I, Christina M Martin Quigley, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Pathobiology & Molecular Medicine.

It is entitled: Characterization of Occult Hepatitis B Virus Infection in HIV-Positive Individuals

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Characterization of Occult Hepatitis B Virus Infection in HIV-Positive Individuals

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of the University of Cincinnati
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of the College of Medicine

by
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Abstract

Hepatitis B Virus (HBV) is a leading cause of both cirrhosis and hepatocellular carcinoma (HCC). With improvements in antiretroviral therapy, liver disease is becoming more apparent in HBV/HIV co-infected patients and is an important cause of morbidity and mortality. Currently 350 million people live with chronic HBV infection (C-HBV) worldwide, which is characterized by high levels of HBV replication and detectable hepatitis B surface antigen (HBsAg) in the serum. Occult HBV infection (O-HBV), in contrast, is defined as persistent low-level HBV replication in the absence of detectable HBsAg. Little is known about the clinical impact of O-HBV or how it develops, although several mechanisms have been postulated. Among these, altered host immunologic response and mutations of the HBV genome appear most likely to contribute and were investigated in this dissertation.

A prevalence study of O-HBV in an HIV-positive cohort identified 12 O-HBV infections and 31 C-HBV infections for characterization. Studies on O-HBV immunology are lacking; therefore, expression of several cytokines and immune markers were used as indicators of immune responses in O-HBV- versus C-HBV-infected patients. Expression levels were similar between O-HBV and C-HBV, indicating weak immune responses and persistent viral infections, although O-HBV infections appear to have decreased levels of apoptotic inhibition, potentially indicating altered pathogenesis in these patients. Studies investigating HBV diversity, especially in HIV-positive patients, are also lacking. We identified recombination within the X gene and investigated HBV diversity, determining that the X region has significantly higher variability than PreS or S. Additionally, immune selection pressures appear to be consistently acting upon
the PreS and S regions in O-HBV- versus C-HBV-infected patients and may result in selection of specific viral mutations. Several mutations associated with O-HBV infections compared to C-HBV infections were identified through genotype-matched mutational analysis, of which, 3 genotype A mutations in the S region – M103I, K122R, and G145A – were determined to be significant by signature sequence analysis. When all 3 mutations were introduced into C-HBV HBsAg expression vectors and transfected into hepatocyte cell lines, both intracellular and extracellular levels of HBsAg were significantly decreased compared to wild-type. This may indicate a synthesis defect or increased HBsAg proteasomal degradation, but may partially explain the lack of detectable HBsAg in the serum of an O-HBV-infected individual.

Taken together, the findings from this dissertation provide additional insight into O-HBV infection and build upon the current body of literature. In addition, it expands our current knowledge of HBV quasispecies variability, which is rarely evaluated. This dissertation provides the first study investigating cytokine and immunologic marker expression performed in O-HBV-infected individuals, the first rigorous investigation of HBV diversity in HIV-positive individuals, includes assessment of O-HBV diversity, and identifies multiple mutations associated with O-HBV infection. Importantly, it includes a functional analysis of 3 HBsAg mutations that may account for the lack of detectable HBsAg in an O-HBV-infected patient, and represents the second study to potentially explain O-HBV based on single viral mutations that directly result in decreased extracellular and intracellular HBsAg levels in vitro.
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The majority of this dissertation is based on manuscripts I – V. In addition to the body of work on occult hepatitis B virus infection, studies assessing viral variability in HCV and HIV were also co-authored during the course of the dissertation work. A brief overview of these studies is included at the end of the Discussion section and the published manuscripts (VI – VIII) are included after the main publications relating to the dissertation. Each individual publication is referred to within the text by its corresponding roman numeral.


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There are many people who have helped me in the past 6 years to complete this project and achieve the goal of obtaining my PhD. First, I must thank Dr. Jason Blackard for taking me as a student in his lab and providing me with every opportunity possible to become the best researcher and teacher I can be. Without him I would not have realized how much I enjoy teaching undergraduate and early graduate students the basics of virology, infectious diseases epidemics and pandemics, and lab techniques.

I would also like to thank my PhD committee members. I thank Dr. Melanie Cushion for being my committee chair and constantly pushing me to think about my project from different angles and broaden my thinking about the scope of the project. Thank you to Dr. Kenneth Sherman for his constant support and guidance, as well as for teaching me as much as possible about clinical aspects of liver disease. I would like to thank Dr. Richard Thompson for providing me with additional virologic methods when troubleshooting during this project and for helping me grasp various concepts within the field of virology. Finally, I would like to thank Dr. Min Liu for providing an outside view of the project and suggesting novel experimental methods to achieve our goals of this project.

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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>anti-HBc</td>
<td>antibodies against HBV core antigen</td>
</tr>
<tr>
<td>anti-HBe</td>
<td>antibodies against HBV e antigen</td>
</tr>
<tr>
<td>anti-HBs</td>
<td>antibodies against HBV surface antigen</td>
</tr>
<tr>
<td>ART</td>
<td>antiretroviral therapy</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>BCP</td>
<td>basal core promoter</td>
</tr>
<tr>
<td>cccDNA</td>
<td>circular covalently closed DNA</td>
</tr>
<tr>
<td>C-HBV</td>
<td>chronic HBV</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CTLs</td>
<td>cytotoxic T lymphocytes (CD8 T cells)</td>
</tr>
<tr>
<td>DCs</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>dN-dS</td>
<td>nonsynonymous-synonymous mutations</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FEL</td>
<td>fixed effects likelihood</td>
</tr>
<tr>
<td>GD</td>
<td>genetic distance</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HAART</td>
<td>highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HBeAg</td>
<td>hepatitis B e antigen</td>
</tr>
<tr>
<td>HBsAg</td>
<td>hepatitis B surface antigen</td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
</tr>
<tr>
<td>HBx</td>
<td>hepatitis B virus X protein</td>
</tr>
<tr>
<td>HCC</td>
<td>hepatocellular carcinoma</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>HDV</td>
<td>hepatitis D virus</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>IDU</td>
<td>injecting drug use/user</td>
</tr>
<tr>
<td>IE</td>
<td>immediate early</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP-10</td>
<td>interferon gamma-inducible protein-10</td>
</tr>
<tr>
<td>LHBsAg</td>
<td>large hepatitis B surface antigen</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>MHBSAg</td>
<td>middle hepatitis B surface antigen</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MSM</td>
<td>men who have sex with men</td>
</tr>
<tr>
<td>NAT</td>
<td>nucleic acid testing</td>
</tr>
<tr>
<td>NS</td>
<td>nonstructural</td>
</tr>
<tr>
<td>O-HBV</td>
<td>occult hepatitis B virus</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>p53</td>
<td>tumor protein 53</td>
</tr>
<tr>
<td>PBMCs</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PCOORD</td>
<td>principal coordinate</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pgRNA</td>
<td>pregenomic RNA</td>
</tr>
<tr>
<td>Pol or P</td>
<td>polymerase</td>
</tr>
<tr>
<td>PreC</td>
<td>precore</td>
</tr>
<tr>
<td>PreS</td>
<td>presurface</td>
</tr>
<tr>
<td>RC-DNA</td>
<td>relaxed circular DNA</td>
</tr>
<tr>
<td>REL</td>
<td>random effects likelihood</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>S</td>
<td>surface</td>
</tr>
<tr>
<td>sFas</td>
<td>soluble Fas</td>
</tr>
<tr>
<td>sFasL</td>
<td>soluble Fas ligand</td>
</tr>
<tr>
<td>SHBsAg</td>
<td>small hepatitis B surface antigen</td>
</tr>
<tr>
<td>STAT-C</td>
<td>specifically targeted antiviral therapies against HCV</td>
</tr>
<tr>
<td>SVR</td>
<td>sustained viral response</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>transforming growth factor-beta 1</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>UCIDC</td>
<td>University of Cincinnati Infectious Diseases Center</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VESPA</td>
<td>viral epidemiology signature pattern analysis</td>
</tr>
<tr>
<td>XAP-1</td>
<td>hepatitis B virus X associated protein-1</td>
</tr>
</tbody>
</table>
**Brief Explanation of the Dissertation Format**

This dissertation is comprised of three main sections: 1) Introduction; 2) Experimental Findings and Discussion; and 3) Related Publications.

