protein (expressed in CHO cells) (McLinden et al., 2006) or a 17 amino acid E2 peptide recognized by mAb M6 (McLinden et al., 2006). Even though the rabbit anti-E2 antibodies do not react with denatured HIV-1 proteins in an immunoblot, they precipitate infectious HIV-1 virions prepared in HOS cells, suggesting that the interaction is conformation dependent.

Identification of the epitope on HIV-1 virions to which the E2 antibody binds is necessary to elucidate the mechanisms of HIV inhibition. Pseudotyped particles with different viral envelope proteins, such as VSV-G and MLV env, can be utilized to determine whether the E2 antibody epitope is HIV gp120, gp41, or another cellular protein carried on HIV-1 particles. In addition, defective VSV particles pseudotyped with the HIV-1 envelope can be used to ascertain whether the HIV-1 envelope is required for E2 antibody HIV neutralization. We utilized the tools of pseudotyped particles and found that E2 antibodies recognize a cellular antigen on retroviral particles and neutralize HIV-1 pseudotyped retroviral particles but not defective VSV particles. In addition, we found that the E2 antibody cellular epitope is exposed only on permeabilized cells, and not on intact cells, suggesting that the E2 antibody epitope is exposed upon viral budding.
and is exposed on the surface of the virus. These findings will be discussed in Chapter III.

**Chapter IV: Animal Models of GBV-C Infection**

GBV-C persistently infects humans, and chimpanzees support persistent infection with human GBV-C upon experimental infection (Bukh *et al.*, 1998). A chimpanzee variant of GBV-C, GBV-C<sub>cpz</sub>, has been detected in captive and noncaptive chimpanzees and not in humans or macaques. While the epidemiology of human GBV-C has been well studied, it is unknown why some individuals support persistent infection for decades and most individuals clear infection after a few years (Alter, 1997; Theodore & Lemon, 1997). Although GBV-C replicates in PBMCs, the level of replication is low, and more robust systems are needed to study GBV-C persistence in humans. These robust systems are also needed to study HIV and GBV-C interactions and to determine whether the protective effect of GBV-C infection can be elicited *in vivo*, as part of an HIV vaccine. Only one study has pursued the chimpanzee as an animal model in which to study GBV-C infection, and this study examined only a few animals (Bukh *et al.*, 1998). No animal models currently exist for studying GBV-C and HIV interactions. To this end, we studied the natural history of GBV-C<sub>cpz</sub> in chimpanzees. With this information, the chimpanzee may be developed as a model in which to study GBV-C persistence and the role of GBV-C vaccines in HIV infection.

We examined the prevalence of GBV-C<sub>cpz</sub> viremia and anti-E2 antibodies in a captive chimpanzee population. Analyses of these isolates demonstrated that the sequences share a high level of sequence identity with the only near full-length sequence of GBV-C<sub>cpz</sub> available. GBV-C<sub>cpz</sub> infection persisted for up to 19 years in one chimpanzee. Chimpanzee subspecies *troglodyte* GBV-C isolates and published subspecies *verus* GBV-C isolates shared a high degree of sequence identity, suggesting that GBV-C in chimpanzees should be identified with a chimpanzee designation (GBV-C<sub>cpz</sub>) rather than the currently used troglodyte designation (GBV-C<sub>trog</sub>). The prevalence
and natural history of GBV-C\textsubscript{cpz} appears to be similar to human GBV-C infection, indicating that the chimpanzee could serve as an animal model in which to study SHIV-GBV-C co-infection. The natural history of GBV-C\textsubscript{cpz} will be discussed in Chapter IV.

*Chapter V: General Discussion*

These chapters explore the multiple facets of the application of GBV-C’s HIV inhibition for HIV therapeutics and vaccine development. Both GBV-C E2 and anti-E2 antibodies directly inhibit HIV and may be used as part of an HIV vaccine, and the study of GBV-C\textsubscript{cpz} is the first step in testing GBV-C as part of an HIV vaccine in a chimpanzee model. Understanding the mechanism of how GBV-C E2 interacts with cellular surfaces, and thus how it inhibits HIV entry, is important to understanding how natural GBV-C infections prolong the survival of HIV co-infected individuals and how to elicit this protection artificially. Although GBV-C viremia provides the highest level of protection in an HIV-infected individual, anti-GBV-C E2 antibodies also inhibit HIV, and this protection may be easier to elicit via vaccine methods. Anti-GBV-C E2 antibodies are elicited by immunization with recombinant GBV-C E2 protein and neutralize HIV infection *in vitro*. Determining whether anti-E2 antibodies can be elicited by immunization and protect against SHIV challenge in primates is the next step in this endeavor. Because chimpanzees support natural infection with GBV-C\textsubscript{cpz} and infection is found in 3.0\% of chimpanzees, the chimpanzee would be a good model in which to study GBV-C persistence and the efficacy of HIV vaccines. However, determining the GBV-C\textsubscript{cpz} viremia and antibody status of the animals participating in GBV-C infection and HIV vaccine trials is important to take into account the influence of GBV-C on HIV disease progression.
Figure 1.1 GBV-C prevalence rates in blood donors from various regions of the world. GBV-C viremia prevalence among 11,391 blood donors was summarized from 50 studies. Viremia was detected by RT-PCR, and studies only included donors who passed screening procedures with the exception of one Scandinavian study that included donors with normal and high ALT (n = 393 high ALT, n = 184 with normal ALT; the GBV-C prevalence rate was similar in both groups). In developed countries, GBV-C RNA prevalence in blood donors ranged from 0.5 to 5%, compared to 5–18.9% in developing countries. *Caribbean (West Indies).
Figure 1.2. Phylogenetic relationships of the RNA-dependent, RNA polymerase sequences of several members of the family Flaviviridae. Three genera (flavivirus, pestivirus and hepacivirus) and the unassigned GB viruses are shown. Representative isolates from the six hepatitis C virus (HCV), five GBV-C, chimpanzee GBV-C$^{cpz}$ variant, and four GBV-A genotypes are depicted. The four GBV-A genotypes were identified in Sanguinus nigrocallis (GBV-A), Sanguinus labiatus (GBV-A$^{lab}$), Aotus Trivirgatus (GBV-A$^{tr}$), and a callithrix hybrid (jacchus-penicillata cross) (GBV-A$^{mx}$). BVDV, bovine viral diarrhea virus; YFV, yellow fever virus (17D vaccine strain); TBEV, tick-borne encephalitis virus; DV, dengue virus (serotype 2) and JEV, Japanese encephalitis virus. 0.2, distance representing 0.2 amino acids substitutions per position.
Figure 1.3. Genome organization and proteolytic processing of hepatitis C virus (HCV) and GB virus C (GBV-C). HCV and GBV-C both contain 5' nontranslated regions containing internal ribosomal entry sites directing translation of polyproteins. The polyproteins are post-translationally processed into structural proteins [core (C), envelope glycoproteins (E1 and E2)] and an ion channel P7 (P5.6 for GBV-C) by cellular signal peptidases. Nonstructural proteins (NS) 2 and 3 are cleaved by NS2 protease, while the remaining cleavage sites are processed by the serine protease domain within NS3, in conjunction with the NS4A cofactor.
Figure 1.4. Comparison of hepatitis C virus (HCV), GB virus C (GBV-C; Accession number AB003291) and GB virus C chimpanzee variant (GBV-Ccpz; Accession number AF070476) structural proteins and glycosylation sites. The relative size and predicted N-linked glycosylation sites on core and envelope glycoproteins (E1 and E2) proteins. The GBV-C core protein is depicted as residing at the N-terminus of the polyprotein. Grey represents the GBV-C core protein if the AUG at position 462 serves as initiation codon, while blue represents initiation at the AUG at 555.
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<th>GBV-C infection in vivo</th>
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<td><strong>Table 1.1. Summary of Mechanisms of HIV Inhibition by GBV-C.</strong></td>
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<td>CCR5 and/or CXCR4 surface expression is decreased on CD4⁺ T cells in GBV-C infected individuals</td>
<td>GBV-C infection reduces the level of activation markers CCR5 and CD38 on CD8⁺ T cells and CD38 on CD4⁺ T cells</td>
<td>Decreased CCR5 surface expression and increased serum levels of RANTES, MIP1α, MIP1β and SDF-1 are associated with prolonged survival in HIV co-infected individuals</td>
<td>Increased interferon activation in dendritic cells in GBV-C - HIV co-infected individuals</td>
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<td>(Nattermann et al., 2003; Schwarze-Zander et al., 2010)</td>
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<td>GBV-C infection of PBMCs inhibits replication of CCR5⁻ and CXCR4-tropic HIV isolates</td>
<td>CCR5 surface expression is decreased in GBV-C infected PBMCs</td>
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<td>GBV-C infection in PBMCs increased secretion of soluble ligands for HIV entry receptors (RANTES, MIP1α, MIP1β, SDF-1).</td>
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<td>Expression of NS5A inhibits HIV replication, downregulates CXCR4 and induces expression of SDF-1 in a CD4⁺ T cell line</td>
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<td>16 amino acid domain in NS5A inhibits HIV infection</td>
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<td>E2-Fc fusion protein inhibits HIV replication and HIV env pseudotyped particle transduction, but not VSV-G pseudotyped particle transduction, in PBMCs</td>
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<td>E2 fusion peptide (269-286) inhibits HIV gp41 fusion, as determined by vesicle leakage assays, lipid mixing assays, NMR spectral analyses of mixed peptides</td>
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<td>E2 peptide (133-150) inhibits gp41-induced fusion in a cell-based fusion assay; multiple other E2 peptides inhibit HIV env pseudotyped particle transduction of TZM-bl cells</td>
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CHAPTER II. GBV-C E2 INHIBITION OF HIV AND CELLULAR INTERACTIONS

Introduction

Both HIV and GBV-C replicate in CD4+ T cells (Xiang et al., 2000) and co-infection of HIV and GBV-C in PBMCs in vitro results in the inhibition of HIV replication (Jung et al., 2005; Xiang et al., 2001; Xiang et al., 2004; Xiang et al., 2005b). Numerous HIV-inhibitory mechanisms induced by GBV-C have been described, including the release of soluble ligands for HIV entry receptors [RANTES, macrophage inflammatory proteins (MIP)-1α and MIP-1β and SDF-1] (Jung et al., 2005; Nattermann et al., 2003; Xiang et al., 2004), downregulation of CCR5 surface expression on PBMCs (Jung et al., 2005; Nattermann et al., 2003; Xiang et al., 2004), and activation of innate immunity through higher levels of interferon activation in dendritic cells (Lalle et al., 2008).

Two GBV-C proteins with HIV-inhibitory activity have been studied; these are the nonstructural protein 5A (NS5A) and envelope glycoprotein E2. GBV-C NS5A induces the release of the chemokine SDF-1 and decreases surface expression of the HIV coreceptor CXCR4, which contributes to the inhibition of HIV in an NS5A-expressing CD4+ T cell line (Xiang et al., 2006). Expression of the other HIV-inhibitory GBV-C protein, E2, in CD4+ T cells or the addition of recombinant E2 protein to cells results in the inhibition of HIV replication (Jung et al., 2007; Mohr et al., 2008; Mohr & Stapleton, 2009; Nattermann et al., 2003). Determining the structure of E2 and how E2 interacts with cells is critical to understanding its role in HIV inhibition.
Although the physical structure of GBV-C E2 has not been elucidated, studies have examined the structure of GBV-C E2 by measuring the ability of a panel of monoclonal antibodies (mAbs) to bind to recombinant E2. In these studies, the antigenic structure of GBV-C E2 was investigated using mAbs generated by DNA immunization (Schmolke et al., 1998). These mAbs are comprised of four specificity groups that, based on competition studies, recognize overlapping epitopes (McLinden et al., 2006). The combination of all four groups of mAbs completely inhibits the binding of human polyclonal anti-E2 antibodies to E2, indicating that this antigenic site is immunodominant during natural infection (McLinden et al., 2006). Three of the four groups of mAbs detect recombinant E2 (secreted by CHO cells) bound to cells, suggesting that the epitopes for the other antibodies may be masked with E2 interactions with cells, or masked by conformation changes upon binding (McLinden et al., 2006).

M6 is the only known mAb to recognize a linear epitope on E2 and all others described to date recognize conformation epitopes (McLinden et al., 2006; Schmolke et al., 1998). Human anti-E2 antibodies elicited during natural infection do not react with denatured E2, indicating that the M6 linear epitope is cryptic and not displayed during natural infection. The M6 epitope (GGAGLTGGFYEPLVRRC) is located from Gly481 to Cys497 of the E2 sequence (McLinden et al., 2006). Sequential deletion analysis at the N- and C-terminus of the linear M6 peptide demonstrates that the M6 mAb recognizes a core of six amino acids (McLinden et al., 2006). M6 only binds the peptide if there are at least four amino acids added at either the C- or N-termini of the core, suggesting that there is a size and sequence requirement, and possible conformational requirement, for optimal interaction with the M6 antibody (McLinden et al., 2006).
Recombinant monomeric GBV-C E2 binds at low levels to a variety of cell types, including Molt4, HeLa, Jurkat, HEK 293 and murine 3T3 cells, but not Chinese Hamster Ovary (CHO) cells, as detected by the M5 monoclonal antibody (McLinden et al., 2006). Thus, multiple cell types may contain a binding/attachment receptor for E2 (Mohr et al., 2008). Although E2 can bind to many cell types, viral entry may not be possible without a different array of receptor(s) for entry, as seen with the closely related virus HCV (Dubuisson et al., 2008).

Transient and inefficient GBV-C replication has been documented in several cell lines including T cells, B cells and hepatocyte cells (George et al., 2003a; Mellor et al., 1998; Tucker et al., 2000). The most efficient system for growing GBV-C is in primary human peripheral blood mononuclear cells (PBMCs), although even in these cells replication can be documented only in a small percentage of cells (Fogeda et al., 1999; George et al., 2003b; Xiang et al., 2000). This indicates that only a subset of PBMCs are permissive for infection or that there are restriction factors preventing GBV-C replication. This may be because only a small subset have the necessary GBV-C E2 cellular receptors to allow binding and entry, a population of cells express the E2 receptor(s) transiently, such as during activation or differentiation, or that there are downstream factors restricting GBV-C replication. GBV-C E2 binding to PBMCs has not been examined.

The incubation of recombinant E2 protein with PBMCs and human osteosarcoma cells expressing CD4 and CCR5 inhibits HIV enveloped pseudoparticle transduction (Fig. 2.1) (Jung et al., 2007; Mohr et al., 2008; Mohr & Stapleton, 2009; Nattermann et al., 2003). Incubation of PBMCs with recombinant E2 at 4°C decreases the entry of
HIV-enveloped retroviral particles, but not VSV-G-enveloped retroviral particles, suggesting that E2 inhibits HIV infection at the step of HIV entry because the HIV envelope proteins are involved (Jung et al., 2007). If HIV envelope proteins must be present for E2 to inhibit HIV, then the HIV-inhibitory step may occur on the cell surface adjacent to HIV cellular receptors and membranes. Multiple peptides from within E2 were found to interact with HIV gp41 and interfere with its interaction with vesicles, and these peptides inhibit HIV infection in CEM-174 and TZM-bl cells (Herrera et al., 2010). The combination of epidemiological studies in HIV-infected individuals and in vitro studies indicate that GBV-C E2 is an HIV-inhibitory protein. Determining the interaction between GBV-C E2 and host cell surfaces may elucidate its role in HIV inhibition.

Preliminary GBV-C E2 receptor studies identified CD81, the HCV E2 attachment receptor, as the GBV-C E2 receptor (Nattermann et al., 2003). The interaction of GBV-C E2 with CD81 was also proposed to elicit HIV inhibitory effects by decreasing the expression of the HIV co-receptor CCR5 (Nattermann et al., 2003). However, follow-up studies did not confirm the identity of CD81 as the receptor for GBV-C E2. GBV-C E2 did not bind to Daudi cells expressing high levels of CD81, and E2 binding was not competitively blocked by soluble human CD81 (Kaufman et al., 2007). The GBV-C E2 cellular receptor has not yet been identified; however, engagement of this receptor is implicated in CCR5 downregulation and HIV inhibition.

Because E2 appears to inhibit HIV infection during HIV entry steps, which occur adjacent to the cell membrane on the surface of the CD4+ cell, examining how GBV-C E2 interacts with the cell surface and identifying cellular binding receptors is important to elucidating how E2 inhibits HIV infection in vivo. In this chapter, we examine GBV-C
E2 binding to human PBMCs and murine bone marrow, spleen, placenta, thymus, and fetal liver cells to identify the subset of cells which bind to GBV-C E2. We also develop GBV-C E1/E2 enveloped lentiviral vectors as a novel approach to studying GBV-C entry. In addition, we screen a panel of transformed cell lines for their ability to bind to recombinant GBV-C E2 and correlate the level of E2 binding with cDNA expression to identify candidate binding receptors. Finally, we characterize the trypsin sensitivities of E2 binding, and further characterize the binding of the anti-E2 monoclonal antibodies to a cell line which binds to E2 at higher levels than the cell lines used in earlier studies.

**Methods**

*Cells and antibodies:* PBMCs were isolated from blood obtained from healthy donors and were maintained in RPMI supplemented with 10% fetal calf serum and antibiotics. NCI-60 cancer cell lines were obtained from the NCI DTP and maintained in RPMI supplemented with 10% fetal calf serum and antibiotics. 293T and murine 3T3 cells were maintained in DMEM supplemented with 10% fetal calf serum and antibiotics. HOS cells were obtained from the NIH Reagent program and contain GFP under the control of HIV-1 LTR (catalog # 3942). Mouse bone marrow and spleen cells were harvested from a wild type C57BL/6 mouse (for Fig. 2.4) and bone marrow, placental, fetal liver and thymic cells were harvested from a Balb/c mouse (for Fig. 2.5). RBCs were lysed with ACK lysis buffer (Quality Biologicals Inc.).

Rabbit polyclonal anti-GBV-C E2 peptide antibody 7066 was generated upon immunization with KLH-conjugated E2 peptide as previously described (Mohr *et al.*, 2010). Rabbit polyclonal anti-GBV-C E2 protein antibody was generated upon immunization with CHO cell supernatants containing GBV-C E2 protein as previously
described (Mohr et al., 2010). Murine anti-GBV-C E2 monoclonal antibodies M5 and M6 (Roche Laboratories, Penzberg, Germany) were generated by DNA immunization and provided by Dr. Alfred Engel. M5 recognizes a conformation-dependent epitope while M6 recognizes a linear epitope on GBV-C E2 (McLinden et al., 2006).

Rabbit isotype control antibodies were purchased from Sigma, Inc. St. Louis, MO and mouse isotype control antibodies were purchased from BD Pharmingen, San Jose, CA. PacBlue-labeled anti-CD3, APC-Cy7-labeled anti-CD14, PerCP-Cy5.5-labeled anti-CD19, Alexa Fluor 700-labeled anti-CD56, APC-labeled anti-CD34, and FITC-labeled anti-CD83 antibodies were purchased from BD Pharmingen and used at the concentrations recommended by the manufacturer. PE-labeled anti-rabbit IgG was purchased from Molecular Probes, Carlsbad CA, and PE-labeled anti-mouse IgG2a and IgG2b antibodies were purchased from BD Pharmingen.

**PBMC stimulation:** Wells were coated with 500 ng/ml anti-CD3 antibody (clone IKT3, eBioscience) diluted in PBS and incubated overnight at 4°C. The anti-CD3 antibodies were removed, and PBMCs resuspended in RPMI supplemented with fetal calf serum, antibiotics, and 0.5 µg/µl anti-CD28 antibody (BD Pharmingen) were added to each well and incubated at 37°C for 24 or 48 hours.