The Introduction reviews the current literature in the field of hepatitis B virus and more specifically, HBV/HIV co-infection and occult hepatitis B virus infection. In addition, the Aims of the Current Investigation are outlined following the Introduction.

The Discussion section reviews the findings of the dissertation project and describes how these findings impact the field of hepatitis B virus research. The Discussion section also includes a General Summary of the body of work provided in the dissertation, as well as Concluding Remarks and a Future Directions section. The Future Directions section provides questions to be answered within the broad field of occult hepatitis B virus, as well as those that directly stem from the current investigation.

Finally, the published and prepared manuscripts related to this body of work are included at the end of the dissertation.
**Introduction**

**HBV Infection**

More than 2 billion individuals worldwide are estimated to have been exposed to hepatitis B virus (HBV) infection [1], although exposure may result in an acute, chronic, or occult HBV infection. The hallmarks of an acute HBV infection include a high HBV DNA level ($>10^5$ IU/mL), elevated alanine aminotransferase (ALT) level, and the presence of hepatitis B surface antigen (HBsAg) and hepatitis B ‘e’ antigen (HBeAg) in the serum [2, 3]. Low levels of ALT are often present in the serum, but elevated levels are considered to be above 30 U/L for men and above 19 U/L for women [4]. Age at the time of infection, as well as immune status, greatly affect whether an individual clears the initial acute HBV infection, or if chronic HBV infection develops. A depiction of acute versus chronic HBV infection is provided in Figure 1.
Figure 1. Progression of Acute versus Chronic HBV Infection. In acute HBV infection (left), as HBV replication increases, an immune response is mounted against HBsAg and HBeAg. Clearance is marked by decreased HBV DNA levels, normalization of ALT level and seroconversion of HBsAg and HBeAg. When chronic HBV infection develops (right), seroconversion of HBeAg may occur, but HBsAg persists. HBV DNA and ALT levels may fluctuate over time and decrease as HBV-specific T-cells increase in number, but the infection is not fully controlled by the host’s immune system (Adapted from [5, 6]).

Clearance of infection is designated by HBsAg and HBeAg seroconversion – or the loss of previously detectable HBV antigens from the serum and the appearance of antibodies against these proteins (anti-HBs and anti-HBe) [2]. Appearance of antibodies against the HBV core protein (anti-HBc) is also common upon clearance of acute HBV infection. When infected as an adult, 1% to 5% of individuals will develop chronic HBV infection. However, those infected as children, for instance at birth via perinatal transmission, have a 90% chance of developing chronic HBV infection [7-10]. Immunocompromised individuals, such as those with HIV
infection, also have a greater risk of developing chronic HBV infection. In HIV-positive individuals, clearance of HBsAg and HBeAg are delayed, resulting in increased rates of chronic HBV infection ranging from 5% up to 55% [11, 12]. At least 350 million individuals worldwide currently live with chronic HBV infection [13], which is characterized by continuously detectable HBsAg and HBV DNA levels, although HBV DNA levels tend to fluctuate over time. Occult HBV infection, on the other hand, is a low-level persistent HBV infection where HBV DNA is detectable in the serum, while HBsAg is not [14]. As of yet, an explanation for this lack of detectable HBsAg in the serum of occult HBV-infected individuals has not been elucidated.

**HBV Immunopathogenesis**

HBV is a noncytopathic viral infection, in which the viral infection itself does not damage infected cells [6]. The host immune response against HBV is responsible for the antiviral activity inducing cell injury and death, clearance or persistence of infection, as well as development of fibrosis that may result in hepatocellular carcinoma (HCC) during chronic HBV infection [15]. During acute HBV infection, clearance of HBV DNA positively correlates with levels of tumor necrosis factor (TNF) α, interferon (IFN) α/β, and IFNγ [16]. These antiviral cytokines are produced by CD4 T-cells and mediate the activities of both Kupffer cells (liver resident macrophages) and dendritic cells (DCs) [17]. Kupffer cells and DCs are then involved in maturation of HBV-specific T-cells [18]. Damage and apoptosis of HBV-infected hepatocytes occurs via cytotoxic CD8 T-cells [19], and higher levels of damage can result in symptomatic hepatitis. In addition, fulminant hepatitis B, during which there is a strong, early immune response with rapid clearance of the virus, results in significant liver damage and sometimes
liver failure [20]. Several factors play a role in HBV persistence and development of chronic HBV infection, including decreased CD4 and CD8 T-cell responses, decreased IFN production, and functional defects of DCs [6, 21, 22]. In addition, the immune response against HBV plays a role in development of liver fibrosis, since transforming growth factor-beta 1 (TGF-β1) is a significant regulator of the fibrotic process [23, 24]. On the other hand, in individuals who are able to clear HBV infection, B cells produce protective antibodies against HBV, which can result in long-term protection against re-infection [25].

**HBV Clinical Features and Diagnosis**

During acute HBV infection, between 30% and 50% of infected individuals develop symptoms of fatigue, nausea, and abdominal pain [9]. In many instances, these general symptoms are accompanied by jaundice – yellowing of the eyes and skin – as a result of decreased liver function [2, 26]. This occurs due to the accumulation of bilirubin – the end-product of heme – in the blood when hepatocytes are incapable of extracting bilirubin from the serum and excreting it into the bile. Acute HBV infection is diagnosed by the presence of detectable HBsAg and anti-HBc immunoglobulin M (IgM) in the serum [3]. Detectable anti-HBc IgM is indicative of an infection within the last 6 months, while anti-HBc IgG persists for long periods of time.

On the other hand, chronic HBV infection is diagnosed by the presence of detectable HBsAg in 2 separate serum samples drawn at least 6 months apart [27]. Symptoms associated with acute HBV infection can be similar in patients with chronic HBV infection during periods of high viral replication with an elevated host immune response, although many individuals with chronic
HBV can remain asymptomatic carriers for decades [28, 29]. Over time, the hepatocyte damage inflicted by the host immune response to chronic HBV infection can lead to scarring of the liver, known as fibrosis. A liver biopsy is often performed when elevated ALT levels are detected to assess the extent of liver fibrosis and monitor the development of cirrhosis [30]. Fibrosis staging is depicted in Figure 2.