**GBV-C E2 binding assay:** GBV-C E2 protein truncated to remove the C-terminal transmembrane domain (nt 1167 to 2162 based on the infectious clone isolate, GenBank number AF121950) (Xiang et al., 2000) was expressed in CHO cells as described (McLinden et al., 2006). For the binding assay, adherent cells were scraped for removal and pelleted, and suspension cells were pelleted. Cells were resuspended in 80 µg/ml GBV-C E2 (or other concentration as noted) diluted in PBSA, CHO cell supernatants
lacking E2 with the same total protein concentration diluted in PBSA, or in PBSA alone and incubated at 4°C for 1 hour. Cells were washed with PBS and incubated with 20 µg/ml 7066 antibody, 8 µg/ml rabbit anti-E2 protein antibody, 10 µg/ml mAb M6 antibody or 0.4 µg/ml mAb M5 antibody diluted in PBSA at 4°C for 1 hour. Cells were washed and incubated with PE-labeled goat anti-rabbit IgG antibody (Molecular Probes), goat anti-mouse IgG2a (Southern Biotechnologies), or goat anti-mouse IgG2b antibody (Southern Biotechnologies) at 4°C for 30 minutes. For the PBMC E2 binding studies, fluorescently labeled anti-CD3, anti-CD14, anti-CD19, anti-CD34, anti-CD56 and anti-CD83 antibodies were incubated with the PBMCs in the step when the PE-labeled antibodies which detect anti-E2 antibody binding were added. PBMC viability was measured with the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen) and the cells were fixed with paraformaldehyde. Fluorescence was measured by flow cytometry on a BD FACScan or BD LSR (University of Iowa Flow Cytometry Core) and analyzed by FlowJo (Tree Star, Ashland, OR, USA). The percent of cells binding to E2 was calculated by subtracting the background binding fluorescence of the antibody from the fluorescence of the cells treated with E2 and antibody. All incubation steps were performed at 21°C for the temperature dependence studies. All E2 binding assays were performed at least twice.

Pseudotyped virus production: 293T cells were co-transfected with pNL4-3.Luc.R-E- (luciferase reporter inserted into pNL4-3 nef gene) and a plasmid that expresses the envelope glycoproteins of either VSV (VSV-G), HIV (pHXB2-env), MLV (Env), GBV-C E1-E2 (nt 555-2479), or no viral envelope protein using CaCl₂ as described (Sinn et al., 2003). Particles were collected 72 hours post-transfection, filtered
with a 0.45 µm filter and concentrated by centrifugation at 12,000 rpm for 20 hours at 4°C. p24 concentration was measured by p24 assay (Zeptometrix).

**GBV-C E2 inhibition assay:** Adherent human osteosarcoma cells (HOS) expressing CD4 and CCR5 were incubated with either CHO cell supernatants containing E2 or lacking E2 at 4°C for 4 hours. The amount of CHO cell supernatants containing E2 was used at 20 µg/ml and 30 µg/ml E2 (based on ELISA assay as previously described) (McLinden *et al.*, 2006). The amount of CHO cell supernatant lacking E2 was normalized to the total protein concentration in the CHO cell supernatant containing E2. The cells were washed and incubated with HIV (HXB2) enveloped retroviral particles, maintaining the E2 or CHO cell supernatant concentration throughout the incubation. Pseudoparticle transduction was measured by luciferase activity (Brightglo; Promega) 3 days post-transduction.

**Pseudoparticle transduction:** Transformed cell lines were plated overnight in RPMI supplemented with fetal calf serum and antibiotics. PBMCs were incubated with RPMI supplemented with fetal calf serum, antibiotics, IL-2 (Zeptometrix) and phytohemagglutinin (PHA; Invitrogen). Pseudoparticles were incubated with cells and transduction was measured by luciferase assay (Promega BrightGlo) 3 days post-transfection. Particle input levels were equalized by p24 concentration. The fold increase of GBV-C particle entry compared to non-enveloped particle entry was calculated by dividing the RLU of the GBV-C enveloped particle-transduced cells by the RLU of the non-enveloped particle-transduced cells.
Statistics: Statistics were performed using SigmaStat software V3.11 (Jandel Scientific, Chicago, IL). T-tests were used for direct comparisons for individual data points.

Results

GBV-C E2 inhibits HIV pseudoparticle transduction

Earlier studies by S. Jung demonstrated that a recombinant E2-Fc fusion protein inhibits HIV infection and HIV pseudoparticle transduction in PBMCs (Jung et al., 2007). We sought to duplicate these results in HOS cells expressing CD4 and CCR5. We plated HOS cells 24h prior to incubation with 20 µg/ml or 30 µg/ml GBV-C E2 at 4°C for 4 hours. The recombinant E2 protein is secreted into the supernatant of CHO cells; an equivalent total protein concentration of CHO cell supernatants lacking E2 was used as a control. HIV enveloped pseudoparticles were added and luciferase activity was measured 3 days post-transduction. GBV-C E2 significantly inhibited HIV pseudoparticle transduction compared to the CHO cell supernatants lacking E2 (Fig. 2.1), confirming the findings by S. Jung (Jung et al., 2007; Mohr & Stapleton, 2009). Both CHO cell supernatants containing and lacking E2 inhibited HIV pseudoparticle entry, although the HIV pseudoparticle transduction was inhibited significantly more by supernatants containing E2 compared to supernatants lacking E2.

GBV-C E2 binds to a small subset of PBMCs

GBV-C replicates in primary B and T lymphocytes (George et al., 2006). Because the level of virus produced during in vitro GBV-C infections is low, it is predicted that GBV-C replicates in a small subset of PBMCs and that only this subset bears GBV-C cellular receptors. To determine whether recombinant GBV-C E2 interacts
with the surfaces of PBMCs and to determine which PBMC subsets (CD3+ T cells, CD19+ B cells, CD14+ monocytes, CD34+ progenitor cells, CD83+ dendritic cells, CD56+ natural killer cells) bear a cellular receptor for GBV-C, GBV-C E2 binding assays were performed with PBMCs from multiple healthy blood donors. Briefly, freshly isolated PBMCs were incubated with GBV-C E2 (or no E2 for the control – the background binding of the antibody alone was measured as the control) at 4°C, washed, incubated with the rabbit anti-E2 peptide antibody 7066, washed, and incubated with a cocktail of fluorescently labeled antibodies, including PE-labeled anti-rabbit IgG and anti-CD3, -19, -14, -34, -83, -56 antibodies. GBV-C E2 bound to a small, distinct subset of PBMCs isolated from a 27 year old donor and this small subset was positive for T cell CD3, B cell CD19 and dendritic cell CD83 markers (Fig. 2.2). The PBMC subset which bound to E2 was not positive for the hematopoietic progenitor CD34 marker, the NK cell CD56 marker or the monocyte CD14 marker. On repeat examination, GBV-C E2 did not bind to PBMCs from a different, middle-aged, healthy blood donor. Because the first donor had received an influenza vaccination 11 days prior to the blood donation, the activation status of the PBMCs was hypothesized to be important.

To determine whether GBV-C E2 binds to activated PBMCs, GBV-C E2 binding assays were performed on PBMCs isolated from a middle-aged donor immediately after isolation and after 1-2 days of stimulation with anti-CD3 and anti-CD28 antibodies (to activate T cells through the T cell receptor) (Fig. 2.3). GBV-C E2 did not bind to either the naïve PBMCs or activated PBMCs, suggesting that even though GBV-C replicates in B and T lymphocytes, the PBMC subset to which it binds is extremely rare and may be influenced by in vivo activation status. Alternatively, the surface expression level of the
cellular receptors may be low or the affinity between E2 and its cellular receptor may be low.

*GBV-C E2 does not bind to primary murine cells*

Early studies of GBV-C replication sites found that spleen and bone marrow biopsies were positive for both negative- and positive strand GBV-C RNA, indicating that GBV-C replicates in these sites in vivo (Tucker *et al.*, 2000). Together with the finding that GBV-C replication in ex vivo PBMCs is low, this suggests that GBV-C may replicate in hematopoietic progenitor cells, and thus, these cells may bear cellular receptors for GBV-C. To determine whether hematopoietic progenitor cells bear cellular receptors for GBV-C E2, E2 binding assays with multiple anti-E2 antibodies (M5, M6, 7066) were performed on adult murine spleen and bone marrow cells (Fig. 2.4). No GBV-C E2 binding was detected with the murine spleen or bone marrow cells with either the monoclonal anti-E2 antibodies M5 and M6 or the polyclonal anti-E2 antibody 7066 (Fig. 2.4). To determine whether hematopoietic progenitor cells from fetal mice bear GBV-C binding receptors, placental, fetal liver and thymic cells were obtained from a pregnant mouse and bone marrow cells were obtained from an adult mouse. E2 binding assays were performed on these cells with the anti-E2 7066 antibody (Fig. 2.5). None of these murine cells bound to GBV-C E2 either, suggesting that either GBV-C does not bind to murine hematopoietic progenitor cells (even though it binds to a murine embryonic fibroblast cell line), or that GBV-C does not bind to hematopoietic progenitor cells in general.

Because of the difficulties encountered with studying GBV-C entry in primary cells, including the lack of GBV-C E2 binding and donor variability, alternative methods
of studying GBV-C entry were pursued. These alternative methods included utilizing GBV-C E1/E2 enve-roparticles and GBV-C E2 binding to transformed cell lines.

*GBV-C E1/E2 enveloped retroviral particles transduce PBMCs*

GBV-C replication in PBMCs is poor and alternative mechanisms of studying GBV-C entry were examined. To this end, GBV-C E1/E2 enveloped retroviral particles (GBV-Cpp) were generated. These GBV-Cpp have GBV-C envelope proteins on their surface because they are immunoprecipitated by anti-E2 protein antibodies (Fig. 3.1), and their immunoprecipitation is competed with CHO cell supernatants containing E2, and not CHO cell supernatants lacking E2 (Fig. 3.2). GBV-Cpp entry was measured in human PBMCs to make sure that GBV-Cpp transduce the same cells which support GBV-C replication. The transduction of GBV-Cpp in PBMCs was compared to the transduction of HIV enveloped retroviral particles (HIVpp) and retroviral particles with no envelope glycoprotein (no env pp). Healthy blood donor PBMCs were stimulated with PHA and IL-2 for 3 days, transduced with GBV-Cpp, HIVpp or no env pp (p24 concentration equalized to 1000 pg), and transduction was measured by luciferase assay 3 days post-transduction. The RLU from GBV-Cpp-transduced cells was approximately 3.5 times higher than the no env pp-transduced cells, suggesting that GBV-Cpp are lymphotropic, as predicted (Fig. 2.6A). The transduction levels of the GBV-Cpp are still low, because the RLU from HIVpp-transduced cells was 250 times higher than the no env pp-transduced cells, which is much higher than the GBV-Cpp-transduced cells (Fig. 2.6A).
GBV-Cpp transduction of multiple transformed cell lines was also examined with the goal of finding a highly permissive cell line for further GBV-Cpp entry studies. The NCI-60 panel of transformed cells consists of cell lines from leukemia, lung tumors, colon cancer, CNS tumors, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer, and these cell lines have been widely characterized. To determine whether GBV-Cpp transduce these cell lines at levels higher than the background entry of no env pp, equivalent p24 concentrations of the pseudoparticles were incubated with the cell lines and luciferase activity was measured 3 days post-transduction. The fold increase of GBV-Cpp transduction compared to no env pp transduction was calculated. None of the 42 cell lines examined supported GBV-Cpp transduction at levels higher than 2.5 times the level of no env pp transduction (Fig. 2.6B). Thus, none of the cell lines in the NCI-60 panel are highly permissive for GBV-Cpp transduction, and further examinations of GBV-C entry using GBV-Cpp would be difficult.

**GBV-C E2 binds to multiple transformed cell lines**

An alternative method to study GBV-C entry and identify candidate cellular receptors is the measurement of GBV-C E2 binding to cell surfaces with anti-GBV-C E2 antibodies. Anti-GBV-C E2 antibodies are no longer made commercially; thus, a rabbit polyclonal anti-GBV-C E2 protein antibody was generated by immunization with CHO cell supernatants containing GBV-C E2. Multiple transformed cell lines from the NCI-60 panel were examined for GBV-C E2 binding using this rabbit anti-E2 protein antibody in the E2 binding assay (as described in the Methods section; Fig. 2.7A). Background binding of the rabbit anti-E2 protein antibody alone was subtracted from the PE fluorescence of the cells treated with E2 and antibody to obtain the percentage of cells
which bound to E2. Because of the nonspecific nature of this anti-E2 protein antibody, the background binding of the antibody to some cell lines was high. It appeared that the majority of transformed cell lines examined bound to E2 at some level when detected with this antibody, and many of the cell lines bound to E2 at very high levels, suggesting that many of the cell lines expressed a GBV-C E2 binding receptor (Fig. 2.7A).

However, upon further examination, and use of CHO cell supernatants lacking E2 (CHO-S) as a control, binding of rabbit anti-E2 protein antibody to CHO-S and E2-containing supernatants was detected at the same level on ACHN and 786-0 cells (Fig. 2.7B). This finding was observed for T-47D and SK-OV-3 cells as well. Thus, the combination of non-purified E2 and a polyclonal anti-E2 IgG preparation did not accurately measure GBV-C E2 binding to cell lines. Rather than repeat these studies using CHO-S as the background control, another more specific anti-E2 antibody was utilized.

Rabbit polyclonal antibody was generated by immunization with a peptide from GBV-C E2 comprising a 17 amino acid region recognized by the M6 monoclonal antibody (McLinden et al., 2006). This antibody is named anti-E2 peptide antibody 7066. Preliminary GBV-C E2 binding assays performed with the 7066 antibody and CHO-S as a control revealed that this antibody detected E2 binding specifically, and did not detect the nonspecific binding of CHO-S to 786-0 cells, a renal cell line included in the NCI-60 panel (Fig. 2.8A). The anti-E2 peptide antibody 7066 alone also did not bind to cells (Fig. 2.8A). To determine the optimal GBV-C E2 concentration for the E2 binding assay with 7066, E2 binding assays were performed on 786-0 cells with E2 concentrations ranging from 0.8 µg/ml to 800 µg/ml. E2 binding reached a maximum level at approximately 80 µg/ml (Fig. 2.8B) and further studies utilized this 80 µg/ml E2
concentration. To determine the optimal anti-E2 peptide antibody 7066 concentration, binding assays were performed with 786-0 and NCI-ADR/RES cells and 7066 concentrations ranging from 10 µg/ml to 100 µg/ml. Maximal E2 binding was detected with 20 µg/ml 7066 (Fig. 2.8C) and this concentration was used for all the following studies with the anti-E2 peptide antibody 7066.

GBV-C E2 binding assays with the anti-E2 peptide antibody 7066 were performed on the 42 transformed cell lines from the NCI-60 panel. The low background binding of the 7066 antibody was subtracted from the fluorescence of the cells treated with both E2 and 7066 antibody to obtain the percentage of cells which specifically bound to E2. GBV-C E2 binds to four cell lines reproducibly at levels higher than 5%: these cell lines are M14 (melanoma), NCI-ADR/RES (ovarian), SK-OV-3 (ovarian) and 786-0 (renal) (Fig. 2.9A). 786-0 and SK-OV-3 bind to GBV-C E2 at the highest level measured, followed by NCI-ADR/RES and M14. The 7066 antibody did not bind to any of the cell lines when they were incubated with CHO cell supernatants lacking E2. Examples of the histograms of a high E2 binding cell line (786-0), medium E2 binding cell line (M14) and low E2 binding cell line (Molt4) are shown in Fig. 2.9B. GBV-C E2 did not bind to any of the leukemia cell lines (CEM, K-562, Molt4, RPMI-8226, SR) at high levels. The lack of E2 binding to multiple leukemia cell lines was unexpected because GBV-C replicates in lymphocytes; however, these transformed cell lines may have significantly different cell surface molecules than primary lymphocytes. Previous studies reported that E2 binds Molt4 cells when detected with the anti-E2 antibody M5 (McLinden et al., 2006), and this was reproducible here (Fig. 2.11A), but the level of binding is much lower than E2 binding with 786-0 and SK-OV-3 cells.
**GBV-C E2 binding receptors are sensitive to trypsin digestion**

The trypsin enzyme cleaves peptide chains mainly at the carboxyl side of arginine and lysine amino acids. In order to characterize GBV-C E2 binding receptors and determine if they were sensitive to trypsin digestion, cell lines which bound to E2 at high levels (786-0 and SK-OV-3 cells) were incubated with trypsin at concentrations ranging from 0.0025% to 0.25% before performing the E2 binding assay with anti-E2 peptide antibody 7066. GBV-C E2 binding was highest in the cells which were not incubated with trypsin, and binding decreased in a dose-dependent manner with increasing trypsin concentrations (Fig. 2.10A). This suggests that the GBV-C E2 binding is dependent on cellular proteins which are sensitive to trypsin digestion.

**E2 binding is not temperature dependent**

To determine whether E2 cellular receptors are internalized at higher temperatures, the incubations of the E2 binding assay were performed at 21°C. There was no change in the level of E2 binding to 786-0, SK-OV-3, M14 or NCI-ADR/RES cells when the incubation steps were carried out at 4°C or 21°C (Fig. 2.10B). This suggests that either the E2 cellular receptors are not internalized, or that they are internalized and recycled back to the cellular membrane during the hour-long incubations.

**GBV-C anti-E2 monoclonal antibodies detect E2 binding**

Previous studies of GBV-C E2 binding used anti-E2 monoclonal antibodies that were generated by DNA immunization. These studies examined the CD4⁺ cell line Molt4 for E2 binding and found that the M6 antibody (IgG2a isotype), which recognizes a linear epitope on E2, blocked E2 binding to cells (as detected by the M5 antibody (IgG2b
isotype)) (McLinden et al., 2006). The level of E2 binding detected with M5 was very low, and further antibody competition studies examining a cell line which binds to E2 at higher levels were pursued. To this end, the binding of E2 to Molt4 and 786-0 cells using the M5 and M6 monoclonal antibodies was measured. M5 detected the highest level of E2 binding to Molt4 and 786-0 cells, and did not bind to CHO cell supernatants lacking E2 (CHO-S) (Fig. 2.11A). In contrast, M6 detected E2 binding at lower levels in the 786-0 cells and no binding was detected in the Molt4 cells (Fig. 2.11A). To determine whether M6 blocks E2 binding to cells, E2 was incubated with M6 (or an IgG2b isotype control antibody) for 1 hour at 4°C, then incubated with 786-0 or Molt4 cells. The cells were washed and incubated with M5 and a labeled secondary antibody. Incubation of E2 with M6 (or IgG2b isotype control antibody) prior to incubation with cells did not reduce M5 detection of E2 bound to 786-0 or Molt4 cells (Fig. 2.11B), which differs from the earlier studies with Molt4 cells which showed that pre-incubation with M6 blocks E2 binding to cells (McLinden et al., 2006). This indicates that the M6 monoclonal antibody probably does not interact with the cellular receptor binding site on E2, as previously thought.

Identification of candidate GBV-C binding receptors

Multiple microarrays have been performed on the NCI-60 panel of cell lines to measure the expression levels of cDNAs. This microarray data is available in a searchable format from the Developmental Therapeutics Program at the National Cancer Institute. Researchers may use an online algorithm, termed COMPARE, to correlate their findings (transduction levels, or in our case, binding levels) from NCI-60 cell line studies with cDNA expression levels from the microarray data. The truncated, soluble form of the
envelope protein E2 from the closely related virus HCV was used to identify the HCV attachment receptor CD81 by examining E2 binding in a nonpermissive cell expressing a cDNA library from a permissive cell line (Pileri et al., 1998). With this background, we hypothesized that we could use the truncated, soluble form of GBV-C E2 to perform binding studies on multiple cell lines from the NCI-60 panel, and correlate levels of GBV-C E2 binding with cDNA expression levels to identify candidate binding receptors.