**Figure 2. Histologic Examples of Fibrosis Stages.** Liver biopsies were stained with hematoxylin and eosin, as well as trichrome to stain connective tissue. Example of a liver biopsy with no indication of fibrosis (top left). Stage 1 contains fibrous expansion of some portal areas, while Stage 2 is indicated by increased fibrous expansion (top center). Stage 3 contains fibrous expansion of most portal areas with occasional portal to portal bridging (top right). Stage 4 contains fibrous expansion of portal areas with marked bridging, both portal to portal and portal to central (bottom left). Stage 5 represents
probable cirrhosis, while Stage 6 represents defined cirrhosis (bottom center). Example of a cirrhotic liver (bottom right). Adapted from a slide courtesy of Gregory Everson, MD.

Development of cirrhosis can lead to several serious complications such as anemia, impaired coagulation, hepatic encephalopathy, ascites (free fluid in the peritoneal cavity), portal vein thrombosis, variceal bleeding, and hemorrhages [31]. Globally, HBV infection is one of the leading causes of both cirrhosis and HCC [1]. The presence of HBeAg and high HBV DNA levels (>20,000 IU/mL) have each been determined as a risk factor for development of cirrhosis and HCC [32]. Additional risk factors for cirrhosis include age, high level of alcohol consumption, HBV genotype C infection, and co-infection with HCV, HIV, and/or HDV [32, 33]. Other risk factors for HCC development include a family history of HCC, male gender, and cirrhosis [34, 35], although up to 50% of HCC cases may appear without presence of cirrhosis [36].

Diagnosis of HBV infection is based on the detection of HBsAg and/or anti-HBc in patient serum by enzyme-linked immunosorbent assays (ELISAs). In this method, anti-HBs or HbcAg are coated on 96-well plates and HBsAg or anti-HBc present in serum samples bind its corresponding antibody or antigen. In regards to HBsAg testing, the anti-HBs that coat the plate can vary in the epitope(s) recognized, most of which are not published. Many commercial HBsAg ELISA kits exist, and it is likely that no two kits utilize the same antibodies and methods. This presents a problem when viral mutations exist in the epitope region for which the antibody binds, several of which reside within the antigenic determinant loops of HBsAg. Over the years, many improvements have been made to HBsAg ELISA kits to include newly
identified mutations within HBsAg that may result in a diagnostic error, although some commercial assays may still produce false negative results [37-40]. After diagnosis of HBV infection, patients will undergo periodic testing to measure HBV DNA levels [27]. While a quantitative real-time polymerase chain reaction (PCR) method is ideal in laboratory and clinical settings to detect and monitor changes in HBV DNA levels, this method is not always accessible, especially in developing countries. Therefore, alternative methods such as qualitative PCR and nucleic acid testing (NAT) exist [41, 42]. While some prevalence studies have utilized qualitative PCR or NAT to identify HBV-infected patients [43, 44], most use the ‘gold standard’ quantitative real-time PCR. Over the past 5 to 10 years, the lower limit of detection for quantitative real-time PCR has steadily decreased [45, 46], from >200 copies/mL down to <50 copies/mL and continues to improve detection of low-level HBV replication, such as those observed in occult HBV infection.

**HBV Transmission**

HBV transmission can occur through exposure to blood and other bodily fluids, via multiple routes. Donated blood, blood products, tissues, and organs were once a significant route for HBV transmission; however, screening procedures have been implemented to detect HBsAg (1971) and anti-HBc (1986), which have greatly decreased transmission through blood transfusion and organ transplantation [47]. In the United States, transmission usually occurs through intravenous drug use (IDU) or sexual contact and results in roughly 330,000 new cases of HBV per year [48]. In other areas of the world, like Asia, the most common route is perinatal transmission from
mother-to-child. Due to the high number of HBV infections acquired at birth, 78% of chronic HBV-infected individuals live in Asia (Figure 3).

**Figure 3. Global Distribution of Chronic HBV Infections.** The majority of chronic HBV-infected individuals reside in Asia (78%), followed by Africa (16%) and South America (3%). North America, Europe, and Oceania each have a lower percentage of chronic HBV infections (1% each) [1].

Significant concern regarding HBV transmission via breast milk arose questioning whether transmission rates increased if HBV-infected mothers decided to breastfeed their newborns. To date, studies have indicated that the risk of HBV transmission from mother-to-child is greatest during birth and that the additional risk of transmission through breastfeeding is negligible [49].

**HBV Vaccination and Treatment**
Fortunately, HBV is an infectious viral disease for which an effective vaccine has been developed. This recombinant DNA vaccine against HBV was first designed in 1982 [50]. It encodes for part of the HBsAg; therefore, vaccinated individuals produce protective anti-HBs, as shown in Table 1. Immunity can last at least 20 years (the length of time the vaccine has been in use) and can be measured in vaccinated individuals using an anti-HBs titer [51].

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HBV DNA</th>
<th>HBsAg</th>
<th>Anti-HBs</th>
<th>Anti-HBc</th>
<th>HBeAg</th>
<th>Anti-HBe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Chronic</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Occult</td>
<td>+</td>
<td>–</td>
<td>Often –</td>
<td>Often +</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Past Infection</td>
<td>–</td>
<td>–</td>
<td>Often +</td>
<td>+</td>
<td>–</td>
<td>+/-</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Methods for manufacturing the HBV vaccine have been modified since the initial version, and the vaccine is currently available in several forms, which require three doses. Two vaccines are produced against HBV alone, while additional combination vaccines exist including protection from *Haemophilus influenzae* type b or hepatitis A virus, as well as one vaccine that protects against HBV, Diphtheria, Tetanus, Pertussis, and Polio [52]. Ongoing research to improve the current vaccine strives to prevent infection with known HBV vaccine escape mutants. HBV vaccination has been recommended in the United States since 1986. While this has never been required, in 2005, a more rigorous vaccination program for all children and adolescents was proposed to eliminate HBV transmission in the United States [52]. Additionally, introduction of mandatory HBV vaccination programs for newborns in numerous countries has greatly decreased the incidence of perinatally-acquired HBV infections [53]. Over time, the number of
chronic HBV infections should continue to decrease due to the effectiveness of HBV vaccination in children.

For individuals who become infected with HBV and develop chronic HBV infection, whether due to lack of vaccination or ineffective antibody production after vaccination, several treatments exist. First, both standard and pegylated interferon alfa – broad-spectrum antivirals – can be effective against HBV under certain circumstances [27]. In addition, since the HBV polymerase contains reverse transcriptase activity, several specific reverse transcriptase inhibitors, including lamivudine, adefovir dipivoxil, entecavir, tenofovir, and telbivudine, are approved by the Food and Drug Administration for HBV treatment [27]. Two experimental drugs – emtricitabine and clevudine – are being tested in clinical trials due to their efficacy against HBV, although the clevudine trial was halted due to safety concerns [54-56]. These reverse transcriptase inhibitors act against the HBV polymerase and are effective in decreasing HBV viral load.