To this end, we performed GBV-C E2 binding assays on 42 of the NCI-60 cells lines and entered the value “percentage of cells bound to E2” (Fig. 2.9A) into the COMPARE algorithm. We searched the Chiron, Novartis and Genelogic (U95 and U133) microarray data for the 100 most correlated cDNAs. The list of 100 cDNAs was narrowed down to include only transmembrane proteins located at least in part at the plasma membrane using GeneCard information. Based on these criteria, candidate receptors were identified from each of the four microarrays (Table 2.1). The Pearson Correlation Coefficient values range from 0.404 – 0.775 for all of the candidate binding receptors. The Target Standard Deviation value corresponds to the expression level range of the particular cDNA within the NCI-60 cell line panel. A high Target Standard Deviation correlates with a high variability of the candidate receptor cDNA expression level across the cell lines examined, and a high value is preferable because there is a high variability of GBV-C E2 binding to the cell lines.

Four of the candidate receptors were notable for their high Pearson Correlation Coefficient values, high Target Standard Deviation values, and expression in primary lymphoid cells (Fig. 2.12; Table 2.2). One of the candidate receptors, HLA-G, is a nonclassical HLA class I molecule, which is involved in maternal-fetal tolerance,
preventing attack of the fetus by the maternal immune system (Carosella et al., 2008). HLA-G is also expressed in immune cells and is upregulated in CD8\(^+\) T cells and monocytes in HIV-infected individuals (Carosella et al., 2008). Another candidate receptor is the related nonclassical HLA class I molecule HLA-F. HLA-F is also predicted to be involved in maternal-fetal tolerance because of its expression in placental tissues (Lee et al., 2010). B cells, NK cells, monocytes and T cells express HLA-F intracellularly and, very early after activation, express HLA-F on the cell surface (Lee et al., 2010). These related nonclassical class I HLA molecules are interesting because of their activities in the immune system, where they are either upregulated after HIV infection or upregulated after immune cell activation.

Another candidate receptor is CD86, a member of the immunoglobulin family which is expressed on antigen-presenting cells (Engel et al., 1994). CD86 is the ligand for two proteins on T cells, and interaction with these cell surface proteins, CD28 and cytotoxic T-lymphocyte-associated 4 (CTLA-4), promotes or negatively regulates T cell activation, respectively. GBV-C may influence T cell activation during HIV infection, so CD86 is an interesting candidate for an E2 binding receptor. The final candidate receptor selected for further examination is ADAM10, a proteinase that mediates ectodomain shedding (Sastre et al., 2008). ADAM10 plays an important role in ectodomain shedding of inflammatory proteins during leukocyte recruitment (Sastre et al., 2008). Multiple approaches are being pursued to determine whether these molecules have a role in GBV-C E2 binding.
Discussion

Recombinant GBV-C E2 protein inhibits HIV enveloped pseudoparticle entry at significantly higher levels than CHO cell supernatants lacking E2. We use recombinant E2 protein in CHO cell supernatants for our experiments; thus, we did not address whether E2 expression in the CHO cells induces secretion of factors that may inhibit HIV pseudoparticle entry. This factors may be absent in the the CHO cells not expressing E2. Thus, development of purified recombinant GBV-C E2 protein is necessary for future studies. However, earlier studies of GBV-C E2 by another research group utilized an E2-Fc fusion protein in studies of HIV and VSV enveloped pseudoparticle entry inhibition, and they purified the protein using the Fc region, so recombinant E2 appears to play a role in HIV inhibition (Jung et al., 2007). Our nonpurified recombinant E2 protein is a monomer and the E2-Fc protein is a dimer. E2 is expected to form a heterodimer with E1, so the E2 monomers and dimers used currently by multiple research groups may not accurately reflect the conformation and arrangement of E2 in the context of the virion. Additional studies of E2 inhibition of HIV should consider utilizing an E1/E2 heterodimer. Despite the fact that monomeric GBV-C E2 may not mimic the E2 structure within the context of an E1/E2 heterodimer, monomeric HCV E2 binds to cells and was used to identify CD81 as an HCV attachment receptor (Pileri et al., 1998). Thus, monomeric GBV-C E2 is also predicted to be in a conformation that allows binding to GBV-C cellular receptors.

GBV-C replicates in B and T lymphocytes (George et al., 2006) and is produced by these cells when removed from infected human ex vivo (George et al., 2003b; George et al., 2006). The specific subsets of lymphocytes which support replication are
unknown. In one donor, we found that recombinant GBV-C E2 binds to a small subset of PBMCs which bear the CD3, CD19 and CD83 markers. This small subset of GBV-C-E2-binding PBMCs is transient, because no binding was detected in the same donor at a later date. Binding may depend on \textit{in vivo} activation status because E2 binding was observed in a single blood donor 11 days after an influenza vaccination, but not in the same donor 28 days post-vaccination. In addition, the E2-binding subset was not found in an older donor more than 2 weeks after influenza vaccination. Activation of the T lymphocytes from the second middle-aged blood donor with anti-CD3 and anti-CD28 antibodies did not produce the E2-binding PBMC population seen in the first donor, suggesting that \textit{in vitro} activation methods we utilized (CD3/CD28 ligation) do not elicit the same conditions seen in the young donor 11 days post-vaccination \textit{in vivo}. \textit{In vitro} activation of T cells via ligation of CD3 and CD28 produces a different activation status than the activation of memory T cells by vaccination or illness \textit{in vivo}, so this could explain why a small PBMC subset bound to E2 11 days post-vaccination but not cells activated by TCR ligation. Activation of T cells with tetanus toxoid or CMV/EBV/Influenza peptide pools may produce a memory T cell activation phenotype which more closely resembles the T cell activation status of recently vaccinated individuals. These alternative stimulation methods may elicit an activation state in which cells that support GBV-C replication are enriched, or one in which the GBV-C cellular receptors are upregulated. This may explain why PBMCs from a recently vaccinated individual bound to GBV-C E2, but PBMCs from the same individual at a later date did not have an E2-binding population. Other variables, such as the cytokine milieu,
infection history and lymphocyte differentiation state, may contribute to the differences seen in GBV-C E2 binding between donors.

GBV-C replicates in the spleen and bone marrow \textit{in vivo} (Tucker \textit{et al.}, 2000) and we hypothesized that hematopoietic progenitor cells in these sites and in the peripheral blood may support GBV-C replication and recombinant E2 binding. To this end, we examined adult murine spleen and bone marrow cells, and fetal liver, thymus and placental cells, for the ability to bind to GBV-C E2 with multiple anti-E2 antibodies (M5, M6 and 7066). We did not observe E2 binding in any of the mouse tissues examined. Thus, it appears that recombinant GBV-C E2 does not bind to detectable levels of murine hematopoietic progenitor cells. GBV-C E2 binds to the murine embryonic fibroblast cell line 3T3 (McLinden \textit{et al.}, 2006), so murine cells can support recombinant E2 binding. Human hematopoietic progenitor cells express the CD34 marker; in the single human PBMC donor with observable E2 binding, the small E2-binding subset did not bear the CD34 marker, only the CD3, CD19 and CD83 markers. Thus, recombinant GBV-C E2 does not appear to bind to human hematopoietic progenitor cells either. However, continued examination of CD34\(^+\) hematopoietic progenitor cells in E2 binding studies is warranted because progenitor cells in cord blood and human bone marrow have not been extensively examined for E2 binding ability. Preliminary studies of PBMCs from a human donor stimulated with GM-CSF did not show E2 binding, but in these studies, only 10,000 cells were examined by flow cytometry. Because of the rarity of the E2 binding population, a total of 3 million PBMCs need to be examined by flow cytometry to determine which subsets bind to E2. Thus, examination of hematopoietic progenitor cells from cord blood or GM-CSF stimulated donors is warranted.
GBV-C E1/E2 pseudotyped retroviral particles (GBV-Cpp) transduce activated PBMCs at higher levels than any of the transformed cell lines examined, including the leukemia cell lines. This indicates that GBV-Cpp bind to and enter a subset of PBMCs, although transduction is still low, which is consistent with the finding that GBV-C replicates to low levels in B and T lymphocytes (George et al., 2006). Although GBV-Cpp transduced PBMCs, no transformed cell lines which supported GBV-Cpp transduction at levels higher than 2-fold above the background were identified. There are multiple explanations for why we did not identify cells permissive for GBV-Cpp transduction. First, no GBV-C entry receptors may be present on the primary cells and cell lines we examined. Secondly, there may be post-entry restriction factors preventing efficient transduction or luciferase gene expression. I did not discern GBV-Cpp binding to cell lines (786-0) at 4°C, as detected by the anti-E2 antibody 7066. However, I have never examined GBV-Cpp attachment to cells by a p24 assay. In these experiments, I would incubate high E2-binding cells (786-0) with GBV-Cpp at 4°C, wash off the unbound virions, and measure how many particles attached to the cells by p24 assay. This may be a more sensitive method of measuring GBV-Cpp attachment than using anti-E2 antibodies for detection because there are multiple copies of p24 per retroviral particle.

Another approach to identifying GBV-C binding receptors involved in GBV-C entry is to identify cell lines which bind to recombinant GBV-C E2. We examined multiple transformed cell lines from the NCI-60 panel for their ability to bind to recombinant E2 with two different anti-E2 antibodies. One of the antibodies used, the anti-E2 protein antibody, was generated by rabbit immunization with non-purified
recombinant E2 protein, and thus, binds to both GBV-C E2 and nonspecific cellular proteins. This antibody detected E2 binding to multiple cell lines from the NCI-60 panel at high levels, but upon further examination, the antibody was found to bind to CHO cell supernatant proteins at levels similar to CHO cell supernatants containing recombinant E2 applied to the same cells.

Instead of repeating the studies with all of the cell lines using the additional required control, we pursued these E2 binding assays with a different anti-E2 antibody. The second antibody that we used was the anti-E2 peptide antibody 7066 which was generated by immunization with an E2 peptide. The 7066 antibody detected E2 binding at high levels in 4 of the NCI-60 cell lines examined (786-0, SK-OV-3, M14, and NCI/ADR-RES). Importantly, unlike the previous anti-E2 protein antibody, the 7066 antibody did not bind to any of the cell lines when they were incubated with CHO cell supernatants lacking E2. The majority of cell lines did not interact with E2, suggesting that either E2 does not bind to these cells or that binding occurs as such a low affinity that E2 is washed off during the assay. It would be interesting to determine whether anti-E2 antibodies could detect E2 cross-linked to the cells at higher levels than non-cross-linked E2.

GBV-C E2 binding was sensitive to trypsin digestion of cell surface proteins, as E2 binding decreased in a dose-dependent manner with increasing trypsin concentration. Preliminary experiments have suggested that E2 binding is sensitive to neuraminidase treatment and further work is necessary to confirm this finding. GBV-C E2 binding was not dependent on temperature, because the same level of E2 binding occurred whether the E2 binding assays performed on the cells (786-0, SK-OV-3, M14, NCI/ADR-RES) were
carried out at 4°C or 21°C. This indicates that either the receptors are not internalized, or that they are endocytosed and recycled back to the plasma membrane within a short period of time.

There are multiple limitations of identifying candidate GBV-C receptors by utilizing an E2 binding assay approach. GBV-C E1 and E2 are predicted to form a noncovalently linked heterodimer on the viral envelope and E2 is predicted to be responsible for initiating virus attachment, as with HCV (Flint et al., 2000; Pileri et al., 1998). Although the C-terminal truncated form of HCV E2 is secreted from cells and binds to CD81, an attachment receptor for HCV, this interaction occurs despite the finding that the majority of the secreted protein does not appear to be in the correct conformation to bind to CD81 and may require interaction with HCV E1 for correct processing and receptor interaction (Flint et al., 2000). In addition, the intracellular portion of C-terminally truncated HCV E2, rather than the extracellular secreted form of HCV E2, has a higher affinity for CD81 (Flint et al., 2000). We utilized similar methods for truncating GBV-C E2 at the C-terminus and obtaining secreted E2 from cell supernatants as did the original HCV E2-CD81 binding studies. Although GBV-C E2 binding is detected in four cell lines, the level of binding is not high when compared to the level of binding of HCV E2 to CD81 (Flint et al., 2000). This may be because GBV-C attachment receptors are expressed at lower levels than HCV’s attachment receptor CD81, or because the majority of secreted GBV-C E2 is not in the correct conformation to bind to attachment receptors, which occurs to some level with secreted HCV E2.

The NCI-60 panel of cell lines has been well-characterized and multiple microarrays have been performed on the panel. We utilized the COMPARE algorithm,
which is available to the public and searches the microarray datasets, to correlate cDNA expression levels with the level of GBV-C E2 binding in order to identify candidate GBV-C E2 binding receptors. We examined 4 of the microarray data sets, limited our search to include only 100 most highly-correlated cDNAs, and further narrowed our list to cDNAs which are transmembrane proteins located at the plasma membrane (Table 2.1). We identified multiple candidate GBV-C E2 binding receptors and studies examining HLA-G, HLA-F, CD86 and ADAM10 are underway. Specifically, siRNAs to these cDNAs will be used to knockdown expression of the candidate receptor in a high E2-binding cell line, 786-0. Then, E2 binding will be measured to determine if the candidate receptor knockdown decreases E2 binding. Additional candidate receptors can be measured in the future with a yet-to-be developed high-throughput E2 binding assay.

There are multiple limitations to the candidate receptors identified by the COMPARE algorithm. First, the microarrays (whose datasets were searched in the COMPARE analysis) were performed on the NCI-60 cell panel after culture in only 5% fetal calf serum; I used 10% fetal calf serum in my studies, so cDNA expression patterns may differ. Secondly, if the recombinant monomeric form of E2 used in our studies is not similar to the correct confirmation of E2, the binding studies will not identify cell lines that express high levels of GBV-C E2 attachment receptors. Instead, cell lines which bind to a mishapen E2 will be identified. However, until additional forms of GBV-C E2 are developed, the C-terminal truncated monomeric form of E2 is the only form available.

In this chapter, we utilized multiple approaches to study the interaction of GBV-C E2 and cell surfaces. We demonstrated that GBV-C E2 binds to a small, transient CD3+,
CD19^+ and CD83^+ subset of lymphocytes and that GBV-C E1/E2 pseudotyped retroviral particles transduce activated PBMCs. In addition, we also identified multiple cell lines to which E2 binds at high levels, which can be used in follow-up E2-cell interaction studies. Examining the interaction of GBV-C E2 with cell surface proteins is important because GBV-C E2-mediated inhibition of HIV pseudotyped particles is predicted to occur at the cell surface. GBV-C E2 may also inhibit HIV via additional mechanisms, such as blocking T cell activation and apoptosis. Nevertheless, with all these mechanisms of HIV inhibition, determining how GBV-C E2 interacts with cell surface and enters the cell by interacting with binding and/or entry receptors is critical.
Figure 2.1. GB virus C envelope glycoprotein 2 (E2) inhibits early steps in the HIV life cycle. HOS cells expressing CD4, CCR5 and CXCR4 were incubated with an equivalent total protein concentration of recombinant E2 (E2) or CHO cell supernatants lacking E2 (E2 neg) at 4°C for 4 h prior to transduction with HIV pseudotyped particles bearing a luciferase reporter. GBV-C E2 inhibited transduction of HIV pseudotyped particles in a dose–dependent fashion, whereas CHO cell supernatants (E2 neg) did not (*P < 0.001 compare to E2 neg of the same concentration; t-test). This graph shows a single experiment with samples in quadruplicate.
Figure 2.2: GBV-C E2 binds to a small subset of PBMCs of a healthy blood donor. Healthy blood donor PBMCs were isolated, incubated with 80 µg/ml E2 (or no E2), washed, incubated with 20 µg/ml anti-E2 peptide antibody 7066, and E2 binding was detected with a PE-labeled secondary antibody. PBMC subsets were identified by staining with anti-CD3, anti-CD19 or anti-CD83 antibodies. GBV-C E2 binds to a small, distinct subset of total PBMCs which is not present in the sample incubated with only anti-E2 antibody 7066. The E2-binding PBMCs are CD3⁺, CD19⁺ or CD83⁺.
Figure 2.3: GBV-C E2 does not bind to naïve or stimulated PBMCs from a middle-aged healthy blood donor. Healthy blood donor PBMCs were either stained for E2 binding immediately after isolation or were cultured with anti-CD3 and anti-CD28 antibodies for stimulation for 24 or 48 hours. The PBMCs were incubated with 80 µg/ml E2, washed, incubated with 20 µg/ml anti-E2 peptide antibody 7066, and E2 binding was detected with a PE-labeled secondary antibody. GBV-C E2 does not bind to a small, distinct subset of total PBMCs, even after stimulation, from this blood donor.
Figure 2.4: GBV-C E2 does not bind to murine bone marrow or spleen cells. Murine bone marrow and spleen cells were harvested, treated with ACK lysis buffer to remove red blood cells, and incubated with recombinant GBV-C E2 protein or PBSA. Cells were washed and incubated with either anti-E2 M5, M6 or 7066 antibody. Antibody binding was detected with a secondary PE-labeled antibody and flow cytometry. The cells incubated with E2 and antibody (dashed line) did not have increased PE fluorescence compared to cells incubated with only the antibody (dotted line), indicating that E2 does not bind to murine bone marrow or spleen cells. The background PE fluorescence of untreated cells is shown with a solid black line.
Figure 2.5: GBV-C E2 does not bind to adult murine bone marrow or fetal liver, thymus or placental cells. Murine bone marrow, placenta, fetal liver and thymic cells were harvested, treated with ACK lysis buffer to remove the red blood cells, and incubated with recombinant GBV-C E2 protein or PBSA. Cells were washed and incubated with anti-E2 7066 antibody. Antibody binding was detected with a secondary PE-labeled antibody and flow cytometry. The cells incubated with E2 and antibody did not have increased PE fluorescence compared to cells incubated with only the antibody, indicating that E2 does not bind to murine bone marrow or fetal liver, thymus or placental cells.
Figure 2.6: GBV-C E1/E2 enveloped retroviral pseudoparticles transduce PBMCs and not transformed cell lines. PBMCs isolated from healthy human donors were transduced with GBV-C E1/E2 (GBV-Cpp), HIV env (HIVpp) pseudotyped retroviral particles and retroviral particles bearing no viral envelope protein (No env pp) (A). This graph shows a single experiment with samples in triplicate which is representative of 2 experiments. Particle input levels were equalized by p24 concentration. Transduction was measured by luciferase activity 3 days post-transduction. A panel of 42 transformed cell lines was transduced with GBV-C E1/E2 enveloped particles and particles with no viral envelope protein, and luciferase activity was measured 3 days post-transduction (B). PBMCs are separated from the NCI-60 cell panel by a dashed line. The fold increase of GBV-C envelope particle transduction compared to no viral enveloped particle transduction was determined by dividing the relative light units (RLU) of the GBV-C sample by the RLU of the no viral envelope protein sample. († p=0.011 GBV-Cpp compared to no env pp; * p=0.002 HIVpp compared to no env pp). Each bar represents an average of 2 experiments, and each experiment was comprised of 3 samples each.
Figure 2.7: Anti-E2 protein antibodies, produced by rabbit immunization of cell supernatants containing E2, do not specifically bind to cell-bound recombinant GBV-C E2. In the binding assay, cells were incubated with 800 µg/ml GBV-C E2, washed, and incubated with 8 µg/ml anti-E2 protein antibody, washed, and binding was detected by a PE labeled secondary antibody. The percentage of cells which bound to E2 was determined by subtracting the background binding of the anti-E2 protein antibody from the fluorescence of the sample treated with E2 and anti-E2 protein antibody (A). Each bar represents an average of at least 2 experiments of single samples. After completing the panel of transformed cells lines, it was discovered that the anti-E2 protein antibody generated with immunization of cell supernatants containing E2 recognized both E2 protein and nonspecific CHO-S cell supernatant proteins in the nonpurified recombinant E2 preparation used in the assays. In two of the cells lines which bound to E2 at high levels, the background binding of anti-E2 protein antibody to CHO-S cell supernatants lacking E2 was higher than the background binding of the anti-E2 protein antibody to cells alone (B). This indicates that the percentage of cells which bound to E2 as determined by subtracting the background binding of the anti-E2 protein antibody alone is not an accurate method of measurement.
Figure 2.8: Titration of GBV-C E2 and anti-E2 peptide antibody 7066 in binding assays. To confirm that the anti-E2 peptide antibody 7066 does not bind to CHO-S supernatants lacking E2, 786-0 cells were incubated with 80 µg/ml E2 or an equivalent total protein concentration of CHO-S supernatants, washed, and incubated with 20 µg/ml anti-E2 peptide antibody 7066 (A). 7066 does not recognize proteins present in the CHO-S supernatants lack E2, as expected. To titrate E2 binding, 786-0 cells were incubated with concentrations of E2 ranging from 0.8 µg/ml to 800 µg/ml or no E2 at 4°C for 1 hour, washed and incubated with anti-E2 peptide antibody 7066 (20 µg/ml) (B). To titrate anti-E2 antibody 7066 binding, 786-0 (triangles) and NCI-ADR/RES (circles) cells were incubated with 80 µg/ml E2 and concentrations of anti-E2 antibody ranging from 10 µg/ml to 100 µg/ml or no antibody (C). Binding was detected with a PE-labeled secondary antibody and flow cytometry. Logarithmic trendlines were calculated in Microsoft Excel.
Figure 2.9: GBV-C E2 binds to transformed cell lines. In the binding assay, cells were incubated with GBV-C E2, washed, and incubated with anti-E2 7066 antibody, washed, and binding was detected by a PE labeled secondary antibody. The percentage of cells which bound to E2 was determined by subtracting the background binding of the 7066 antibody from the fluorescence of the sample treated with E2 and 7066 (A). Each bar represents an average of at least 2 experiments of single samples. Histograms of a cell line which bound to E2 at a high level (786-0), mid level (M14), and a cell line which did not bind to E2 (Molt4) are provided for illustration (B).
Figure 2.10: GBV-C E2 cellular receptors are sensitive to trypsin digestion and not to temperature. 786-0 and SK-OV-3 cells were incubated with 0.0025%, 0.025%, or 0.25% trypsin-EDTA at room temperature for 15 seconds. The reaction was neutralized with fetal calf serum, and the cells were incubated with GBV-C E2, anti-E2 antibody 7066, and a PE-labeled secondary antibody. The percentage of PE positive cells was measured by flow cytometry (A). GBV-C E2 was incubated with 786-0, SK-OV-3, NCI-ADR/RES and M14 cells at 4°C or 21°C for 1 hour and E2 binding was measured with the anti-E2 7066 antibody and a PE-labeled secondary antibody. The percentage of cells which bound to E2 was determined by subtracting the background 7066 binding from the fluorescence of the cells incubated with E2 and 7066 (B). * p>0.150; t-test. Each bar represents a single experiment with samples done in triplicate and is representative of at least 2 experiments.
Figure 2.11: Monoclonal anti-E2 antibodies detect cell-bound E2 on transformed cell lines. 786-0 and Molt4 cells were incubated with 80 µg/ml E2 or an equivalent amount of CHO-S supernatant based on total protein concentration, washed, and incubated with either 10 µg/ml anti-E2 M6 antibody (IgG2a isotype) or 0.4 µg/ml anti-E2 M5 antibody (IgG2b isotype), and a PE-labeled goat anti-mouse secondary antibody (A). The optimal concentrations of M5 and M6 were determined by titration and the antibody concentrations which detected the highest levels of E2 binding were used in these experiments. Fluorescence was measured by flow cytometry. 786-0 and Molt4 cells were incubated with E2 (E2 & M5), E2 mixed with M6 (E2/M6 & M5) or E2 mixed with a mouse IgG2b isotype control antibody (E2/IC & M5), washed, and incubated with anti-E2 M5 antibody and a PE-labeled secondary antibody. The background binding of M5 antibody alone to the cells was subtracted to calculate the percentage of cells bound to E2 in each condition. * p>0.05 compared to E2 & M5; t-test. Each bar represents a single experiment with samples done in triplicate and is representative of at least 2 experiments.
Table 2.1. Candidate GBV-C E2 Binding Receptors Identified in a Comparative Genomic Analysis.