Although guidelines for treatment of patients with HBV exist, drug regimens are determined on a patient-specific basis. Acute HBV is usually not treated, since 95% of adults will spontaneously clear the infection. However, in the case of fulminant hepatitis B, it has been reported that treatment with lamivudine improves survival rates [57]. For those with chronic HBV infection, several factors play a role in the decision to initiate HBV therapy and which medications to use, including ALT levels, HBeAg positivity, HBV genotype, and HBV DNA level, as well as the presence of risk factors for cirrhosis and HCC development. In addition, in patients with chronic HBV infection not undergoing treatment, consistent monitoring of ALT and HBV DNA levels is
important. Discontinuation of a particular medication or of all HBV treatment will depend on several factors, including the development of viral treatment resistance mutations or adverse events. Anti-HBV therapy can also be discontinued if HBV infection is resolved, as indicated by a long-term HBV DNA viral load below detectable limits, HBsAg seroconversion, and normalization of ALT levels [27].

**HBV/HIV Co-Infection**

Due to shared transmission routes, 3 million of the 40 million HIV-positive individuals worldwide are co-infected with HBV [58]. Development of chronic HBV infection in HIV-positive individuals was more common before the introduction of effective antiretroviral therapy (ART) due to severe impairment of immune responses that are otherwise necessary for HBV clearance. HIV patients are living longer today due to advances in ART, which allows the progression of liver disease to become more apparent in co-infected individuals. Liver disease is now a leading cause of morbidity and mortality in several HIV-positive cohorts [59].

It is already known that HIV co-infection has a significant impact on the natural history of HBV, since HBV-related pathogenesis and seroconversion are immune-mediated. HBV/HIV co-infection is associated with increased HBV replication, decreased probability of acute HBV clearance, delayed HBeAg seroconversion, as well as faster progression of liver fibrosis [60]. The observed increase in HBV replication may be indirectly due to increases in HIV RNA levels, since increased HIV RNA levels correlate with decreased CD4 cell counts [61, 62]. Lower CD4 levels indicate decreased overall immune function resulting in a decreased ability to clear HBV.
infection and correlate with decreased production of protective antibodies against HBV. Although overall immune function may be lower, immune-mediated killing of HBV-infected hepatocytes occurs via CD8 cells; therefore, HBV pathogenesis and liver damage may not be significantly decreased [63].

Vaccination against HBV should be considered for all HIV-positive individuals, although those with severely impaired immune responses may not produce sufficient anti-HBs; thus, anti-HBs titer should be measured after completion of the vaccination series. Fortunately, in regards to treatment, several reverse transcriptase inhibitors are effective against both HBV and HIV, including lamivudine, tenofovir, and emtricitabine [64, 65]. The ability to use the same drugs against both HBV and HIV somewhat eases the burden of treating co-infected individuals, but also makes treatment more complicated if drug-resistant HBV and/or HIV variants emerge. Several combination medications exist that are approved for HIV that likely impact HBV, but are not currently used in HBV monoinfected individuals. These include Atripla (tenofovir + emtricitabine + efavirenz), Combivir (lamivudine + zidovudine), Epzicom (lamivudine + abacavir), Trizivir (lamivudine + zidovudine + abacavir), and Truvada (tenofovir + emtricitabine). Unfortunately, effective ART may also accelerate liver damage directly through hepatotoxicity of medications, as well as indirectly through immune reconstitution, during which improvement in immune responses coincides with a strong response against HBV [66, 67]. Interferon or reverse transcriptase inhibitors with effectiveness against only HBV, can also be used to treat HBV in HIV-positive patients not on anti-HIV therapy, although these are only recommended at CD4 cell counts above 500 cells/μL to avoid immune reconstitution [27].
While much is known about the indirect effects of HIV infection on HBV, less is known about the effects of HBV infection on HIV. Studies suggest that these viruses can infect both hepatocytes and CD4 cells, the primary infection sites for HBV and HIV, respectively [68-70]. If both viruses infect the same cell, virus-virus interactions may occur and could directly affect the natural progression of each infection. *In vitro* studies investigating the functions of the hepatitis B virus X protein (HBx), which can act as a nuclear co-activator, have determined that HBx is capable of inducing the transcriptional activity of the HIV long terminal repeat (LTR) [71, 72]. Similarly, HBx is an inducer of NF-κB, an important regulator of HIV LTR activity [72]. As previously stated, increased HIV replication may indirectly increase HBV replication [60], potentially contributing to both persistence of HBV infection and accelerated liver disease progression.

**HBV Genome and Life Cycle**

Unlike hepatitis viruses A, C, D and E, which are RNA viruses, HBV is a DNA virus belonging to the *Hepadnaviridae* family. Hepadnaviruses are hepatotropic, yet non-cytopathic to infected hepatocytes [6, 15]. One trait of hepadnaviruses is their narrow host range; other members of this family include the woodchuck hepatitis virus and duck hepatitis virus. The circular HBV genome consists of 3.2 kilobases of partially double-stranded DNA that encode for four partially overlapping open reading frames (ORFs) (Figure 4) [73].
**Figure 4. HBV Genome Structure.** The HBV DNA genome contains 4 ORFs: 1) P, which encodes the viral polymerase; 2) PreS/S, which encodes the surface glycoproteins; 3) PreC/C, which encodes the core proteins; and 4) X, which encodes a regulatory protein of largely unknown function.

The polymerase ORF encodes the viral polymerase. The surface ORF contains the PreS and S genes, which are translated into the three forms of HBsAg that constitute 80% of the viral envelope (large (L)HBsAg, middle (M)HBsAg, and small (S)HBsAg). The core ORF, contains the PreCore and Core regions, which encode for the HBeAg and structural capsid proteins. Finally, the X ORF encodes HBx, a regulatory protein with many predicted functions (Table 2) [74, 75].
Table 2. HBV Proteins and Functions.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Functions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymerase</td>
<td>Reverse transcription of pgRNA into RC-DNA – the functional genome of infectious virions</td>
<td>[73, 76]</td>
</tr>
<tr>
<td>LHBsAg</td>
<td>Receptor binding</td>
<td>[77]</td>
</tr>
<tr>
<td>MHBsAg</td>
<td>Receptor binding and membrane fusion</td>
<td>[77]</td>
</tr>
<tr>
<td>SHBsAg</td>
<td>Receptor binding and contains the antigenic determinant region</td>
<td>[77, 78]</td>
</tr>
<tr>
<td>Core</td>
<td>Structural proteins that form the inner core of the virion</td>
<td>[73, 76]</td>
</tr>
<tr>
<td>HBeAg</td>
<td>Soluble protein that marks high levels of HBV replication (possible repression of host immune response)</td>
<td>[73, 76]</td>
</tr>
<tr>
<td>HBx</td>
<td>Direct and indirect modulation of cellular processes including: apoptosis, cell cycle progression, protein degradation, signal transduction, and transcription (possible assistance in G0 exit in arrested cells, as well as activation of nuclear factor-kB, extracellular signal-related kinases, and c-Jun N-terminal kinases)</td>
<td>[74, 79-81]</td>
</tr>
</tbody>
</table>

() indicate predicted functions

The hepatitis B surface antigen was first documented in 1965 as the “Australia antigen” [82]. In 1970, the hepatitis B virus was identified as the 42nm “Dane particle” by electron microscopy [83]. HBV virions – the infectious viral particles capable of infecting human hepatocytes – consist of an inner core containing the HBV genome, which is surrounded by an outer envelope (Figure 5).
Figure 5. HBV Virion Structure. The infectious HBV virion consists of an outer envelope – containing large, middle, and small forms of hepatitis B virus surface antigen (HBsAg) – surrounding HBV core proteins which form an icosahedral core to surround the relaxed circular DNA genome (RC-DNA). When the genome is packaged within the core, one copy of the viral polymerase is also enclosed.

The three forms of HBsAg in the outer envelope – large HBsAg (LHBsAg), middle HBsAg (MHBsAg) and small HBsAg (SHBsAg) – are capable of binding receptors on the cell membrane of hepatocytes, which compose up to 80% of cells of the human liver [31]. IgA-binding receptor and asialyglycoprotein receptor are among the postulated receptors for HBV entry [77]. LHBsAg and MHBsAg are responsible for receptor binding, as well as fusion of the viral envelope with the target cell’s plasma membrane, once the virion has entered the target cell via endocytosis. Membrane fusion triggers uncoating of the outer envelope from the inner core, which is released into the cytoplasm of the host cell. The HBV genome contained within the core of an infectious virion is then released from the core in its relaxed circular DNA form (RC-
DNA), which contains a nick in the positive strand and a gap in the negative strand of the genome, and is translocated to the nucleus of the host cell. The nick and gap regions must be repaired in the nucleus, using a host cell polymerase and ligase, to form the covalently closed circular DNA (cccDNA) necessary for transcription. At any point while in the nucleus, any segment of cccDNA can integrate at random into the host cell genome. The cellular RNA polymerase II is responsible for transcription of the 4 HBV mRNAs from the cccDNA: 1) pregenomic RNA (pgRNA), 2) PreS1 mRNA, 3) PreS2/S mRNA, and 4) X mRNA. HBx does not get packaged into virions, but has several potential functions affecting both viral and cellular processes that ensure efficient HBV replication [73].

The pgRNA is translated into several HBV proteins. These include: 1) HBeAg, a soluble antigen secreted from hepatocytes during periods of increased HBV replication, 2) the structural core protein, used for the capsids of newly forming virions, and 3) the viral polymerase. One copy of the pgRNA is packaged into new capsids along with one copy of the viral polymerase. The viral polymerase, which has reverse transcriptase capabilities, then converts the pgRNA into the RC-DNA form needed for infectious virions. When the capsid is assembled, it can move into the endoplasmic reticulum (ER) to be enveloped, or if in excess, can be recycled and the RC-DNA genome can be translocated back to the nucleus to form additional cccDNA.

Once the capsid reaches the ER, it is surrounded by all forms of HBsAg, which have been translated by the PreS1 and PreS2/S mRNAs in the ER, to form the outer envelope of the virion. Enveloped virions are then able to bud from the host cell to infect additional hepatocytes. In addition to virion budding, two types of HBsAg-containing subviral particles – spheres and
filaments – are released from infected hepatocytes. The round 22nm spheres contain only MHBsAg and SHBsAg, while the long, oval-shaped 22nm filaments can also contain small amounts of LHBsAg. These subviral particles are detected in the serum of acute and chronic HBV-infected patients at a ratio of 100-1000 to 1 virion. The excess of subviral particles stimulates an immune response against the immunogenic SHBsAg; however, these particles are also thought to distract the immune system and increase the chances of infectious virions escaping the immune response and continuing the HBV life cycle [73] (Figure 6).
**Figure 6. The HBV Life Cycle.** Virion envelope proteins bind unspecified receptors on the cell surface and enter the cell through endocytosis. Uncoating of the viral envelope and core surrounding the RC-DNA genome occurs and the RC-DNA enters the nucleus. The RC-DNA gaps are repaired in the nucleus, possibly by host cell factors, to form cccDNA. The cccDNA is used as the template for the transcription of the four viral mRNAs. The X mRNA is translated in the cytoplasm to form the regulatory HBx protein. The pgRNA is used to make HBeAg, which is secreted from cells, the viral polymerase, core proteins and is also packaged in new capsids. Once the capsid is formed, the viral
polymerase reverse transcribes the packaged pgRNA into RC-DNA. The large HBsAg, from the PS1 mRNA, and the middle and small HBsAgs, from the PS2/S mRNA, are synthesized in the ER and are released as subviral particles at a ratio of 100:1 to virions. New capsids travel through the ER and are enveloped in HBsAgs to form a complete virion. New virions bud from the cell to start the cycle again [73].

While the hepatocyte is the primary cell type infected by HBV, infection of extrahepatic cell types has also been reported [84]. Peripheral blood mononuclear cells (PBMCs), bone marrow cells, and several additional cell types have been reported to contain HBV transcripts, although some are not convinced that productive HBV replication occurs in all of these cell types. To date, PBMCs are the most likely extrahepatic cells to harbor replicating HBV [70], even though the topic remains controversial.

**HBV Diversity**

Due to the low fidelity of the HBV polymerase during reverse transcription, the mutation rate is high compared to most DNA viruses, with $2 \times 10^{-5}$ nucleotide substitutions per year [85]. Although this mutation rate is 10-fold higher than most DNA viruses, it is still 10- to 100-fold lower than the mutation rate of RNA viruses and retroviruses [73]. The result of an increased HBV mutation rate is a higher degree of genetic diversity compared to other DNA viruses. HBV exists within an infected individual as a population of similar, yet distinct viral variants known as the viral quasispecies. This degree of HBV variation allows the virus to persist in spite of the host’s defenses and/or antiviral therapy.
In addition to quasispecies diversity, 8 HBV genotypes (A-H) have been characterized that differ by at least 8% at the nucleotide level [86]. Within several genotypes, further differentiation into subtypes has been described, with at least 4% nucleotide divergence (Figure 7).

**Figure 7. Phylogenetic Diversity of HBV Genotypes.** GenBank references utilized in the current studies were aligned; genotypes are defined by >8% difference at the nucleotide level, while subgenotypes are defined by >4% but <8% difference at the nucleotide level.

Genotypes and subtypes exhibit some geographic restriction, likely due to differential evolution in primitive human populations (Figure 8). In the United States, due to the diverse nature of its human population, it is possible to encounter all HBV genotypes, although genotypes A and C predominate [87-89].
Figure 8. Global Distribution of HBV Genotypes. In the United States, while all genotypes could be identified, genotypes A and C are the most common. Rare genotypes (F, G, and H) are found in distinct regions in Central and South America.

Individuals are not limited to infection with one genotype of HBV. In regions of the world where 2 or more genotypes are prevalent, multiple exposures can result in HBV infections with multiple genotypes. In addition, recombinant viruses have emerged within individuals infected with viruses from more than one genotype [90].

Several methods exist for determining HBV genotype, such as restriction fragment length polymorphisms (RFLP) analysis, hybridization assays, and phylogenetic analysis of HBV sequence [91]. RFLP is a cheap and fast assay using restriction enzyme fragments to determine genotype, but is error-prone in the presence of single nucleotide mutations. Hybridization assays,
like the Inno-LiPA assay (Innogenetics), that uses amplification of the HBV PreS1 region followed by hybridization to a membrane strip containing genotype-specific oligonucleotides, are also faster, but expensive [92]. Phylogenetic analysis is the most reliable, especially if a mixed or recombinant HBV infection is encountered, although it is often avoided due to the time necessary to perform DNA amplification, cloning and sequencing, as well as the large cost required.