<table>
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<th>Array name</th>
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<th>Target Standard Deviation</th>
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Table 2.1. Continued

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<td>Latrophilin-2</td>
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**Bold** = Target standard deviations at or above the median target standard deviation for an individual array dataset.

**Colors** = cDNAs that appear multiple times in the same dataset or another array are marked by the same color.
Figure 2.12: GBV-C E2 binding profile and microarray-derived gene expression profiles for CD86, HLA-G, HLA-F and ADAM10. The GBV-C E2 binding profile was determined by incubating recombinant GBV-C E2 with cells and measuring the percentage of cells which bound to E2, as described in Fig. 2.9 (A). Relative cDNA expression levels for CD86 (B), HLA-G (C), HLA-F (D) and ADAM10 (E) were obtained from the microarray dataset on the COMPARE website. The relative level for the indicated measured value (GBV-C E2 binding or CD86, HLA-G, HLA-F or ADAM10 expression) is indicated by bar height in each of the graphs. Each bar on the graphs represents a different cell line in the panel. The order of the cell lines is the same in each graph.
Table 2.2. Description of Select Candidate GBV-C E2 Binding Receptors.

<table>
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<tr>
<th>Candidate Binding Receptor</th>
<th>Full Name</th>
<th>Also known as</th>
<th>Tissue Distribution</th>
<th>Gene ID Summary</th>
<th>Immunological Properties</th>
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<tbody>
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<td>HLA-G, alpha chain</td>
<td>Major histocompatibility complex, class I, G</td>
<td>MHC-G</td>
<td>Trophoblast, thymus, cornea, nail matrix, pancreas, erythroid and endothelial precursors</td>
<td>HLA-G belongs to the HLA class I heavy chain paralogues. This class I molecule is a heterodimer consisting of a heavy chain and a light chain (beta-2 microglobulin). The heavy chain is anchored in the membrane.</td>
<td>Multiple immunosuppressive properties occur through interactions with receptors differentially expressed on NK cells, T cells and antigen-presenting cells.</td>
</tr>
<tr>
<td>HLA-F, alpha chain</td>
<td>Major histocompatibility complex, class I, F</td>
<td>CDA12; HLA-5.4; HLA-CDA12</td>
<td>B cells, T cells, NK cells, monocytes, trophoblasts</td>
<td>HLA-F belongs to the HLA class I heavy chain paralogues. It encodes a non-classical heavy chain that forms a heterodimer with a beta-2 microglobulin light chain, with the heavy chain anchored in the membrane. It contains a divergent peptide-binding groove, and is thought to bind a restricted subset of peptides for immune presentation.</td>
<td>HLA-F is expressed under activating conditions and may provide a signal that indicates an activated immune response to Treg cells as part of a communication between activated cells and regulatory cells.</td>
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<tr>
<td>CD86</td>
<td>CD86 molecule</td>
<td>B70; B7-2; LAB72; CD28LG2; MGC34413</td>
<td>B cells, dendritic cells, endothelial cells, macrophages, monocytes, T cells, fibroblasts, eosinophils and others</td>
<td>This gene encodes a type I membrane protein that is a member of the immunoglobulin superfamily. This protein is expressed by antigen-presenting cells, and it is the ligand for two proteins at the cell surface of T cells, CD28 antigen and cytotoxic T-lymphocyte-associated protein 4.</td>
<td>Binding of this protein with CD28 antigen is a costimulatory signal for activation of the T-cell. Binding of this protein with cytotoxic T-lymphocyte-associated protein 4 negatively regulates T-cell activation and diminishes the immune response.</td>
</tr>
<tr>
<td>ADAM10</td>
<td>ADAM metalloproteinase domain 10</td>
<td>kuz; AD10; MADM; CD156c; HsT18717</td>
<td>Monocytes, NK cells, T cells, dendritic cells, B lymphoblasts, cardiac myocytes, CNS, microglia and others</td>
<td>Members of the ADAM family are cell surface proteins with a unique structure possessing both potential adhesion and protease domains. This gene encodes an ADAM family member that cleaves many proteins including TNF-alpha and E-cadherin.</td>
<td>ADAM proteinases mediate ectodomain shedding, the proteolytic release of extracellular domains from their membrane-bound precursors. Ectodomain shedding is a mechanism whereby cells can regulate the proteins expressed on their cell surface. ADAM10 appears to play a particularly prominent role in ectodomain shedding of inflammatory proteins at all stages of leukocyte recruitment.</td>
</tr>
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CHAPTER III: GBV-C E2 ANTIBODY INHIBITION OF HIV

Introduction

HIV-1 vaccine development has relied primarily upon the use of HIV-1 proteins as immunogens in an attempt to elicit either neutralizing antibodies or cellular immune responses to prevent or modify HIV-related disease (Fauci et al., 2008; Johnston & Fauci, 2008). Due to the high replicative rate of HIV-1 and the error prone RNA-dependent DNA polymerase, neutralization and T-cell escape mutants are generated on a daily, if not hourly, basis in infected individuals. HIV-infected individuals usually elicit high titers of HIV-1-specific antibodies, but HIV neutralizing antibodies are rare (Nelson et al., 2007).

However, a few broadly neutralizing human HIV monoclonal antibodies have been isolated from HIV-infected individuals (Buchacher et al., 1994) (Table 3.1). These broadly neutralizing human mAbs include an antibody directed against the HIV envelope glycoprotein 120 (gp120) (e.g. antibody 2G12) that interferes with CD4 binding and antibodies that react with the membrane proximal ectodomain region (MPER) of the HIV envelope glycoprotein 41 (gp41) (e.g. antibodies 2F5 and 4E10) (Buchacher et al., 1994; Dennison et al., 2009). The 2F5 and 4E10 antibodies are predicted to disrupt MPER fusogenic functions, required for viral entry into CD4+ T cells and macrophages, by preventing hinge motion or by perturbing MPER orientation in the lipid bilayer (Song et al., 2009). The 2F5 and 4E10 antibodies also react with a 36-residue peptide that overlaps with the MPER called T-20 (Wild et al., 1993). T-20 inhibits HIV replication by preventing virus envelope fusion with cell membranes, and T-20 is an effective and licensed antiretroviral treatment (Fuzeon) (Ferrantelli et al., 2003; Wild et al., 1993).
The 2F5 and 4E10 antibodies are polyspecific, and cross-react with cellular antigens including several lipids (Coutant et al., 2008; Haynes et al., 2005; Haynes & Alam, 2008; Matyas et al., 2009a; Matyas et al., 2009b; Sanchez-Martinez et al., 2006). Although antigens that interact with these antibodies have been identified, active immunization with gp41, MPER or T-20 does not elicit broadly neutralizing HIV antibodies (Koff, 2010; Ruprecht et al., 2003). Immunization with the 2F5 and 4E10 epitopes results in poor immunogenicity, which is predicted to be a result of immunological tolerance-suppression of autoantigen reactivity (Haynes et al., 2005). Clearly, new approaches to HIV-1 vaccines are needed (Fauci et al., 2008; Johnston & Fauci, 2008).

GB virus C (GBV-C) is a common human infection and due to shared modes of transmission, the prevalence of GBV-C in HIV-infected people is high (17% to 42%) (Stapleton et al., 2004). Although persistent GBV-C viremia is associated with the best survival in epidemiological studies (Williams et al., 2004), one study found that subjects without viremia who have GBV-C E2 antibodies survived longer than those without E2 antibodies (Tillmann et al., 2001). Similar data were observed in HIV seroconverters participating in the Multicenter AIDS Cohort Study. Specifically, subjects with GBV-C viremia measured between 5-6 years post HIV-1 seroconversion (GBV-C RNA+; n=50) survived significantly longer than those without viremia. However, among subjects without GBV-C viremia, those with detectable antibody to GBV-C E2 protein (GBV-C Ab+; n=59) survived longer than did those with neither E2 antibody nor viremia (n=29).

Human GBV-C E2 antibodies from natural infection and all but one of characterized GBV-C E2 murine mAbs are conformation-dependent (McLinden et al.,
One mAb (M6) recognizes a linear epitope on E2 (Schmolke et al., 1998). A GBV-C E2 peptide encompassing this epitope has been proposed to be involved in GBV-C – cell membrane fusion, based on findings that it forms an amphipathic helix in the presence of lipids and model membranes (Larios et al., 2005a; Larios et al., 2005b). In addition, another E2 peptide which overlaps the putative fusion peptide prevents oligomerization of the HIV-1 gp41 fusion peptide and membrane fusion in an *in vitro* model (Herrera et al., 2009). Finally, incubation of PBMCs or CD4+ T cell lines with the GBV-C envelope glycoprotein E2 competitively inhibits HIV-1 entry *in vitro* (Jung et al., 2007; Mohr & Stapleton, 2009).

These findings suggest that the structure of GBV-C E2 may structurally mimic a protein found on HIV-1 particles and that anti-E2 antibodies recognize both E2, as expected, and proteins with E2-like structures on HIV particles. The E2 protein structure may mimic the structure of the HIV-1 proteins present on virions, such as gp41 or gp120, or one of the many cellular transmembrane proteins that are present on the surface of HIV particles. Many cellular surface adhesion molecules are present on HIV particles and increase HIV infectivity by virus attachment to T cells (ICAM-1, HLA-II, Galectin-1, CD80, CD86) and dendritic cells (LFA-1) (Ott, 2008). In addition, the presence of other cell adhesion molecules on HIV virions (CD62L and CD40L) increases the capture of virions by endothelial cells and B cells, respectively, which then expose the captured HIV virions to T cells (Ott, 2008). The AIDS and Cancer Program at the NCI has a database of all the cellular proteins detected in HIV particles, and so far, 306 cellular proteins have been detected in HIV particles, and 100 of these are on the surface of the particles, which would be accessible to antibodies in the serum. If a structure present on
HIV particles mimics the structure of E2, then antibodies directed against GBV-C E2 may interfere with HIV attachment or fusion, and potentially modify HIV-1 disease progression.

Recent findings from J. Xiang support the hypothesis that GBV-C E2 antibodies inhibit HIV infection in vitro (Mohr et al., 2010). GBV-C E2 antibody positive healthy blood donor sera and purified IgG neutralize both CCR5- and CXCR4-tropic HIV isolates in a PBMC-based neutralization assay. The effect is specific for E2 antibodies, because three murine GBV-C E2 mAbs M1(VS), M5 and M6 also inhibit HIV-1 isolates in a TZM-bl cell-based neutralization assay. Three broadly neutralizing HIV-1 mAbs (2G12, 2F5, and 4E10) neutralize HIV-1 infection to similar levels as the anti-E2 antibodies. In addition, HIV neutralizing antibodies were elicited by immunization of mice and rabbits with recombinant GBV-C E2 protein (McLinden et al., 2006). However, rabbit antibodies directed against a 17 amino acid E2 peptide recognized by the mAb M6 did not neutralize in this assay (McLinden et al., 2006), suggesting that an immunogen containing a larger portion of E2 must be used to elicit HIV neutralizing antibodies. Post-immune sera from the mice and rabbits immunized with E2 protein neutralize HIV-1 infectivity in MT-2 cells. Interestingly, HIV-1 inhibition is diminished when the antibodies are not maintained in the cell culture media for the duration of the infection. Thus, initial infection appears to be incompletely inhibited by the antibodies, and HIV-1 cell-to-cell spread is suppressed, though not completely eliminated, in the presence of GBV-C E2 antibodies.

Even though the rabbit anti-E2 antibodies do not react with denatured HIV-1 proteins in an immunoblot, they pull down infectious HIV-1 virions, suggesting that the
interaction is conformation dependent. Immune precipitation of HIV-1 virions is blocked by recombinant CHO cell culture supernatant fluid containing E2 protein, but not CHO supernatant without E2, in a dose-dependent manner. The rabbit GBV-C E2 protein antibodies do not precipitate or neutralize YFV or mumps virus prepared in MT-2 cells. Like HIV-1, mumps virus has a class I envelope glycoprotein. YFV prepared in Vero cells, and mumps prepared in BHK cells, are also not neutralized nor precipitated by the E2 antibodies. Taken together, the data suggest that the anti-E2 antibody interactions are specific for HIV-1 virions and that the epitope is not exposed on flavivirus or paramyxovirus particles.

Finally, to determine which step of HIV infection is inhibited by GBV-C E2 antibodies, HIV-1 and E2 antibodies were incubated in various combinations with cells. Pre-incubation of MT-2 cells with GBV-C E2 antibody does not inhibit HIV-1 replication. When E2 antibodies were mixed with HIV-1 particles prior to infection, the anti-GBV-C E2 antibody blocked HIV-1 attachment to MT-2 cells in a dose-dependent manner. Thus, interaction of the antibody with the virus prior to the interaction of the virus with the cell appears to be necessary for HIV-1 infection inhibition. To determine if E2 antibodies inhibit HIV post-attachment entry steps in addition to inhibiting HIV attachment, HIV-1 was incubated with MT-2 cells at 4°C to facilitate viral attachment and E2 antibodies were subsequently added to allow binding at 4°C. When the medium was replaced with media containing E2 antibodies, HIV-1 replication was inhibited three days post-infection. When the replacement medium did not contain E2 antibodies, HIV-1 replication was not inhibited. Because HIV-1 virion infection is productive and the surrounding cells are infected in cell-to-cell spread, it is difficult to distinguish between
inhibition of entry during initial infection and subsequent inhibition of HIV-1 cell-to-cell spread. These findings suggest that E2 antibodies inhibit HIV-1 attachment and that E2 antibodies need to be maintained in the culture media to inhibit cell-to-cell spread.

Identification of the epitope on HIV-1 virions to which E2 antibody binds is necessary to elucidate the mechanisms of HIV inhibition. Pseudotyped particles with different viral envelope proteins, such as VSV-G and MLV env, can be utilized to determine whether the E2 antibody epitope is HIV gp120, gp41, or another cellular protein carried on HIV-1 particles. In addition, defective VSV particles pseudotyped with the HIV-1 envelope can be used to ascertain whether the HIV-1 envelope is required for E2 antibody HIV neutralization. We utilized the tools of pseudotyped particles to find that E2 antibodies recognize a cellular antigen on retroviral particles and neutralize HIV-1 pseudotyped retroviral particles but not defective VSV particles. In addition, we found that the E2 antibody cellular epitope is only exposed on permeabilized cells (capable of producing infectious and pseudotyped virions), and not intact cells, suggesting that the E2 antibody epitope is exposed upon viral budding and is exposed on the surface of the virus.

Methods

Antigens and antibodies: GBV-C E2 protein truncated to remove the C-terminal transmembrane domain (nt 1167 to 2162 based on the infectious clone isolate, GenBank number AF121950) (Xiang et al., 2000) was expressed in CHO cells as described (McLinden et al., 2006). Rabbit anti-GBV-C E2 antibodies were elicited upon immunization with 50 µg E2 in IFA (Invitrogen, Carlsbad, CA) and boosted 6 and 12 weeks later by the Iowa State University Hybridoma Facility. Sera were collected before
immunization (pre-immune) and after the final boost (17 weeks after initial immunization) for analysis and IgG purification as described (McLinden et al., 2006).