Regions where 2 HBV genotypes are common, such as Southeast Asia (B and C) and India (A and D), have made it easier for researchers to evaluate the clinical importance of HBV genotype. For instance, genotype can impact both the clinical course of disease, as well as treatment efficacies. In regards to clinical course of disease, genotype C has been associated with increased liver dysfunction compared to genotype B [86, 93]. Additionally, patients with genotype A have an increased risk of developing chronic HBV infection, but genotype D infection correlates with increased progression to HCC [94]. Finally, studies on recurrence of HBV after liver transplantation have also shown that genotype A infections have the least risk, while genotype D is associated with increased recurrence of disease [94].

Several studies on HBV genotype and its impact on treatment efficacy have indicated that genotype C infection correlates with failure of interferon therapy [86, 95, 96]. This decrease in efficacy results in an increased success rate of interferon therapy in areas like Northwestern Europe, where genotype A is common, compared to Southeast Asia with a high prevalence of genotype C. Spontaneous Pre-core mutants containing stop codons or deletions in the region encoding for the HBeAg also appear to be associated with interferon therapy failure [91].
Pre-core mutants are only one example of viral mutations that impact the clinical outcome of HBV infection. Immune selection pressure can trigger evolution of the S and core regions, via alteration of B- and/or T-cell epitopes, resulting in immune escape and viral persistence. In addition, prolonged monotherapy with one reverse transcriptase inhibitor may result in HBV viral variants with resistance mutations [97, 98]. Due to the overlap between the surface and polymerase ORFs, several mutations that arise due to antiviral resistance may also result in mutations of HBsAg. This phenomenon has resulted in the discovery of vaccine escape mutations that render the current HBV vaccine ineffective [91]. Despite the current vaccine and treatments available for HBV prevention and therapy, continued research is necessary to improve their efficacy against HBV mutants, as well as the methods used to detect these mutants.

**Occult HBV Infection**

Occult HBV infection (O-HBV) has often been referred to as “silent HBV infection” or “anti-HBc alone” due to the observed serologic pattern of undetectable HBsAg while often having detectable anti-HBc along with persistent HBV DNA detection [14]. O-HBV infection was initially thought to represent the “window period” between acute or chronic HBV infection and clearance, where HBsAg was diminished but anti-HBs were still below detectable levels [99]. This theory is not applicable for all HBsAg-negative individuals due to the observed detection of HBV DNA in several cases [100-102]. In some patients who have cleared HBV infection, anti-HBs may never be detectable; therefore, anti-HBc is used as a measure of past infection [3]. In individuals with positive anti-HBc alone, HBV DNA levels must be measured to differentiate between past infection and occult HBV infection.
O-HBV infection is found most often in the settings of HBV/HIV or HBV/HCV co-infection, although they have been identified in HIV− / HCV− blood donors and in liver biopsies from patients undergoing surgery for unrelated reasons without a history of liver disease [103-105]. Published prevalence rates of occult HBV infection in HIV-positive cohorts range from 0% – 89.5% (Table 3).

<table>
<thead>
<tr>
<th>Country</th>
<th>N tested</th>
<th>N (%) O-HBV</th>
<th>% HCV+</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iran</td>
<td>106</td>
<td>3 (2.8%)</td>
<td>NA</td>
<td>[106]</td>
</tr>
<tr>
<td>Netherlands</td>
<td>191</td>
<td>9 (4.7%)</td>
<td>13.6</td>
<td>[107]</td>
</tr>
<tr>
<td>Italy</td>
<td>115</td>
<td>17 (14.8%)</td>
<td>38.3</td>
<td>[108]</td>
</tr>
<tr>
<td>South Africa</td>
<td>502</td>
<td>38 (7.6%)</td>
<td>NA</td>
<td>[109]</td>
</tr>
<tr>
<td>Switzerland</td>
<td>57</td>
<td>51 (89.5%)</td>
<td>57.9</td>
<td>[100]</td>
</tr>
<tr>
<td>Spain (HIV+)</td>
<td>159</td>
<td>8 (5%)</td>
<td>55.3</td>
<td>[110]</td>
</tr>
<tr>
<td>Spain</td>
<td>238</td>
<td>15 (6.3%)</td>
<td>100</td>
<td>[111]</td>
</tr>
<tr>
<td>United States</td>
<td>179</td>
<td>17 (9.5%)</td>
<td>55</td>
<td>[43]</td>
</tr>
<tr>
<td>South Africa</td>
<td>192</td>
<td>34 (17.7%)</td>
<td>NA</td>
<td>[112]</td>
</tr>
<tr>
<td>China</td>
<td>105</td>
<td>32 (30.5%)</td>
<td>43.8</td>
<td>[69]</td>
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<tr>
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<td>167</td>
<td>22 (13.2%)</td>
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<td>[113]</td>
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<tr>
<td>France</td>
<td>160</td>
<td>1 (0.6%)</td>
<td>52.6</td>
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<td>48 (14%)</td>
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<tr>
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<td>NA</td>
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<td>France</td>
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<td>100</td>
<td>[117]</td>
</tr>
<tr>
<td>France</td>
<td>368</td>
<td>5 (1.4%)</td>
<td>100</td>
<td>[118]</td>
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<td>42 (41.6%)</td>
<td>100</td>
<td>[120]</td>
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<td>Lebanon</td>
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<td>24 (23.8%)</td>
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<td>[121]</td>
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<tr>
<td>Multinational (including US)</td>
<td>866</td>
<td>0 (0%)</td>
<td>100</td>
<td>[122]</td>
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<tr>
<td>Brazil</td>
<td>170</td>
<td>16 (9.4%)</td>
<td>NA</td>
<td>[123]</td>
</tr>
<tr>
<td>United States</td>
<td>240</td>
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<td>[102]</td>
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<tr>
<td>United States</td>
<td>909</td>
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<td>13.2</td>
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<tr>
<td>United States</td>
<td>452</td>
<td>8 (1.8%)</td>
<td>77.4</td>
<td>[124]</td>
</tr>
</tbody>
</table>

Representative studies including greater than 50 HIV-infected individuals; NA – not applicable; 1 denotes percentage HCV seropositive among persons with O-HBV.
Nunez et al. did not observe any occult HBV infections among 85 HIV+ / HBsAg– / anti-HBc+ IDUs [125]. On the other hand, Hofer et al. identified 51 individuals with detectable HBV DNA out of 57 patients (89.5%) with the anti-HBc+ alone serology [100]. This highly variable range is due to study-specific differences in region, experimental techniques, and inclusion criteria. In regards to experimental techniques, HBV DNA levels observed in the serum of occult HBV-infected individuals have been significantly lower compared to chronic HBV infections [123, 126]. This reduced HBV viral load requires sensitive PCR assays to reliably detect all occult HBV infections. Additionally, most studies may have excluded seronegative individuals (HBsAg– / anti-HBs– / anti-HBc–) or those with anti-HBs alone, by performing serologic testing before quantitative HBV DNA measurement. Nonetheless, occult HBV infections have been identified in individuals with all possible antibody patterns.

Occult HBV infection is transmissible through blood transfusion, both human-to-human and human-to-chimpanzee [127, 128]. In human-to-human studies, the recipients developed either chronic or occult HBV infections [127-130]. The risk of occult HBV transmission through blood donation has gained significant interest within the past year. Although occult HBV infection is found more often in co-infection with HIV or HCV in Europe and the United States, there is concern that transmission of occult HBV may occur more frequently in regions with a higher prevalence of HBV. Therefore, multiple prevalence studies of occult HBV in blood donors have recently been published from Northern Africa [131], the Middle East [132], and throughout Asia [130, 133-138]. In addition, transmission of occult HBV has been observed through organ transplantation, in which chronic HBV infection developed in a liver transplant recipient from an occult HBV-infected donor [129].
Almost all published studies on occult HBV have been retrospective in nature; therefore, little is known about the clinical outcome of occult HBV infection. While a direct clinical impact is lacking, several retrospective studies have associated occult HBV infection with increases in cirrhosis and HCC [139-141]. In occult HBV-infected individuals, severity of liver cirrhosis was increased and patients had a higher incidence of HCC development compared to HBV-negative patients. Additionally, two recent studies have prospectively assessed development of HCC in occult HBV-infected individuals, confirming increased incidence of HCC in occult HBV-infected patients compared to those without HBV infection [142, 143].

The reasons for the lack of detectable HBsAg in occult HBV infection are unclear, though several possibilities have been proposed: 1) persistence after reactivation from extrahepatic cell types; 2) interference of HBV replication by other viruses; 3) HBsAg is contained within immune complexes with anti-HBs rendering it undetectable; 4) altered host immune responses to HBV infection; and 5) altered HBsAg expression as the result of HBV mutation(s). Infection of extrahepatic cell types is a controversial topic [144, 145], but has not been excluded as a potential reservoir for HBV reactivation. However, a direct connection to occult HBV infection has yet to be established. Along similar lines, virus-virus interactions between HBV and HCV, as well as HBV and HIV have been described [71, 72, 146-149], but mechanisms affecting HBsAg expression are lacking. HBsAg in complex with anti-HBs presents a logical explanation, although immune complexes were not identified in occult HBV-infected patient serum [150]. Thus, the most relevant explanations appear to be altered host immune response and/or mutations of the HBV genome.
The vast majority of occult HBV studies have documented prevalence in various countries or populations (Table 4, above), but only one has analyzed immune responses in occult HBV-infected patients and only a few have analyzed HBV mutations associated with occult HBV infection (Table 5). These published investigations often describe mutations identified in full-length genome sequences from 1 or 2 individuals, most of whom harbor infection with genotype A, B, C, or D, and fewer have included functional analyses of occult HBV-associated mutations to determine the impact of specific mutations on HBsAg expression/synthesis (Table 4).

### Table 4. O-HBV Studies Including Mutational and/or Functional Analysis.

<table>
<thead>
<tr>
<th>Country</th>
<th>N tested</th>
<th>HBV Genotypes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>India</td>
<td>50</td>
<td>A, C, D</td>
<td>[151]</td>
</tr>
<tr>
<td>India</td>
<td>9</td>
<td>A, C, D</td>
<td>[152]</td>
</tr>
<tr>
<td>Multinational (Europe and S. Africa)</td>
<td>20</td>
<td>D</td>
<td>[153]</td>
</tr>
<tr>
<td>Taiwan</td>
<td>10</td>
<td>NA</td>
<td>[93]</td>
</tr>
<tr>
<td>Canada</td>
<td>9</td>
<td>NA</td>
<td>[154]</td>
</tr>
<tr>
<td>Brazil</td>
<td>15</td>
<td>A</td>
<td>[155]</td>
</tr>
<tr>
<td>India</td>
<td>66</td>
<td>A, C, D</td>
<td>[44]</td>
</tr>
<tr>
<td>Indonesia</td>
<td>25</td>
<td>B, C</td>
<td>[136]</td>
</tr>
<tr>
<td>Italy</td>
<td>3</td>
<td>NA</td>
<td>[156]</td>
</tr>
<tr>
<td>Germany</td>
<td>33</td>
<td>A, C, D, E</td>
<td>[78]</td>
</tr>
<tr>
<td>China</td>
<td>30</td>
<td>B, C</td>
<td>[137]</td>
</tr>
<tr>
<td>China</td>
<td>22</td>
<td>B, C</td>
<td>[138]</td>
</tr>
<tr>
<td>United States</td>
<td>1</td>
<td>NA</td>
<td>[157]</td>
</tr>
<tr>
<td>Germany</td>
<td>2</td>
<td>A, E</td>
<td>[159]</td>
</tr>
<tr>
<td>India</td>
<td>9</td>
<td>A, D</td>
<td>[103]</td>
</tr>
<tr>
<td>Germany</td>
<td>1</td>
<td>A+D</td>
<td>[160]</td>
</tr>
<tr>
<td>France</td>
<td>7</td>
<td>A, B, D</td>
<td>[38]</td>
</tr>
<tr>
<td>Japan</td>
<td>1</td>
<td>C</td>
<td>[161]</td>
</tr>
<tr>
<td>Italy</td>
<td>13</td>
<td>A, D</td>
<td>[162]</td>
</tr>
<tr>
<td>Germany</td>
<td>1</td>
<td>A</td>
<td>[101]</td>
</tr>
<tr>
<td>France</td>
<td>25</td>
<td>A</td>
<td>[163]</td>
</tr>
<tr>
<td>Brazil</td>
<td>4</td>
<td>A</td>
<td>[164]</td>
</tr>
<tr>
<td>India</td>
<td>2</td>
<td>A, D</td>
<td>[165]</td>
</tr>
</tbody>
</table>

NA – not applicable; Black – studies including only mutational analysis; Red – studies including both mutational and functional analyses; Blue – studies including only functional analysis (based on a previous mutational analysis); * indicates HIV-positive patients
Mutations in any of the four HBV ORFs could decrease HBV replication and/or affect HBsAg expression. For example, mutations in the basal core promoter and two enhancer regions can directly decrease overall HBV replication. X gene mutations may alter any of the regulatory functions of the HBx protein and could disrupt or create interactions between the protein and various transcription factors involved in HBV replication. Polymerase mutations, potentially due to drug resistance, can directly impact the fidelity of the enzyme and decrease viral replication. Polymerase mutations can also alter the S ORF and consequently, HBsAg synthesis. Mutations throughout the S ORF can affect HBsAg expression in several ways. First, mutations in the S region could affect detection of HBsAg by commercial methods. HBsAg is detected through antibody-based methods such as enzyme-linked immunosorbant assays (ELISAs), immunoprecipitation, or western blotting. HBsAg amino acid changes have the potential to alter antibody epitopes directly or alter HBsAg secondary structure inhibiting accessibility of antibody epitopes. Second, surface promoter mutations can directly reduce HBsAg through a decrease in PreS1 and PreS2/S mRNA levels, or can lead to virus retention or protein misfolding. Third, PreS mutations resulting in a stop codon can also eliminate production of any or all three forms of HBsAg. The three forms of HBsAg, L, M and S, are constantly kept in balance with the M and S forms in excess of L. An increase in L compared to the other two could disrupt this balance resulting in retention of HBV virions and subviral HBsAg particles, as well as ER stress [Xu, 1997 #66]. Finally, HBsAg amino acid changes that alter the secondary structure of the protein have the potential to affect protein folding and processing. Misfolded proteins could either be degraded normally by the cellular proteasome or trigger the unfolded protein response and disrupt normal cellular processes.
This dissertation utilizes a prospective HIV-positive cohort from the University of Cincinnati Infectious Diseases Center, previously utilized to determine prevalence of chronic and occult HBV infections (I) to: 1) assess immune responses in occult HBV-infected individuals; 2) investigate HBV diversity through rigorous phylogenetic analysis; 3) identify occult HBV mutations compared to chronic HBV infection; and 4) functionally characterize the impact of mutations associated with occult HBV infection on HBsAg expression, secretion, and retention.
Aims of the Present Investigation