Murine anti-GBV-C E2 mAb M6 (Roche Laboratories, Penzberg, Germany) was provided by Dr. Alfred Engel. Isotype control antibodies were purchased from Sigma, Inc. St. Louis, MO. M6 recognizes a linear epitope on GBV-C E2 (McLinden et al., 2006). HIV-neutralizing human mAb 2G12 was obtained from the NIH AIDS Research Reference and Reagent program (Catalog # 1476).

**Cells and viruses:** PBMCs were isolated from blood obtained from healthy donors. All human subjects provided written informed consent and the project was approved by the University of Iowa Institutional Review Board. The CD4\(^+\) cell line (human osteosarcoma [HOS] cells expressing human CD4, CCR5, and CXCR4) was maintained as previously described (Montefiori, 2004; Wuenschmann & Stapleton, 2000; Xiang et al., 2004). The HOS cells were obtained from the NIH Reagent program, and also contained GFP under the control of HIV-1 LTR (catalog # 3942). 293T cells were maintained in DMEM supplemented with 10% fetal calf serum and antibiotics.

**Pseudotyped virus production:** 293T cells were co-transfected with pNL4-3.Luc.R-E- (luciferase reporter inserted into pNL4-3 nef gene), and a plasmid that expresses the envelope glycoproteins of either VSV (VSV-G), HIV (pHXB2-env), MLV (MLV env), GBV-C E1-E2 (nt 555-2479), or no viral envelope protein using CaCl\(_2\) as described (Sinn et al., 2003). Particles were collected 72 hours post-transfection, filtered with a 0.45 \(\mu\)m filter and concentrated by centrifugation at 12,000 rpm for 20 hours at 4°C. Alternatively, 293T cells were transfected with an HIV envelope (pHXB2) protein-expressing plasmid and transduced with defective VSV particles with a GFP reporter
gene kindly provided by Dr. Wendy Maury, University of Iowa. Pseudotyped VSV particles were collected from supernatants and filtered with a 0.45 µm filter prior to use.

**Viral infections and neutralization assays:** Defective HIV or VSV particles (produced in 293T cells) were incubated with a range of concentrations of GBV-C E2 antibody-positive IgG preparations for 1 hour at 37°C prior to adding to HOS cell cultures. E2 antibody negative IgG preparations served as the negative control antibodies. Viral particle transduction was measured by reporter gene expression 72 hrs post-transduction. Pseudoparticle transduction was measured by luciferase activity (BrightGlo Luciferae Assay System, Promega) or by GFP expression (flow cytometry). All neutralization experiments were performed in quadruplicate, and independently repeated at least once with consistent results.

**Immune precipitation:** Defective HIV-1 pseudoparticles were mixed with antibodies at various concentrations and incubated while mixing overnight at 4°C. All pseudoparticles were prepared in 293T cells. Heat-killed, formalin fixed *Staphylococcus aureus* cells with a coat of protein A (Pansorbin, Calbiochem, Inc., San Diego, CA) were added and incubated for 2 hr at 4°C. Cells were pelleted, washed 3 times in PBS containing 0.5% Tween-20, resuspended in PBS, and viral particle precipitation was measured (p24 antigen ELISA). For the E2 competition assay, GBV-C E2 or E2 negative CHO-S cell supernatants were incubated with E2 antibody for 1 hr at 37°C prior to incubating the antibody with virus particles.

**Flow cytometry:** HOS cells, 293T cells and PBMCs were incubated with anti-GBV-C M6 mAb or IgG2a isotype control antibody (BD Biosciences) for 1 hr at 4°C. Antibody was added to intact cells or permeabilized cells (permeabilized using the BD
Cytofix/Cytoperm kit as recommended by the manufacturer (BD Biosciences, Franklin Lakes, NJ). Cells were washed and PE-labeled anti-mouse secondary antibody (Southern Biotech, Birmingham, AL) was applied for 30 min at 4°C. Antibody binding was assessed by flow cytometry as described (Kaufman et al., 2007; Wunschmann et al., 2000).

Statistics: Statistics were performed using SigmaStat software V3.11 (Jandel Scientific, Chicago, IL). T-tests were used for direct comparisons for individual data points

Results

**GBV-C E2 antibodies precipitate HIV retroviral particles**

HIV-1 envelope protein (gp120/41), GBV-C envelope protein (E1 and E2) and VSV envelope protein (G) were used to pseudotype defective HIV-1 retroviral particles generated in 293T cells as described in the Methods section. Retroviral particles without a viral envelope protein were also generated in 293T cells. Rabbit anti-GBV-C E2 IgG precipitated HIV env and GBV-C env pseudotyped retrovirus particles significantly more than pre-immune IgG or no antibody control (Fig. 3.1). Interestingly, rabbit anti-GBV-C E2 IgG also precipitated retrovirus particles pseudotyped with the VSV-G protein or with no viral envelope (Fig. 3.1), indicating that precipitation did not involve the viral envelope proteins. GBV-C enveloped retroviral pseudoparticles (GBV-C env) were precipitated to a significantly greater extent than the other defective particles by anti-E2 antibody, suggesting that, in addition to the retroviral particle antigen recognized by these antibodies, interactions with the GBV-C E2 protein contributed to the immune precipitation (Fig. 3.1). Immunoprecipitation of retroviral particles (HIV env and GBV-
C env) was specific for the E2 antibody, because addition of E2-containing CHO-S supernatants (50 µg/ml total protein concentration) blocked immunoprecipitation, whereas addition of CHO-S supernatants without E2 (50 µg/ml total protein concentration) did not block immunoprecipitation (Fig. 3.2). Although M6 and the anti-gp120 (2G12) mAb precipitated HIV-1 pseudotyped retroviral particles (HIV env particles) more than the IgG2a and human polyclonal IgG control antibodies, the percent of input of defective HIV env particles and GBV-C env particles precipitated by all of these mAbs was very low (< 2% of input virus), suggesting that their reactivity with HIV-1 retroviral particles is significantly weaker than the rabbit polyclonal anti-GBV-C E2 antibody.

*E2 antibody neutralizes HIV gp160 pseudotyped retroviral particles*

Single cycle replication assays allow assessment of viral envelope specificity, and may provide information regarding the step(s) of HIV replication which are inhibited. The HIV-1 envelope protein (gp120/41) was used to pseudotype defective HIV-1 retroviral particles or defective VSV particles generated in 293T cells as described in the Methods section. The ability of the rabbit anti-GBV-C E2 IgG to neutralize these pseudotyped particles was assessed in a HOS cell-based assay (HOS cells expressing human CD4, CXCR4, and CCR5). IgG was mixed with the particles prior to addition to cells. The anti-GBV-C E2 IgG preparation neutralized HIV env pseudotyped retrovirus particles in a dose-dependent fashion; however, this antibody did not neutralize HIV env pseudotyped VSV particles (Fig. 3.3). Pre-immune rabbit IgG did not neutralize either particle type. This suggests that anti-E2 antibody does not need to interact specifically with HIV gp120/41 for HIV neutralization. These findings also suggest that the
environment surrounding the HIV envelope protein may be important for neutralization.

The HIV envelope proteins on defective VSV particles are expected to enter cells via the
same route (CD4 and coreceptor attachment, then fusion) as the HIV envelope proteins
on retroviral particles; thus, there should be no different in entry methods. But because
HIV and VSV particles may bud from different lipid rafts on the plasma membrane, the
cellular lipid and protein environment around the HIV envelope proteins may differ.

To determine whether E2 antibody neutralization of retroviral particles is
independent from the HIV envelope protein and the transmembrane proteins surrounding
the HIV envelope protein on HIV-1 retroviral particles, the ability of the rabbit anti-
GBV-C E2 IgG to neutralize retroviral particles pseudotyped with the HIV gp120/41,
VSV-G or MLV envelope proteins was measured. The anti-E2 IgG preparation
neutralized HIV-1 gp120/41 pseudotyped retroviral particles in a dose-dependent fashion,
but not the VSV-G or MLV env pseudotyped retroviral particles (Fig. 3.3). Pre-immune
rabbit IgG did not neutralize any of the particle types. This suggests that although the
HIV gp160 envelope protein is not required for neutralization, the environment
surrounding the HIV gp160 envelope protein is important. This environment may
include transmembrane proteins or lipids.

Together, these data indicate that the anti-E2 antibodies neutralize HIV-1
retroviral particles (Table 3.2). Because retroviral particles pseudotyped with the VSV-G
or MLV envelope protein were not neutralized, this suggests that neutralization with E2
antibodies is dependent upon the HIV-1 retroviral particle environment surrounding the
HIV-1 envelope protein. The VSV-G and MLV envelope proteins may not be arranged
in the same transmembrane protein environment as HIV gp160, perhaps due to budding
from different areas of the cellular membrane or from lipids rafts of differing composition. These data are consistent with the hypothesis that neutralizing E2 antibodies may bind to a non-gp120/41 protein on the retroviral particle, and that this prevents gp120 from interacting with cellular receptors, but not VSV-G or MLV env from interacting with their cellular receptors.

*Anti-GBV-C E2 antibodies interfere with HIV-1 particle binding*

Rabbit anti-GBV-C E2 antibodies inhibit HIV-1 attachment to MT-2 cells. Previous work in the lab aimed to determine whether E2 antibodies inhibited HIV post-attachment entry steps in addition to inhibiting HIV attachment. Because the surrounding cells are infected in cell-to-cell spread in a productive HIV-1 virion infection, it was difficult to distinguish between inhibition of entry during initial infection and subsequent inhibition of HIV-1 cell-to-cell spread. Thus, we assessed this in a single cycle of infection with pseudotyped particles. HIV env pseudotyped retroviral particles generated in 293T cells were added to HOS cells for 2 hr at 4°C. Pre- or post-immune (anti-E2) IgG was added to the cells following viral attachment and the cells were warmed to 37°C to allow viral entry. Luciferase activity was measured 72 hrs later, and HIV-1 transduction was not inhibited (Fig. 3.4). Thus, once HIV particles are bound to CD4, access to the cross-reacting antigen on viral particles was blocked, and the antibodies were not able to block subsequent entry steps.

*Characterization of E2 antibody cellular antigen*

To characterize the cellular molecule interacting with anti-GBV-C E2 antibodies on viral particles, we compared the presence of the molecule on intact cells to the presence of the molecule on permeabilized cells. Infectious HIV particles were generated
in HOS cells and PBMCs, and pseudotyped retroviral particles were generated in 293T cells, so these cells were used for analysis. We utilized the murine anti-E2 M6 mAb for these studies, which was generated by DNA immunization, and not the polyclonal rabbit anti-E2 antibodies because the rabbit anti-E2 protein antibody used in the previous studies binds to cell surfaces at high levels. The rabbit antibody was generated by immunization with CHO cell supernatants containing E2, so it was expected that it would bind to cell surfaces. The murine anti-E2 M6 mAb did not interact with intact HOS cells, PBMCs or 293T cells as measured by flow cytometry, but did react with HOS cells, PBMCs and 293T cells following fixation and permeabilization (Fig. 3.5). This suggests that E2 antibodies interact with a cellular antigen that is exposed upon permeabilization, or perhaps budding of viral particles, and not on the surface of intact cells. The fact that multiple HIV particle-producing cell types contain a cellular antigen recognized by E2 antibodies upon permeabilization indicates that the cellular antigen is conserved in many cell lines. No additional cell types have been examined; thus, no virus-producing cell types which do not contain the E2 antibody-interacting antigen have been identified.

**Discussion**

Anti-GBV-C E2 antibodies precipitated defective HIV-1 particles pseudotyped with many different viral envelope proteins, including HIV-1 gp120/41, VSV-G, GBV-C E1/E2, and retroviral particles that bud from 293T cells with no viral envelope protein. This suggests that a cellular antigen, common to all of these retroviral particles, is present on the particle surface and that E2 antibodies interact with this cellular antigen. The incorporation of cellular molecules on HIV-1 virions is well known and Gag protein assembly at lipid rafts influences which proteins are incorporated into the budding virus
(Waheed & Freed, 2009). The cellular molecules incorporated into the viral particle are important because they influence virus infectivity. Cell adhesion molecules (ICAM-1, HLA-II and Galectin-1, among others) increase HIV attachment to T cells and dendritic cells (Ott, 2008). Other cellular adhesion molecules enhance HIV infection by increasing the capture of virions by endothelial cells and B cells for presentation to T cells (Ott, 2008).

Determining the role of the specific virus particle type in immunoprecipitation is important; in these experiments, only retrovirus particles were used. Defective VSV particles with different viral envelope proteins should also be examined for precipitation by anti-E2 antibodies to determine whether these particles bear the cellular antigen which interacts with E2 antibodies. We attempted to immunoprecipitate defective VSV particles bearing the HIV envelope, but did not continue with the experiments after the positive control particles, defective VSV particles bearing the VSV-G envelope protein, were not immunoprecipitated by anti-VSV-G antibodies. VSV particles also bud from the plasma membrane, as does HIV, but may bud from different lipid rafts. YFV and mumps virus were not immune precipitated by E2 antibodies, so defective VSV particles may not be immunoprecipitated by anti-E2 antibodies either.

In addition to immune precipitating HIV particles, the GBV-C E2 antibodies elicited by immunization with E2 protein neutralized HIV-1 env pseudotyped retroviral transduction in vitro (Table 3.2). Because entry of defective VSV particles bearing the HIV envelope glycoproteins is dependent on the HIV envelope glycoprotein and VSV particles displaying HIV-1 envelope glycoproteins were not neutralized by the anti-GBV-C E2 antibodies, this indicates that the anti-E2 antibodies do not specifically require HIV
gp120/41 protein interaction for neutralization. However, retroviral particles pseudotyped with VSV-G or MLV envelope proteins were not neutralized either, suggesting that neutralization is not completely independent from the HIV env protein. Thus, although the E2 antibody binds a cellular antigen on all the retroviral particles examined, it appears that only the HIV gp120 envelope protein is prevented from binding to its cellular receptors. This could be explained by the blocking of the HIV gp120 receptor-binding site when the E2 antibody interacts with the adjacent cross-reacting cellular antigen. The receptor-binding sites of VSV-G or MLV env proteins may not be blocked by the E2 antibody interaction with the cellular antigen. This mechanism of inhibition agrees with our finding that HIV-1 neutralization required incubation of anti-E2 antibodies with the viral particles before interaction with the cell surface, since the antibodies did not block entry following viral attachment in single cycle infections. Pre-incubation of the E2 antibodies with HIV-1 particles reduced virus-cell attachment, thus HIV-1 replication is inhibited at least in part by decreasing HIV-1 attachment.

One of the first steps to characterizing the cellular antigen which interacts with E2 antibodies is to determine where the antigen is located in cells which produce infectious HIV virions and pseudotyped retroviral particles. We found that anti-GBV-C E2 antibodies interacted with cellular antigen(s) following cell permeabilization, suggesting that anti-E2 antibodies cross-react with an antigen that is not exposed on the cell surface. This cellular protein may be located on the inner layer of the plasma membrane and its orientation may be reversed during viral budding, or it may normally be located in the Golgi and HIV infection results in its relocation to the plasma membrane.

Phosphatidylserine is an example of a lipid which is flipped from the inner layer of the
plasma membrane to the outer layer, although this occurs in response to apoptosis triggers from vaccinia infection (Fairn & Grinstein, 2008). Phosphatidylserine also plays a role in HIV infection because it is present on the viral envelope (Fairn & Grinstein, 2008), so other cellular lipids and proteins may also be flipped in response to HIV budding. The finding that HIV neutralizing anti-E2 antibodies recognize a cellular antigen is similar to the findings of studies of well-characterized and broadly neutralizing HIV-1 antibodies 2F5 and 4E10 (Table 3.3), which react with both HIV-1 gp41 peptides and permeabilized cells (Haynes et al., 2005). Studies are underway to further characterize the cellular antigen recognized by the anti-GBV-C E2 antibodies. These studies will likely include examining the cellular location of the antigen recognized by anti-E2 antibodies with confocal microscopy in HIV infected and uninfected cells. In addition, nonhuman cell lines which do not support HIV replication should be examined for their ability to interact with E2 antibodies when permeabilized. If specific human cellular proteins directly involved in HIV budding are the cellular antigen for E2 antibodies, then the nonhuman cell types would be good negative controls.

GBV-C E2 protein neutralizes HIV-1 by inhibiting entry (Jung et al., 2007; Mohr & Stapleton, 2009); thus, viremia and E2 antibody both influence HIV-1 replication in vitro. During persistent viremia, the GBV-C concentration in infected humans is typically >50 million genome equivalents per mL of plasma, and GBV-C is produced by B lymphocytes and both CD4+ and CD8+ T lymphocytes (George et al., 2006). Since each virus particle is predicted to have multiple copies of E2 protein on the surface, lymphocytes are constantly exposed to low concentrations of E2 protein, which may explain in part why persistent viremia is associated with the best survival outcome, while
E2 antibodies are associated with an intermediate survival. In addition, the serum concentration of GBV-C E2 antibodies in humans varies over time, and may drop to levels below the limit of detection during longitudinal follow up (Linnen et al., 1996; Tillmann et al., 2001). It is likely that the E2 antibody concentration is an important variable in determining the magnitude of any potential clinical effect of GBV-C E2 antibodies.

No significant amino acid sequence identity between GBV-C E2 protein and either HIV-1 or cellular proteins was identified in a protein-protein Blast search. Several studies have identified HIV-1 neutralizing antibodies in approximately 20% of humans with HIV-1 infection, and a lower percentage in HIV-uninfected people (Doria-Rose et al., 2010; Galea et al., 1999; Hasselrot et al., 2009; Hasselrot et al., 2010; Lopalco et al., 2010; Mazzoli et al., 2010; Sei et al., 1988). There are few data suggesting a beneficial clinical course in those with HIV neutralizing antibodies compared to those without. However, a modest association between GBV-C E2 antibody and prolonged survival was observed by Tillmann et al. (Tillmann et al., 2001), and in the MACS cohort (Mohr et al., 2010). There are clearly neutralizing antibodies present in HIV-1 infected people that are not due to GBV-C E2 antibodies; thus, measuring survival on the basis of the presence of HIV-1 neutralizing antibodies independent of the presence of GBV-C E2 antibody will confound the analysis. It will be interesting to determine what proportion of HIV-1 neutralizing antibodies in HIV-infected individuals is directed against the GBV-C E2 protein.

A concern related to the use of non-HIV-1 antigens to invoke HIV-1 neutralizing antibodies is that these may induce auto-antibodies. Since approximately 10% of healthy
U.S. blood donors have antibody to GBV-C E2 protein (Heuft et al., 1998; Pilot-Matias et al., 1996; Tacke et al., 1997b), it appears that GBV-C antibodies do not induce any ill effects in humans. Furthermore, the GBV-C E2 antibodies appear to recognize cellular proteins carried on HIV-1 particles that are not displayed on the surface of cell plasma membranes.

Currently, no commercial GBV-C E2 mAbs or hybridoma cell lines producing GBV-C E2 mAbs are available ((Blankson et al., 2008) and personal communication George Hess, Roche Diagnostics, Penzberg, Germany, and Biodesign, Inc.). Consequently, the development of additional GBV-C E2 mAbs to allow further characterization of the antigen(s) that elicits the HIV-1 cross-reacting neutralizing antibodies is needed. Characterization of the immunogenic domain(s) on the GBV-C E2 protein and its interactions with HIV particles may provide novel HIV-1 candidate vaccines.

Since a high proportion of HIV-1 infected people have GBV-C E2 antibodies, it is unlikely that this antibody will, by itself, prove highly protective. Nevertheless, identification of immunogens that elicit broadly HIV-1 neutralizing antibodies may contribute to protection induced by a multivalent HIV-1 vaccine, or they may delay or modify HIV-1 disease progression. Characterization of the E2 antigenic structure responsible for eliciting HIV-1 neutralizing activity may identify conserved targets for drug design, and testing of GBV-C E2 protein as a candidate HIV-1 vaccine antigen appears warranted.
Table 3.1. Summary of HIV Neutralizing Antibodies.

<table>
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<tr>
<th>HIV neutralizing antibody</th>
<th>Target</th>
<th>Method of HIV neutralization</th>
<th>Special features</th>
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<tbody>
<tr>
<td>2G12</td>
<td>gp120</td>
<td>Interferes with CD4 binding</td>
<td></td>
</tr>
<tr>
<td>2F5</td>
<td>MPER of gp41 with lipids</td>
<td>Disrupts MPER fusogenic functions</td>
<td>Cross-reacts with cellular antigens</td>
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<tr>
<td>4E10</td>
<td>MPER of gp41 with lipids</td>
<td>Disrupts MPER fusogenic functions</td>
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Figure 3.1. Rabbit anti–GBV-C E2 Abs precipitate retroviral particles. Defective HIV retroviral particles displaying HIV, VSV, or GBV-C envelope glycoproteins, or retroviral particles with no virus envelope glycoproteins, were precipitated by postimmune rabbit anti-E2 IgG and not by preimmune rabbit IgG (*p < 0.01 compared with preimmune IgG; †p < 0.05 compared with other postimmune IgG); t-test. Each bar represents a single experiment with samples done in triplicate and is representative of at least 3 experiments.
Figure 3.2. Rabbit anti–GBV-C E2 Abs precipitation of retroviral particles is specific. Post-immune IgG (Post) precipitated HIV env and GBV-C env pseudotyped retroviral particles at higher levels than pre-immune IgG (Pre). CHO cell culture supernatants containing E2 (E2) protein (at 50 µg/ml total protein concentration) inhibited precipitation with post-immune IgG, whereas CHO cell culture supernatants without E2 (E2 neg) (at 50 µg/ml total protein concentration) did not. (*p < 0.05; †p > 0.5); t-test. Each bar represents a single experiment with samples done in triplicate and is representative of at least 2 experiments.
Figure 3.3. Rabbit anti–GBV-C E2 Abs neutralize HIV–enveloped retroviral particles but not HIV–enveloped VSV particles or other retroviral particles. Post-immune anti-E2 rabbit IgG neutralized HIV enveloped retroviral particles (HIV env Gag particle) but not HIV enveloped defective VSV particles (HIV env VSVΔG particle), and pre-immune rabbit IgG did not neutralize either. Post-immune anti-E2 antibody also did not neutralize VSV enveloped retroviral particles (VSV env Gag particle) or MLV enveloped retroviral particles (MLV env Gag particle). The rectangles outline the specific particle type. The dashed line denotes particles with HIV envelopes and the dotted line denotes retroviral particles. †p<0.001 compared with no Ab control and pre-immune antibody 10 µg/ml; ‡p=0.021 compared with no Ab control. Each bar represents a single experiment with samples done in triplicate and is representative of at least 2 experiments.
Table 3.2. Summary of Defective Particle Neutralizations and Immunoprecipitations by Anti-E2 Antibody.

<table>
<thead>
<tr>
<th>Defective Particle Backbone</th>
<th>Viral Envelope Protein</th>
<th>Neutralized by anti-E2 protein antibody</th>
<th>Immunoprecipitated by anti-E2 protein antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1</td>
<td>HIV-1 gp120/41</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>VSV</td>
<td>HIV-1 gp120/41</td>
<td>No</td>
<td>Not examined</td>
</tr>
<tr>
<td>HIV-1</td>
<td>VSV-G</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>HIV-1</td>
<td>MLV env</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Figure 3.4. GBV-C Abs do not block HIV-1 entry following HIV-1 attachment to cells. Following attachment of HIV enveloped retroviral particles to HOS cells for 2 h at 4°C, pre- or postimmune rabbit GBV-C E2 IgG was added at the concentrations indicated, the cells were warmed to 37°C and transduction (luciferase activity) was measured 3 days later. Each bar represents a single experiment with samples done in quadruplicate and is representative of at least 2 experiments.
Figure 3.5. Anti–GBV-C E2 Abs interact with a cellular antigen in permeabilized cells, and not intact cells. Intact PBMCs, HOS cells and 293T cells were incubated with the murine anti-E2 M6 mAb to measure surface staining by flow cytometry. M6 reactivity increased in all cells tested following fixation and permeabilization. IC, isotype control Ab.
Table 3.3. Summary of HIV Neutralizing Antibodies Including Anti-E2 Antibodies.

<table>
<thead>
<tr>
<th>HIV neutralizing antibody</th>
<th>Target</th>
<th>Method of HIV neutralization</th>
<th>Special features</th>
</tr>
</thead>
<tbody>
<tr>
<td>2G12</td>
<td>gp120</td>
<td>Interferes with CD4 binding</td>
<td></td>
</tr>
<tr>
<td>2F5</td>
<td>MPER of gp41 with lipids</td>
<td>Disrupts MPER fusogenic functions</td>
<td>Cross-reacts with cellular antigens</td>
</tr>
<tr>
<td>4E10</td>
<td>MPER of gp41 with lipids</td>
<td>Disrupts MPER fusogenic functions</td>
<td>Cross-reacts with cellular antigens</td>
</tr>
<tr>
<td>Anti-E2 antibodies</td>
<td>GBV-C E2 &amp; cellular antigen</td>
<td>Binds to cellular antigen on HIV virion and blocks attachment</td>
<td>Cross-reacts with cellular antigen which is only detected in permeabilized cells</td>
</tr>
</tbody>
</table>
CHAPTER IV: NATURAL HISTORY OF CHIMPANZEE GBV-C

Introduction

Following the discovery of Hepatitis C virus (HCV) in 1989, virus discovery groups searched for novel etiological agents responsible for non-A, non-B, non-C hepatitis. In the process, human and primate viruses related to HCV were identified. Abbott laboratories identified two viruses in tamarins which they named GBV-A and GBV-B (Schaluder et al., 1995). The tamarins in which these viruses were identified had been inoculated with the 12th passage of tamarin plasma. The initial tamarin had been inoculated with serum from a surgeon with non-A, non-B hepatitis whose initials were G.B. (Schaluder et al., 1995). GBV-A and GBV-B were not identified in any human sera (Schaluder et al., 1995); however, using degenerate oligonucleotides to amplify related viral sequences, these investigators discovered a human virus which they named GBV-C (Simons et al., 1995a). Concurrently, Genelabs Inc. discovered a virus in a patient with HCV infection that they called hepatitis G virus (HGV) (Linnen et al., 1996). Sequence comparisons revealed that HGV and GBV-C were different isolates of the same species (Linnen et al., 1996). Based on phylogenetic relationships, the GB viruses and HCV are classified as members of the Flaviviridae. GBV-A is closely related to GBV-C, and neither virus is associated with hepatitis or any other disease. GBV-B is more closely related to HCV, and causes hepatitis in tamarins and owl monkeys (Adams et al., 1998;Schaluder et al., 1995).

GB virus C (GBV-C) is a lymphotropic virus associated with improved survival in HIV-infected individuals (Stapleton & Chaloner, 2004;Zhang et al., 2006). The prevalence of GBV-C viremia ranges from 1-5% in healthy human blood donors and is significantly higher (up to 42%) in individuals with other blood borne or sexually transmitted infections (Mohr & Stapleton, 2009;Rey et al., 2000;Stapleton, 2003;Thomas et al., 1998;Williams et al., 2004). GBV-C may cause persistent infection, and
approximately 80% of HIV-infected individuals maintain GBV-C viremia for at least 5 years (Williams et al., 2004). However, the majority of immune competent individuals appear to clear GBV-C viremia within 2 years (Hitzler & Runkel, 2004; Theodore & Lemon, 1997; Thomas et al., 1998). Unlike HCV, antibodies to GBV-C are not readily detected during viremia (Heuft et al., 1998); however, antibodies to the GBV-C envelope glycoprotein E2 are detected in individuals following clearance of viremia. Concurrent detection of GBV-C E2 antibodies and viremia is uncommon, with fewer than 7% of individuals having concurrent anti-E2 antibody and GBV-C viremia (Lefrère et al., 1997; Sauleda et al., 1999). Anti-E2 antibodies appear to partially protect against reinfection (Hassoba et al., 1998; Thomas et al., 1998; Tillmann et al., 1998). Based on the prevalence of E2 antibody to GBV-C RNA in healthy blood donors, it appears that approximately 75% to 80% of GBV infections are cleared (Heuft et al., 1998).

Abbott Laboratories identified a variant of GBV-C (GBV-C_trog) in an HCV-infected chimpanzee with resolving hepatitis (Birkenmeyer et al., 1998), and reported a near-complete genome sequence (GenBank accession number AF070476) (Birkenmeyer et al., 1998). Adams et al. also identified GBV-C RNA in 3 of 39 noncaptive chimpanzees (subspecies troglodytes and verus) that they called GBV-C_cpz (Adams et al., 1998). For the remainder of the thesis, the chimpanzee variant of GBV-C will be noted with the designation GBV-C_cpz rather than GBV-C_trog. GBV-C_cpz infection was not found in human or macaque monkey blood samples (Birkenmeyer et al., 1998). The GBV-C_cpz polyprotein shares 83.6% amino acid identity with GBV-C, while human GBV-C isolates are >95% identical (Mohr & Stapleton, 2009; Muerhoff et al., 1995; Pavesi, 2001). Based on a limited phylogenetic analysis of sequences from the 5’ntr region, helicase and RNA dependent, RNA polymerase (RdRp), all of the GBV-C_cpz sequences are monophyletic within a group of GBV-C viruses from humans and chimpanzees (Adams et al., 1998). Thus, GBV-C_cpz is considered a chimpanzee variant of GBV-C rather than a separate genotype. Sequence analyses of all available chimpanzee GBV-C sequences from Abbott
laboratories (named GBV-C<sub>trog</sub>) and Adams et al. (named GBV-C<sub>cpz</sub>) demonstrated that these viruses were different isolates of the GBV-C<sub>cpz</sub> variant (Adams <i>et al.</i>, 1998; Birkenmeyer <i>et al.</i>, 1998).

The structure of human GBV-C and GBV-C<sub>cpz</sub> envelope proteins have not been described but are predicted to be similar to HCV envelope protein structure based on sequence identity. HCV envelope proteins E1 and E2 contain multifunctional transmembrane regions (TM) in their C-terminal regions. The TM domains function as membrane anchors, ER retention signals and are involved in E1E2 heterodimerization (Cocquerel <i>et al.</i>, 2002). The same is predicted for GBV-C envelope proteins, although there are no experimental studies which examine the topology of the transmembrane domains of human GBV-C and GBV-C<sub>cpz</sub>.

Although Adams et al. detected GBV-C<sub>cpz</sub> viremia in 3 of 39 noncaptive chimpanzees and generated partial sequences for one chimpanzee with samples 24 months apart, the prevalence and natural history of GBV-C<sub>cpz</sub> has not been otherwise examined (Adams <i>et al.</i>, 1998). At the time GBV-C<sub>cpz</sub> was identified, serological reagents to detect GBV-C E2 antibodies were not available, so there are no data published on the presence of E2 antibodies in chimpanzees. In this study, we examine the prevalence and natural history of GBV-C<sub>cpz</sub> in a large cohort of captive chimpanzees and predict the topology of the transmembrane domains of human and chimpanzee GBV-C using computer analyses.

**Methods**

<i>Sample identification: </i>Chimpanzee (<i>Pan troglodytes troglodytes</i>) serum samples (frozen serum samples) were obtained from repositories located at the Southwest Foundation for Biomedical Research [SFBR; NHBLI colony and Southwest National Primate Research Center colony (n=81)], San Antonio, TX and from University of Texas MD Anderson Cancer Center (n= 154), Bastrop, TX. A single serum sample from each animal was tested for GBV-C RNA, and longitudinal samples were studied in animals
that tested positive when available. Samples were also tested for the presence of anti-GBV-C E2 antibodies. Demographic information, infection history and blood product history for GBV-C RNA+ animals are shown in Table 4.1.

**GBV-C RNA detection:** RNA was extracted from chimpanzee serum samples using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). RNA was stored at -80°C until use. Reverse transcription was performed using a Moloney murine leukemia virus reverse transcriptase mutant with reduced RNase H activity (SuperScript II; Invitrogen, Carlsbad, CA), and PCR was performed using high-fidelity Taq polymerase (Platinum Taq DNA Polymerase High Fidelity; Invitrogen). Oligonucleotide primers employed are shown in Table 4.2.

PCR products were purified using the QIAquick PCR purification kit (Qiagen), ligated with pCR2.1 (TA Cloning Kit, Invitrogen), and INVFA or DH5α competent cells (Invitrogen) were transformed. Six colonies were randomly selected to study sequence diversity. Plasmid DNA was purified (WizardPlus SV Miniprep DNA Purification System, Promega) and sequenced (ABI sequencer, University of Iowa DNA Facility). Nucleotide sequences were entered into GenBank with accession numbers HM626487 – HM626506, HM638234 - HM638236, and HM769722.

Sequence analysis was performed using DNAmam (Linnen, Biosoft) and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007). Sequences were aligned with the Clustal W method, evolutionary histories were inferred using the UPGMA method (Sneath & Sokal, 1973) and bootstrap consensus trees were inferred from 2000 replicates (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) or the Poisson correction method (Zuckerkandl & Pauling, 1965). dN and dS values were estimated using the Nei-Gojobori method in MEGA4 (Nei & Gojobori, 1986; Tamura et al., 2007). GBV-C isolates representing the different genotypes were: AB003291, genotype 1; AF121950, genotype 2; U94695, genotype 3;
AB003292, genotype 4; AY949771, genotype 5. Representative isolates of three HCV genotypes were: AF0011753, genotype 1a; AF333324, genotype 1b; D14853, genotype 1c; D00944, genotype 2a; D10988, genotype 2b; AF046866, genotype 3a. GBV-A (U94421) and GBV-B (AJ277947) sequences were also analyzed. Hydrophobic cluster analyses were performed with the online program available from the Ressource Parisienne en Bioinformatique Structurale server.

For real-time PCR, RNA was amplified using 5’-ntr primers and a 6-carboxyfluorescein/6-carboxytetramethylrhodamine-labeled probe as previously described (Souza et al., 2006) (Table 4.2) using the SuperScript II Platinum One-step Quantitative RT-PCR (Invitrogen) as recommended by the manufacturer. A standard curve was generated using an 842nt GBV-C 5’ntr (AF121950) synthetic RNA and was confirmed for GBV-C amplification by terminal dilution. This standard curve was also used for GBV-C<sub>cpz</sub>. Results were analyzed with 7500 System SDS Software.

**E2 antibody detection:** Serum samples were tested for E2 antibodies with either the µPlate anti-HGenv test (kindly provided by Dr. Georg Hess, Roche Diagnostics, Mannheim, Germany), or by using an in-house assay when the commercial assay was no longer available. The sensitivity and specificity of the µPlate anti-HGenv test and in-house assay correlated (overall regression = 0.76). For the in-house assay, Nunc Immobilizer plates were coated with recombinant GBV-C E2 expressed in CHO cells as described (McLinden et al., 2006), blocked with PBS containing 0.02% Triton X, 0.02% Azide, 1% BSA and 2.5% FCS. Serum diluted 1:50 was added to wells for 1 hour at 37°C. Wells were washed and bound antibody was detected using alkaline phosphatase labeled anti-human Fc antibodies (Sigma) followed by incubation with P-nitrophenylphosphate diluted in diethanolamine buffer for 1 hour at 37°C. The absorbance was measured at 405nm after 30 min.
Results

GBV-C\textsubscript{cpz} prevalence and natural history in captive chimpanzees

Serum samples from 235 captive chimpanzees were tested by nested RT-PCR using primers designed from a human GBV-C 5’ non-translated region (ntr) sequence (AF121950) or by real-time RT-PCR using primers and probe designed to amplify GBV-C\textsubscript{cpz} (Table 4.2). Seven of the 235 (3.0%) samples contained GBV-C RNA. One of these samples came from the Southwest Foundation for Biomedical Research (SBRF) and the remaining six samples came from the University of Texas MD Anderson Cancer Center. Sequence analysis was successful for 5 of the samples (Candie, 1855, P187, 3915, 3912) and alignments of 4 of the sequences demonstrated that the sequences aligned more closely with GBV-C\textsubscript{cpz} than with human GBV-C and one sequence shared the highest sequence identity with human GBV-C (Fig. 4.1A). Sequence analysis was not successful for 2 of the 7 animals, although GBV-C\textsubscript{cpz} viremia is presumed because the amplification was successful only when GBV-C\textsubscript{cpz} specific primers were utilized. Two of the GBV-C\textsubscript{cpz} positive samples tested positive in all available samples, demonstrating persistent infection of at least 4 or 19 years, respectively (chimpanzees Candie and 1855) (Fig. 4.1A). The remaining four animals had transient viremia with only one sample containing GBV-C\textsubscript{cpz} RNA (Fig. 4.1A).

E2 antibody was detected in 26 of the 235 chimpanzee serum samples (11.1%). The two persistently infected chimpanzees did not have E2 antibody detected in any of their samples, and one chimpanzee with transient viremia did not develop E2 antibodies (Fig. 4.1A). In contrast, E2 antibodies were detected in the other 3 GBV-C\textsubscript{cpz}-transiently infected chimpanzees. One of these animals had detectable E2 antibody levels after GBV-C\textsubscript{cpz} viremia, consistent with seroconversion, while the other 2 transiently viremic animals had E2 antibody detected before and after GBV-C\textsubscript{cpz} viremia. The chimpanzee with transient human GBV-C viremia was also positive for E2 antibody on multiple
sample dates surrounding the period of viremia. None of the chimpanzees received human blood products (Table 4.1).

Among the chimpanzees with persistent viremia (Candie and 1855), the serum viral load remained constant with an average of $5.3 \times 10^8$ genome equivalents (GE)/ml for chimpanzee Candie and $7.3 \times 10^8$ GE/ml for chimpanzee 1855 over 4 years or 19 years, respectively (Fig. 4.1B).

Sequences from the 5’ ntr and NS5A/B coding region were determined at early and late infection time points in the animals with persistent infection, chimpanzees 1855 and Candie (Fig. 4.2). No nucleotide changes were observed in a 329 nucleotide (nt) sequence from the 5’ntr of chimpanzees 1855 and Candie (HM626487, HM626488, HM626489, HM626490). The rate of substitution in a 394 nt segment in the NS5A/B coding region was similarly low, with Candie showing only 1 nucleotide substitution over a 4 year period and 1855 showing 6 nucleotide substitutions over a 16 year period (Fig. 4.2). The amino acid sequences of this NS5A/B region from these longitudinal samples were identical in both animals.

**Sequence diversity among GBV-C$_{cpz}$ isolates**

To study GBV-C$_{cpz}$ NS5A/B sequence heterogeneity, 6 clones each from chimpanzees Candie and 1855 were compared with the consensus sequence (the sequence which occurs with the highest frequency for each nucleotide position; (Ruiz et al., 2010)). Chimpanzee Candie had only 1 of 6 clones identical to the consensus sequence after 4 years of infection, resulting in a heterogeneity index of 0.83 (the proportion of GBV-C$_{cpz}$ clones not bearing the predominant sequence) (Fig. 4.3A). Chimpanzee Candie demonstrated an average of 2.8 substitutions per clone in the 394 nt sequence examined, with transitions (A$\leftrightarrow$G or C$\leftrightarrow$T) accounting for 64.7% of the total number of substitutions. At sixteen years post-infection, chimpanzee 1855 did not have a predominant nucleotide sequence (Fig. 4.3B), which accounted for a heterogeneity index of 1.0, consistent with the prediction of an error prone RNA dependent RNA polymerase.
(RdRp) and the generation of quasispecies in serum. Chimpanzee 1855 demonstrated an average of 4.2 substitutions per clone, and transitions accounted for 84% of these substitutions (Fig. 4.3B). Comparison of non-synonymous to synonymous substitutions (dN/dS ratio) in the six RdRp sequences demonstrated a ratio of <0.25 for chimpanzees Candie and 1855, indicating that there was not positive selection. Sequence diversity was not detected in another chimpanzee (3915) with transient GBV-C cpz infection, with all 5 clones having an identical sequence (heterogeneity index of 0; HM626492).

**Phylogenetic relationships of GBV-C cpz isolates**

Human GBV-C isolates can be grouped into five, and possibly six, genotypes (Muerhoff et al., 2006). GBV-C, HCV, GBV-A and GBV-B 5’ntr sequences were compared with the published GBV-C cpz 5’ntr sequence (AF070476), and the GBV-C cpz and human GBV-C 5’ntr sequences identified in this captive chimpanzee population. As predicted, the newly generated GBV-C cpz sequences and AF070476 form a monophyletic group separate from the human GBV-C sequences (Fig. 4.4A).

A GBV-C isolate with a 5’ntr sequence that aligned more closely with human GBV-C sequences from genotype 1 was identified in one chimpanzee (number 3912) (Fig. 4.4). This GBV-C isolate was genotype 1, which correlates with African human isolates (Muerhoff et al., 2006). This animal did not receive human blood products, and the mode of transmission is not known. Since chimpanzees can support experimental human GBV-C infection (Bukh et al., 1998), and other animals in the colony may have received human blood products including blood from humans with HIV and HCV infection, it is possible that the animal acquired human GBV-C via intra-colony transmission.

Adams et al. published partial 5’ntr sequences from 3 noncaptive chimpanzees (subspecies troglodytes and verus), including one animal with two samples obtained 24 months apart (Adams et al., 1998). The 5’ntr sequences of these isolates share less sequence identity with the published GBV-C cpz sequence (AF070476) and the GBV-C cpz...
sequences that we characterized (Fig. 4.4B), suggesting that GBV-C<sub>cpz</sub> sequences from noncaptive chimpanzees differ from captive chimpanzees. 5’ntr sequences obtained from the chimpanzee of the subspecies <i>verus</i> (chimpanzee 30), are more similar to the GBV-C<sub>cpz</sub> sequence AF070476 obtained from the subspecies <i>troglodytes</i> than are the sequences from the remaining noncaptive chimpanzees (23 and 33), which were also obtained from <i>troglodytes</i> subspecies hosts. Thus, GBV-C<sub>cpz</sub> infects both subspecies of chimpanzee, <i>troglodytes</i> and <i>verus</i>, and does not strictly cospeciate with either animal host. However, because the newly studied chimpanzees are captive animals, it is possible that the virus was transmitted in captivity, and our results may not accurately reflect the species diversity of GBV-C<sub>cpz</sub> infection found in the wild.

Phylogenetic relationships are best determined by comparing highly conserved functional domains including regions of the RdRp. The deduced amino acid sequences of GBV-C<sub>cpz</sub> isolates were determined and compared to the published GBV-C<sub>cpz</sub> sequence (AF070476), human GBV-C, HCV, GBV-A and GBV-B sequences. The GBV-C<sub>cpz</sub> NS5B sequences we characterized shared considerable sequence identity with AF070476 and, like the 5’ntr sequences, formed a monophyletic group separate from the human GBV-C genotypes (Fig. 4.5A). NS5B sequences generated from noncaptive chimpanzees by Adams et al. (Adams <i>et al.</i>, 1998) diverged from the GBV-C<sub>cpz</sub> sequences obtained from captive chimpanzees (Fig. 4.5B).

The chimpanzee GBV-C<sub>cpz</sub> RdRp functional motifs, as defined by Koonin et al. (Koonin & Dolja, 1993), were highly conserved with human GBV-C sequences. The NS5B sequences from chimpanzees 1855, 3915, Candie and the published GBV-C<sub>cpz</sub> sequence (AF070476) were identical within the eight RdRp conserved motifs. The partial GBV-C<sub>cpz</sub> NS5B sequences from noncaptive chimpanzees only contain sequence for RdRp motifs III and IV and differed from the GBV-C<sub>cpz</sub> AF070476, chimpanzee 1855, 3915 and Candie sequences (Fig. 4.5C) (Adams <i>et al.</i>, 1998; Koonin & Dolja, 1993). The 4 non-captive chimpanzee sequences were identical to the AF070476
sequence in RdRp motif III, and 2 of the 4 non-captive chimpanzee sequences (23 and 33) had one amino acid substitution in the RdRp motif IV compared to AF070476 (arginine to threonine; Fig. 4.5C). The substitutions were identical to the human GBV-C RdRp motif IV amino acid sequences instead of the GBV-C<sub>cpz</sub> AF070476 sequence. In contrast, human GBV-C sequences differ from the AF070476 sequence by up to 3 amino acid substitutions in RdRp motif III and 2 amino acid substitutions in RdRp motif IV. The observation that two of the chimpanzees have one amino acid substitution in the RdRp motif IV when compared with the other chimpanzee GBV-C<sub>cpz</sub> sequences suggests that there is sequence diversity among GBV-C<sub>cpz</sub> sequences. More GBV-C<sub>cpz</sub> sequences from captive and noncaptive chimpanzees are necessary to determine whether there are multiple genotypes as with human GBV-C.

**GBV-C envelope protein hydrophobicity and C-terminal domain topology**

HCV and GBV-C envelope proteins each contain a single transmembrane domain in their fully processed form. Deletion of the GBV-C E2 C-terminal domain and insertion of an N-terminal secretory peptide results in secretion of the protein from CHO cells (McLinden et al., 2006). During the biogenesis of HCV and GBV-C envelope proteins, multiple membrane-spanning domains are possible. Kyte and Doolittle hydrophobicity profiles of the entire E1 and E2 proteins of HCV, GBV-C and GBV-C<sub>cpz</sub> show multiple hydrophobic regions at the C-termini, suggestive of a transmembrane domain with two potential membrane-spanning domains. The predicted GBV-C E2 transmembrane domain (TM) of the fully processed E2 resides between amino acids 343 and 371 (Fig 4.6A). Two putative fusion peptides are located within (347–364 region) and adjacent to (267–298 region) the E2 TM region (Fig. 4.6A). The M6 monoclonal antibody recognizes a linear epitope encompassing amino acids 277–293 within the putative fusion peptide located at from amino acids 267 to 298 (Fig. 4.6A).

During HCV envelope protein biogenesis, the two potential membrane-spanning domains of E1 and E2 appear to be too short to serve as classical transmembrane α-
helices, and are predicted to fold as two antiparallel β-strands in a hairpin structure, and an amphiphilic α-helix upstream of the hairpin is predicted to stabilize the hairpin on the ER membrane (Fig. 4.6B). Although the topology of the transmembrane domains of human and chimpanzee GBV-C have not been experimentally studied, computational analyses predict that the C-terminal domain of GBV-C E1 may adopt an amphipathic α-helix followed by a β-hairpin topology and GBV-C E2 may adopt a β-hairpin topology, similar to that predicted for HCV (Fig. 4.6B) (Jordan et al., 2007).

The HCV envelope proteins are processed in the following manner: after translation of the HCV E1 and E2 proteins, the N-terminus of E1 is translocated in the ER lumen (Cocquerel et al., 2002). Because the E1 TM domain is also the signal sequence of E2, the E1 C-terminus shifts orientation toward the ER lumen, forming a hairpin structure within the translocon and enabling E2 to be translocated into the ER lumen (Cocquerel et al., 2002). Following signal sequence cleavage between E1 and E2, the E1 TM domain is reoriented to form a single membrane-spanning domain where the N-terminal ectodomain of E1 is located within the ER lumen and the C-terminal domain is in the cytosol (Cocquerel et al., 2002). This conformational change of the TM domain from a hairpin structure to a single membrane-spanning domain following signal sequence cleavage occurs for HCV E2 as well (Cocquerel et al., 2002). The biogenesis of the GBV-C transmembrane domain topology is unknown, but based on sequence comparisons it appears to be formed in a similar manner to the HCV TM domains. This processing mechanism explains why there are two potential membrane-spanning regions detected in the amino acid sequence even though there is only a single transmembrane domain in the envelope protein’s fully processed form.

GBV-C and GBV-C_{cpz} E1, and possibly E2, are predicted to have a similar hairpin to single transmembrane-spanning domain conformational change based on a hydrophobic cluster analysis (HCA) (Fig. 4.6C). Specifically, the hydrophobic cluster analysis of the C-termini of HCV E1 and E2 suggests that the envelope glycoproteins
share a similar pattern of a long amphiphilic region (18 or 19 residues) followed by two short hydrophobic stretches (7 to 10 residues) surrounded by hydrophilic residues (Charloteaux et al., 2002). Human and chimpanzee GBV-C E1 and E2 C-termini show the same pattern of two short hydrophobic stretches (suggested by the short vertical clusters on the HCA profile), and GBV-C E1 shares the putative amphiphilic α-helix seen with HCV E1 and E2 (suggested by the long horizontal cluster on the HCA profile) (Jordan et al., 2007). The HCV hydrophobic regions are too short to be classical transmembrane α-helices, and are predicted to fold as two antiparallel β-strands in a hairpin structure (Charloteaux et al., 2002). The amphiphilic α-helix upstream of the hairpin is predicted to stabilize the hairpin (Charloteaux et al., 2002). Because of the similarity of the cluster shapes and location in human and chimpanzee GBV-C E1 when compared to HCV E1, the human and chimpanzee GBV-C E1 C-termini are also predicted to form a membrane-spanning hairpin structure (Fig. 4.6C). Although human and chimpanzee GBV-C E2 and HCV E2 share a putative hairpin structure, the GBV-C E2 proteins do not contain a predicted amphiphilic α-helix domain, suggesting that there may be different membrane topologies compared to HCV E2 (Jordan et al., 2007). Further experimental studies are necessary to determine the precise timing and location of the putative β-hairpin structures of these transmembrane domains during envelope protein biogenesis.

Discussion

Human GBV-C infection may persist in human hosts for decades even though the majority of humans studied clear infection within 2 years following infection (Alter, 1997; Hitzler & Runkel, 2004; Theodore & Lemon, 1997). Although most GBV-C
infections were transient in captive chimpanzees, persistent infection was documented for up to 19 years in one animal. Serum GBV-C
viral loads were high and constant in persistently infected animals (~1x10^8 GE/ml). Thus, GBV-C
viral loads are similar to that observed for human GBV-C (Sauleda et al., 1999; Tillmann & Manns, 2001). The
fact that the animals did not have a documented exposure to human blood products or tissues, and that their viral genome sequences align most closely with chimpanzee GBV-C sequences, suggests that chimpanzee infection was acquired via intra-colony transmission (Brook, 1998).

As with human GBV-C infection, seroconversion may occur with GBV-C<sub>cpz</sub> clearance, although E2 antibody may be intermittent and was detected before and after GBV-C<sub>cpz</sub> viremia. The detection of E2 antibodies prior to the detection of GBV-C<sub>cpz</sub> viremia may reflect a GBV-C<sub>cpz</sub> viral load below the limit of detection of the assay. Of the 4 chimpanzees that had both viremia and E2 antibodies detected, 3 animals had concurrent detection of GBV-C E2 antibodies and viremia. This prevalence is higher than the prevalence of concurrent viremia and E2 antibody in humans (7%) (Lefrère et al., 1997; Sauleda et al., 1999). E2 antibodies have not been examined in chimpanzee serum prior to this study, and more information is needed to provide a clear understanding of the relationships between E2 antibody and GBV-C<sub>cpz</sub> viremia. Nevertheless, the increased frequency of coexisting E2 antibody and viremia may reflect the limited sample size or suggest that the immune mechanism for clearing human and chimpanzee GBV-C infections differs.

Like human GBV-C, GBV-C<sub>cpz</sub> quasispecies are detected in serum (Sauleda et al., 1999; Thomas et al., 1998; Tillmann et al., 1998). GBV-C<sub>cpz</sub> NS5A/B sequence diversity was detected in chimpanzees with persistent infection and not in a chimpanzee with transient GBV-C<sub>cpz</sub> infection, suggesting that the generation of sequence diversity may require persistent infection. Human GBV-C quasispecies have nucleotide substitution rates of up to 8.7% in the 5’ntr region, 2.0% in the E2 region and 3.3% in the NS3 region (Ruiz et al., 2010; Zampino et al., 1999). We found lower rates of GBV-C<sub>cpz</sub> NS5A/B nucleotide substitution in chimpanzees, with rates of 0.7% (Candie) and 1.0% (1855) in the NS5A/B region. None of the substitutions in NS5A/B quasispecies correlated with the mutations observed by Bukh et al. during the experimental human GBV-C infection
of chimpanzees (Bukh et al., 1998), and most GBV-C_{cpz} nucleotide substitutions in the NS5A/B region were silent mutations. Positive selection was not detected (dN/dS ratio < 1) in the two chimpanzees with persistent infection, suggesting a lack of immunologic selective pressure. Even though sequence diversity was detected, a minority of sequences predominated in human GBV-C and GBV-C_{cpz} during persistent infection (Radkowski et al., 1999; Ruiz et al., 2010). The mutation rate observed between early and late samples during persistent infection was only 0.2% - 1.5%, and none of the mutations resulted in a change in the amino acid sequence. RNA secondary structure constraints in the NS5A and NS5B regions of the GBV-C genome may contribute to the low mutation rate over time (Davis et al., 2008; Thurner et al., 2004).

Given the worldwide distribution and presence of quasispecies of human GBV-C, there is a surprising lack of genetic diversity among human GBV-C isolates. Although few GBV-C_{cpz} sequences are available, the extent of sequence diversity observed between GBV-C_{cpz} isolates may be similar to that of human GBV-C genotypes. More GBV-C_{cpz} sequences need be examined to determine if the GBV-C_{cpz} sequence diversity is significant enough to form separate genotypes as with human GBV-C.

The biogenesis of the transmembrane domains of the human and chimpanzee GBV-C envelope proteins appears to be similar, based on a hydrophobic cluster analysis. As with HCV E1 and E2, both human and chimpanzee GBV-C E1 and E2 proteins are predicted to form a β-strand hairpin structure transiently during biogenesis. The human and chimpanzee GBV-C E1 protein hydrophobic cluster analysis (HCA) suggested the presence of an amphipathic helix which stabilizes the hairpin structure, as with HCV. However, the HCA did not show a putative amphipathic helix in human or chimpanzee GBV-C E2 proteins, suggesting that the biogenesis of these proteins may differ from HCV. Further experimental studies of envelope protein transmembrane domain biogenesis are needed to determine the precise timing and orientation of these transient structures.
Adams et al. found that GBV-Ccpz isolates from noncaptive chimpanzees of the *Pan troglodytes* subspecies *verus* aligned as a separate group from those found in *Pan troglodytes* subspecies *troglodytes* (Adams *et al.*, 1998). The *Pan troglodytes verus* chimpanzees originated from West Africa and *Pan troglodytes troglodytes* animals originated from Cameroon and Nigeria (Adams *et al.*, 1998). Alignment of the previously published GBV-Ccpz sequences and the sequences we characterized does not confirm that GBV-Ccpz segregates into separate *verus* and *troglodytes* subspecies groups. The GBV-Ccpz sequences we studied aligned more closely with GBV-Ccpz sequences from noncaptive chimpanzees of the subspecies *verus*, and to a lesser extent with noncaptive chimpanzees of the subspecies *troglodytes*, raising the possibility of interspecies transmission. Chimpanzee inter-subspecies transmission of GBV-Ccpz is feasible because human GBV-C can infect both chimpanzees (Bukh *et al.*, 1998) and humans. Our data suggest that it may be more appropriate to call the chimpanzee variant GBV-Ccpz to signify that this virus infects both *verus* and *troglodytes* subspecies as suggested by Adams et al. (Adams *et al.*, 1998).

Another GB virus, GBV-A, has a species-specific pattern of sequence divergence, and the levels of sequence variation between GBV-A found in different species is similar to the relative sequence distance between human GBV-C and GBV-Ccpz. This is consistent with the hypothesis that GBV-C may have evolved with a common ancestor of humans and chimpanzees into the distinct GBV-C and GBV-Ccpz variants (Adams *et al.*, 1998). The similarities of human GBV-C and GBV-Ccpz (serum viral load, seroconversion, persistence) and the length of time that each virus has existed in its host since humans and primates evolved separately suggests that chimpanzee GBV-Ccpz infection could serve as an animal model of GBV-C – SHIV interaction *in vivo*. Chimpanzees also support experimental infection with human GBV-C with viral loads of $10^6$-$10^7$ GE/ml (Bukh *et al.*, 1998). Thus, SHIV co-infection with either GBV-C or GBV-Ccpz could be used to examine SHIV-GBV-C interactions *in vivo*. In this animal
model, it would be prudent to determine GBV-C\textsubscript{cpz} viremia and E2 antibody status, especially in trials involving HIV. Finally, it is unclear why human GBV-C or GBV-C\textsubscript{cpz} viremia persists in some hosts but not others. The chimpanzee may provide a model to study host factors related to clearance and persistence of infection.
Table 4.1. Chimpanzee Demographic Information, Blood-Product Exposure History and Virus Exposure History.

<table>
<thead>
<tr>
<th>Chimpanzee ID</th>
<th>GBV-C RNA+ sample date (day/month/year)</th>
<th>Sex</th>
<th>DOB (day/month/year)</th>
<th>HIV Exposure Status</th>
<th>HCV Exposure Status</th>
<th>Blood-product/virus exposure history</th>
</tr>
</thead>
<tbody>
<tr>
<td>5855</td>
<td>22/5/2006</td>
<td>M</td>
<td>24/7/1992</td>
<td>No</td>
<td>Negative</td>
<td>Respiratory syncytial virus; hepatitis B virus</td>
</tr>
<tr>
<td>9713</td>
<td>27/1/2005</td>
<td>F</td>
<td>24/8/1999</td>
<td>No</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>3912</td>
<td>11/9/2006</td>
<td>M</td>
<td>18/4/1978</td>
<td>No</td>
<td>Negative</td>
<td>Hepatitis E virus; Respiratory syncytial virus</td>
</tr>
</tbody>
</table>

M = male; F = female; DOB = date of birth; HIV = human immunodeficiency virus; HCV = hepatitis C virus; RBC = red blood cells
Table 4.2. Oligonucleotide Primer Sequences Utilized to Detect GBV-C RNA.

<table>
<thead>
<tr>
<th>Region</th>
<th>RT-PCR product size (bp)</th>
<th>GBV-C primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBV-C 5’ NTR</td>
<td>92</td>
<td>+GGGACCGCGGAAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-GTTAAGACCGAACCTATAGTCCTACC</td>
</tr>
<tr>
<td>GBV-C&lt;sub&gt;genovirus&lt;/sub&gt; 5’ NTR</td>
<td>77</td>
<td>+AATGCCATGGCCCACC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-ATGCCACCGGAGACCTC</td>
</tr>
<tr>
<td>GBV-C 5’ NTR</td>
<td>203</td>
<td>+AGGCCCAATAAGGCCAGGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-TGAGCCGCAAGGAGGTCTAC</td>
</tr>
<tr>
<td>GBV-C&lt;sub&gt;genovirus&lt;/sub&gt; 5’ NTR</td>
<td>364</td>
<td>+TTGGCAGCTGTTAAATCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-GCCCAACAGTTTGTGAGG</td>
</tr>
<tr>
<td>GBV-C&lt;sub&gt;genovirus&lt;/sub&gt; NSSA/B</td>
<td>388</td>
<td>+GGGACCAATGGGGCGTGCAGGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-TCTGATGCAAAAAAGGAGTAGCATAGTCCTC</td>
</tr>
<tr>
<td>GBV-C&lt;sub&gt;genovirus&lt;/sub&gt; NSSB</td>
<td>781</td>
<td>+GAACACCTGAAAGGAAAATGGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-GGTGGCAAAGGAGTAGACAACAA</td>
</tr>
</tbody>
</table>

RT-PCR = reverse transcription polymerase chain reaction; 5’ntr = 5’ nontranslated region; bp = base pairs; NS5A = nonstructural protein 5A; NS5B = nonstructural protein 5B; + = sense primer; - = antisense primer; Pr = probe sequence.
Figure 4.1. Evolution of GBV-C viremia and E2 antibody in chimpanzees with GBV-C infection. Open circles indicate a negative result for GBV-C E2 antibody and closed circles a positive result (A). Closed bars indicate a positive result for GBV-C cpz RNA, dashed bars indicate a positive result for human GBV-C RNA, and open bars indicate a negative result for either RNA in serum (A). GBV-C cpz viremia titers (genome equivalents (GE) per ml) were measured by real-time PCR at multiple time points during persistent infection for chimpanzees Candie (♦) and 1855 (□) (B). The first available samples are marked at t=0.
Figure 4.2. Nucleotide alignment of GBV-C_{cpz} partial NS5A/B sequences obtained at early and late time points during persistent infections of chimpanzees Candie (early time point; HM638235) and 1855 (early time point; HM638234). Consensus sequences of 6 quasispecies (as described in Fig. 4.3) from the late infection time points were compared. Arrowhead denotes the putative start side of NS5B and dots represent identical bases.
Figure 4.3. Nucleotide alignment of GBV-C<sub>cpz</sub> partial NS5A/B sequences recovered from individual clones (1-6) isolated from serum from two chimpanzees 4 and 16 years post-infection: Candie (A; HM626501 – HM626506) and 1855 (B; HM626495 – HM626500), respectively. Arrowhead denotes the putative start site of NS5B and dots represent identical bases.
Figure 4.4. Phylogenetic relationships of GB viruses and Hepaciviruses 5’ ntr. (A) 5’ ntr nucleotide sequences from GBV-C<sub>cpz</sub>, GBV-C, HCV, GBV-A and GBV-B, and the sequences from chimpanzees P187 (HM638236), 1855 (HM626488), Candie (HM626490), 3912 (HM769722) and 3915 (HM626491) were aligned with Clustal W. There are 308 nucleotides in the final dataset, although only 137 nucleotides were available for sequence alignment with 3912. (B) GBV-C<sub>cpz</sub> 5’ntr sequences from noncaptive chimpanzees (noted in italics; see text) were included in the comparison. There are 98 nucleotides in the final dataset except for 3912, for which there were 45 nucleotides available. The evolutionary distances were computed using the Maximum Composite Likelihood method. Bootstrap values are shown for each branch point. The units represent the number of base substitutions per site.
Figure 4.5. Phylogenetic relationships of GB viruses and Hepaciviruses RdRp. (A) RdRp amino acid sequences from GBV-C<sub>cpz</sub>, GBV-C, HCV, GBV-A and GBV-B, and chimpanzees 1855 (HM626494), 3915 (HM626492) and Candie (HM626493) were aligned with Clustal W. There are 231 amino acids in the final dataset. (B) GBV-C<sub>cpz</sub> RdRp sequences from noncaptive chimpanzees (noted in italics; see text) were included in the comparison. There are 61 amino acids in the final dataset. The evolutionary distances were computed using the Poisson correction method. Bootstrap values are shown for each branch point. The units represent the number of amino acid substitutions per site. (C) NS5B functional motifs III and IV are marked as described by Koonin et al. from the NS5B alignment of Fig. 4.5B.
Figure 4.6. Functional mapping of the GB virus C envelope glycoprotein E2. The predicted GBV-C E2 transmembrane domain (TM) resides between amino acids 343 and 371 (A). Two regions within E2 encode putative fusion peptides: 347–364 (within the TM) and 279–298 (this overlaps with the M6 monoclonal antibody linear epitope which encompasses amino acids 277-293) (A). HCV hydrophobic TM regions on both E1 and E2 appear to be too short to serve as classical transmembrane α-helices during biogenesis, and thus are predicted to fold as two antiparallel β-strands in a hairpin structure transiently during biogenesis (B). The amphiphilic α-helix upstream of the hairpin is predicted to stabilize the hairpin on the ER membrane (B). Hydrophobic cluster analysis of HCV, human and chimpanzee variants of GBV-C (GBV-C_{hum} and GBV-C_{cpz} respectively) found that the E1 C-termini of these three viruses are similar (C) and share the putative structure shown in panel B. However, only the HCV E2 protein had the predicted structure of a β-hairpin and α-helix. Long horizontal clusters on an HCA plot are consistent with helical structures and vertical clusters are consistent with β-strand secondary structures. GBV-C E2 contains helix-breaking proline residues upstream from the TM region, so an α-helix structure may be unlikely (C).
CHAPTER V: GENERAL DISCUSSION

These chapters explore very different aspects of the inhibition of GBV-C by HIV, and are linked by the common goal of gaining insight into how GBV-C influences HIV disease progression by improving the understanding of GBV-C natural history, GBV-C E2 protein interactions with cells, and anti-GBV-C E2 antibody inhibition of HIV.

In the first chapter, I studied the interaction of the GBV-C envelope glycoprotein E2 with cell surface receptors. Because recombinant GBV-C E2 inhibits the entry of HIV env pseudotyped particles but not VSV-G pseudotyped particles (Jung et al., 2007), we predicted that GBV-C E2 inhibits HIV transduction at the step of entry, when HIV envelope proteins interact with cellular receptors and membranes (Fig. 5.1). It is unknown whether GBV-C E2 inhibits HIV attachment to cellular receptors or fusion, although recent work with E2 peptides indicates that HIV fusion may be inhibited. Thus, understanding how GBV-C E2 interacts with cellular surfaces and identifying E2 binding and entry receptors is critical to understanding how E2 inhibits HIV. GBV-C E2 may bind to a small transient subset of PBMCs, and PBMCs support GBV-C E1/E2 pseudotyped particle transduction, which explains why GBV-C replicates to such low levels in B and T cells (George et al., 2006; Xiang et al., 2000). However, additional studies examining the transduction of GBV-C into PBMC subsets are required to confirm this hypothesis. GBV-C E1/E2 pseudotyped particles do not transduce any of the transformed cell lines examined at levels higher than 2-fold above background, which suggests that these cell lines lack GBV-C entry receptors (which were present in PBMCs) or restrict transduction via restriction factors, and makes further examination of GBV-C entry and identification of GBV-C entry receptors difficult. However, we found that
recombinant GBV-C E2 binds to multiple transformed cell lines, and we pursued this route to identify candidate E2 binding receptors and characterize E2 binding. I found that GBV-C E2 binding is sensitive to trypsin digestion of cellular surface proteins and is not sensitive to temperature alterations.

Utilizing a comparative genomics approach, multiple candidate E2 binding receptors were identified by correlating the level of E2 binding with the cDNA expression levels in multiple cell lines from the NCI-60 panel. Multiple candidate binding receptors, including HLA-G, HLA-F, CD86 and ADAM10, are being examined further with siRNA knockdowns and binding competitions with anti-candidate receptor antibodies and recombinant E2. If we find that knockdown of a candidate receptor reduces E2 binding to a cell line which normally binds to E2, we will have to determine whether the candidate receptor is also involved in GBV-C E2 binding to PBMCs and GBV-C infection. To determine if a candidate receptor is involved in E2 binding to a specific PBMC subset, the candidate receptor can be knocked down in the specific PBMC subset which binds to E2. Conversely, the candidate receptor can also be expressed in a PBMC subset which does not normally support E2 binding. This same PBMC subset approach can be used to determine if the candidate receptor is involved in GBV-C infection. However, even though the candidate receptor may be involved in E2 binding, knockdown of the candidate receptor may not necessarily hamper GBV-C infection because other cellular receptors may compensate for the lack of one cellular receptor. This occurs with HCV infection: although CD81 is involved in HCV attachment, knockdown of CD81 does not completely inhibit HIV infection.
It is interesting that GBV-C E2 binds to cell lines at higher levels than PBMCs, and that GBV-C E1/E2 pseudotyped particles transduce PBMCs at higher levels than cell lines. This discrepancy suggests that there may be multiple receptors involved in GBV-C entry, as occurs in HCV entry, and that the cell lines have high levels of an attachment receptor and lack an entry receptor, and that a small subset of PBMCs has the correct ratio of attachment and entry receptors to allow transduction.

Although no E2 cellular receptors were definitively identified, the advances described here will give future studies a firm foundation for multiple reasons. First, I identified cell lines which bind to E2 at significantly higher levels than the previous cell lines examined. Secondly, I characterized E2 binding with an anti-E2 antibody (7066) which is plentiful, unlike the anti-E2 antibody (M5) for which there are insufficient quantities available for high-throughput experiments. Third, I identified promising candidate binding receptors which are interesting because of their role in immune system activation and differentiation. Finally, I found that GBV-C E2 binds to a small subset of PBMCs, which had not been shown before. This may explain why GBV-C replication is so poor in vitro. With these advances, future studies to identify GBV-C E2 cellular receptors have a greater chance of being successful. Future studies may include developing a high-throughput E2 binding assay to assess E2 binding levels of cells transfected with global siRNA pools, or identifying GBV-C E2 binding partners from E2 cross-linked to plasma membrane preparations of cell lines which bind E2 at high levels (786-0 cells) or of PBMCs.

In the second chapter, I examined how GBV-C E2 antibodies inhibit HIV infection by utilizing the tool of pseudotyped particles. I found that E2 antibodies pull
down retroviral particles pseudotyped with GBV-C E1/E2, HIV gp120/41, VSV-G envelope proteins, and particles with no viral envelope proteins, which suggests that E2 antibodies interact with a cellular protein present on the particles (Fig. 5.2). However, only HIV env pseudotyped particles are neutralized; VSV-G and MLV enveloped particles were not neutralized. This suggests that the E2 antibody interaction with cellular proteins on the particle blocks the interaction of HIV gp120 with its cellular receptors, and not the interaction of VSV-G or MLV envelope proteins with their receptors. Finally, the cellular antigen recognized by E2 antibodies is only found in permeabilized cells and is not located at the cell surface, suggesting that the cellular antigen may be exposed during virus budding. Future directions for this research include identifying the location of the cellular antigen by confocal microscopy and cross linking the cellular antigen to anti-E2 antibodies and identifying the bound proteins by mass spectrometry. Determining which cellular antigen structurally mimics E2 and thus interacts with anti-E2 antibodies is necessary to determine how E2 antibodies neutralize HIV infection because the cellular antigen is carried on HIV virions. We are also immunizing mice and primates with recombinant GBV-C E2 to elicit anti-E2 antibodies. Previous immunization of E2 in rabbits demonstrated that E2 immunization elicits HIV neutralizing anti-E2 antibodies and we will determine whether post-immune IgG from the mice and primates will also neutralize HIV infection. Finding an immunogen which elicits broadly neutralizing HIV antibodies is important because, so far, immunization with HIV gp120 and gp41 has not produced broadly neutralizing antibodies which are necessary for an effective vaccine.
In the third chapter, I studied the natural history of GBV-C_{cpz} in a captive chimpanzee population, which had not been previously described. The prevalence of GBV-C_{cpz} viremia (2.5%) and anti-E2 antibodies (11%) was similar to the prevalence of GBV-C viremia (1.8%) and anti-E2 antibodies (11%) in healthy human blood donors. The serological response to GBV-C_{cpz} infection is less clear than GBV-C infection, because anti-E2 antibody seroconversion and clearance of viremia occurs in most humans and some chimpanzees, but in many of the chimpanzees, E2 antibody occurred concomitantly with viremia in animals with transiently detected viremia. The GBV-C_{cpz} 5'ntr and RdRp sequences from this captive chimpanzee population shared a high level of sequence identity with each other and with the only nearly full length GBV-C_{cpz} sequence published. Also, the GBV-C_{cpz} sequences from chimpanzee subspecies *troglodytes* and *verus* shared significant sequence identity, so the GBV-C_{cpz} sequences from chimpanzees should be identified with the GBV-C_{cpz} designation rather than the currently used GBV-C_{trog} designation. This new understanding of the natural history of GBV-C_{cpz} in a captive chimpanzee population provides the background research necessary for development of the chimpanzee as an animal model in which to study GBV-C infections and its interaction with HIV *in vivo*. This animal model can be used to address questions such as why some people are infected with GBV-C for decades and others for only an average of two of years. In addition, the *in vivo* interaction between GBV-C and HIV (or SHIV) can be studied in this model. Chimpanzees involved in clinical research trials are not currently tested for GBV-C_{cpz} viremia or anti-E2 antibodies. Because both GBV-C viremia and anti-E2 antibodies impact the clinical course in humans co-infected with HIV, we hypothesize that GBV-C_{cpz} may alter the chimpanzee’s response to HIV/SHIV
infection or immunization. Thus, we propose that determining the GBV-C<sub>cpz</sub> viremia and anti-E2 antibody status is important to the study of HIV vaccine candidates in chimpanzees and especially to the study of GBV-C E2 immunizations as HIV vaccines.

In conclusion, GBV-C inhibits HIV infection via multiple mechanisms. These include downregulation of HIV coreceptors, induction of coreceptor ligand expression, and increased interferon activity in dendritic cells. The studies I described in this dissertation describe additional mechanisms of how GBV-C inhibits HIV infection. I found that recombinant GBV-C E2 protein inhibits HIV entry and that anti-GBV-C E2 antibodies inhibit HIV attachment to cells by interaction with a cellular antigen present on the virions (Table 5.1). Additional studies are necessary to further elucidate the mechanisms of GBV-C inhibition of HIV and to develop GBV-C as a novel therapeutic for HIV infection.
Figure 5.1. Model of GBV-C E2 inhibition of HIV. Incubation of recombinant GBV-C E2 with cells at 4°C prior to addition of HIV particles inhibits HIV entry. There are multiple steps in HIV entry (CD4 attachment, chemokine receptor binding, membrane insertion and fusion), and it is unknown which step GBV-C E2 specifically inhibits. Studies by Herrera et al. suggest that E2 peptides inhibit gp41 fusion in cell-cell fusion assays (Herrera et al., 2010), so E2 may be more likely to inhibit an HIV fusion step than attachment step.
Fig. 5.2. Model of anti-E2 antibody inhibition of HIV. Anti-E2 antibodies must be incubated with HIV virions prior to infection of permissive cells for HIV attachment inhibition (step 2). Inhibition of anti-E2 antibodies with cells prior to HIV infection will not result in HIV inhibition. Anti-E2 antibodies recognize a conserved cellular antigen on HIV virions, because retroviral particles with multiple viral envelope proteins are immunoprecipitated, but only HIV gp120/41 enveloped particles are neutralized. This suggests that the anti-E2 antibody interaction with the cellular antigen on the viral particle blocks access of HIV gp120 to its cellular receptors, but not VSV-G or MLV env to their cellular receptors. Multiple cellular antigens become part of the virus particle during budding (step 1). Because the cellular antigen is only detected in permeabilized cells, and individuals with anti-E2 antibodies do not have autoimmune diseases, this suggests that the cellular antigen recognized by anti-E2 antibodies is not present on the cell surface. The cellular protein may be in a different orientation in the cell and HIV infection changes its orientation in the lipid raft from which the virion buds (B; for inner leaflet plasma membrane proteins), or it may normally be located in the Golgi apparatus and HIV infection increases its presence in the lipid rafts (A; for proteins retained in the Golgi apparatus).
1. HIV particle budding

- CD4
- CCR5
- Anti-GBV-C E2 antibody
- gp160
- HIV capsid
- Cellular transmembrane protein retained in the Golgi apparatus
- Cellular transmembrane protein at the plasma membrane

2. Neutralization by anti-E2 antibodies
Table 5.1. Summary of Mechanisms of HIV Inhibition by GBV-C.

<table>
<thead>
<tr>
<th>GBV-C infection</th>
<th>Mechanisms of HIV inhibition</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>in vivo</strong></td>
<td>CCR5 and/or CXCR4 surface expression is decreased on CD4+ T cells in GBV-C infected individuals</td>
<td>(Nattermann et al., 2003; Schwarze-Zander et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>GBV-C infection reduces the level of activation markers CCR5 and CD38 on CD8+ T cells and CD38 on CD4+ T cells</td>
<td>(Maidana Giret et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Decreased CCR5 surface expression and increased serum levels of RANTES, MIP1α, MIP1β and SDF-1 are associated with prolonged survival in HIV co-infected individuals</td>
<td>(Rowland-Jones, 1999)</td>
</tr>
<tr>
<td></td>
<td>Increased interferon activation in dendritic cells in GBV-C - HIV co-infected individuals</td>
<td>(Lalle et al., 2008)</td>
</tr>
<tr>
<td><strong>in vitro</strong></td>
<td>GBV-C infection of PBMCs inhibits replication of CCR5- and CXCR4-tropic HIV isolates</td>
<td>(Jung et al., 2005; Xiang et al., 2001; Xiang et al., 2004; Xiang et al., 2005b)</td>
</tr>
<tr>
<td></td>
<td>CCR5 surface expression is decreased in GBV-C infected PBMCs</td>
<td>(Jung et al., 2005; Nattermann et al., 2003; Xiang et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>GBV-C infection in PBMCs increased secretion of soluble ligands for HIV entry receptors (RANTES, MIP1α, MIP1β, SDF-1).</td>
<td>(Jung et al., 2005; Nattermann et al., 2003; Xiang et al., 2004)</td>
</tr>
<tr>
<td>GBV-C NS5A</td>
<td>Expression of NS5A inhibits HIV replication, downregulates CXCR4 and induces expression of SDF-1 in a CD4+ T cell line</td>
<td>(Chang et al., 2007; Xiang et al., 2005a)</td>
</tr>
<tr>
<td></td>
<td>16 amino acid domain in NS5A inhibits HIV infection</td>
<td>(Xiang et al., 2007)</td>
</tr>
<tr>
<td>GBV-C E2 protein</td>
<td>E2-Fc fusion protein inhibits HIV replication and HIV env pseudotyped particle transduction, but not VSV-G pseudotyped particle transduction, in PBMCs</td>
<td>(Jung et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Recombinant E2 protein inhibits HIV env pseudotyped particle transduction in a dose-dependent manner in an HIV permissive human osteosarcoma cell line</td>
<td>(Mohr et al., 2008)</td>
</tr>
<tr>
<td>GBV-C E2 peptides</td>
<td>E2 fusion peptide (269-286) inhibits HIV gp41 fusion, as determined by vesicle leakage assays, lipid mixing assays, NMR spectral analyses of mixed peptides</td>
<td>(Herrera et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>E2 peptide (133-150) inhibits gp41-induced fusion in a cell-based fusion assay; multiple other E2 peptides inhibit HIV env pseudotyped particle transduction of TZM-bl cells</td>
<td>(Herrera et al., 2010)</td>
</tr>
<tr>
<td>GBV-C E2 antibodies</td>
<td>E2 antibodies neutralize HIV infection by interacting with cellular proteins carried on the virus particle and inhibiting HIV virion attachment to cells</td>
<td>(Mohr et al., 2010)</td>
</tr>
</tbody>
</table>

Bold = inhibition mechanisms examined in this dissertation.
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