Hepatitis B Virus (HBV) is an important cause of morbidity and mortality among HIV-positive individuals, since HBV/HIV co-infection occurs frequently due to shared transmission routes. Before antiretroviral therapy (ART), HIV-positive patients were more likely to develop chronic HBV infection due to impaired immune responses that would otherwise be capable of clearing HBV. HBV is a leading cause of both cirrhosis and hepatocellular carcinoma (HCC). With improvements in ART, HBV/HIV co-infected patients are living longer and liver disease is becoming more apparent. Despite available HBV vaccines and treatments, there are currently 350 million people living with chronic HBV infection worldwide. Chronic HBV infection is characterized by high levels of HBV replication and detectable hepatitis B surface antigen (HBsAg) in the serum twice over more than 6 months. Occult HBV infection, on the other hand, is defined as persistent low-level HBV replication in the absence of detectable HBsAg. Occult HBV infection has not been well-studied to date; therefore, several questions remain regarding the mechanisms leading to its development and clinical relevance. Several mechanisms have been postulated to explain the development of occult HBV infection. Among these, altered host immunologic response and mutations of the HBV genome appear most likely to contribute. Immunologic assessment of occult HBV is almost non-existent in the literature, with only one study performed thus far. In addition, few studies have included virologic characterization of occult HBV, especially in HIV-positive individuals. Identification of mutations that distinguish occult from chronic HBV infection is necessary, along with studies to determine the impact of specific mutations on HBV replication and HBsAg expression.
The goals of this dissertation were to assess immune responses in occult HBV-infected patients and to identify and characterize viral mutations, particularly in the surface (S) and polymerase (P) open reading frames (ORFs), that could contribute to the hallmarks of occult hepatitis B virus infection. To achieve these goals, three specific aims were developed, to be completed within an HIV-positive cohort, previously assessed for prevalence of chronic and occult HBV infection (I):

Specific Aim 1 – To utilize serum cytokines/markers to assess immune responses in chronic versus occult HBV-infected individuals (II).

To date, only one study related to immune responses in O-HBV-infected individuals has been performed [105]. This previous investigation assessed cell-mediated immune responses in patients with occult HBV and chronic HCV. Weak HBV-specific T cell responses were observed among patients without detectable anti-HBc but appeared normal in patients with detectable anti-HBc. Since liver biopsy and PBMC samples were not available from our cohort, we measured serum expression levels of several regulatory, apoptotic, and fibrotic/anti-fibrotic cytokines and markers as indicators of immune responses in individuals with chronic versus occult HBV infections. In addition, potential correlations between cytokine expression and patient variables, such as age, gender, race, and HBV DNA level, were evaluated.

Specific Aim 2 – To assess viral quasispecies diversity and identify virologic differences between chronic and occult HBV infection (III, IV).

Few studies have assessed HBV viral quasispecies diversity, let alone in an HIV-positive cohort or in occult HBV-infected individuals. In addition, a limited number of studies have investigated
mutations associated with O-HBV infection, only one of which was performed in HIV-positive patients [159], and two of which were performed on one patient each in the United States [157, 158]. In this aim, we first assessed HBV genotypes, as well as viral quasispecies diversity in the PreS, S, X, and Pol regions. Chronic and occult HBV diversity were also compared for the PreS, S, and Pol regions. In addition, mutations associated with occult HBV infection were identified through a rigorous analysis comparing occult HBV sequences to genotype-matched chronic HBV sequences and GenBank reference sequences.

**Specific Aim 3 – To determine the effects of S gene mutations on HBsAg expression, secretion and retention *in vitro* (V).**

A limited number of studies have included functional analysis of mutations associated with occult HBV infection, utilizing varying methods and suggesting several distinct mechanisms for the development of occult HBV infection. In this aim, chronic HBV sequences were used to create wild-type HBsAg expression vectors. Mutations associated with occult HBV infection were introduced into the wild-type HBsAg expression vectors by site-directed mutagenesis and transfected into Huh7 and HepG2 cells to measure extracellular and intracellular HBsAg levels at days 3, 5, and 7 post-transfection. Ratios of intracellular HBsAg to extracellular HBsAg were also calculated to assess intracellular retention of HBsAg.
Experimental Findings and Discussion

This section summarizes the findings of the five publications related to this dissertation project and describes how these findings impact the field of hepatitis B virus, specifically occult hepatitis B virus research. The five publications related to the dissertation are referenced by their corresponding roman numeral (I – V, as described in the Copyright Notice) and are provided at the end of the dissertation with specific descriptions of the methods utilized and results obtained for each study.

Occult Hepatitis B Virus Prevalence

An HIV-positive patient population from the University of Cincinnati Infectious Diseases Center (UCIDC) was utilized for the studies included in this dissertation. This cohort had been screened for prevalence of chronic hepatitis B virus (C-HBV) infection and occult hepatitis B virus (O-HBV) infection prior to the start of the main body of work described as part of the dissertation. In I, 909 HIV-positive patient sera were first screened for HBV DNA by real-time PCR, before determining presence or absence of serologic markers (HBsAg, anti-HBs, and anti-HBc) by ELISA, to ensure that any individual with O-HBV infection was identified in the current population. Thirty-one individuals had detectable HBsAg and HBV DNA, indicating C-HBV infection (3.4%), while 12 had detectable HBV DNA, but HBsAg levels were below detectable limits of the ELISA (0.5 ng/mL), indicating O-HBV infection (1.3%). Interestingly, this study identified 5 O-HBV-infected individuals who lacked detectable HBsAg, anti-HBs, and anti-HBc, making HBV DNA the only detectable marker of infection (see I, Table 1). When comparing the
two groups of patients, several differences were observed. First, CD4 and CD8 cell counts were lowest in the O-HBV group compared to the C-HBV and HBV-negative groups, although the trends were not significant. Second, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were significantly higher in the C-HBV group compared to the O-HBV or HBV-negative groups ($p < 0.001$; see Figure 2). Importantly, HBV DNA levels were significantly lower in O-HBV infections compared to C-HBV infections ($p < 0.001$; see Figure 1).

As reviewed in the Introduction, to date, several studies have been published determining the prevalence of O-HBV infection in HIV-positive individuals (see Introduction, Table 3). O-HBV prevalence varied greatly between studies (0% to 89.5%) [100, 122]; likely due to differences in experimental methods, inclusion/exclusion criteria, and geographical location, affecting transmission routes, host genetic factors, and prevalent HBV genotypes. Prior to I, only 3 studies had investigated O-HBV prevalence in greater than 50 HIV-positive individuals in the United States [43, 102, 124], with an additional multinational study including patients from the United States [122]. The majority of studies in HIV-positive individuals screened between 50 and 600 patients for O-HBV infection. To date, I represents the second largest set of HIV-positive patients examined and the largest O-HBV prevalence study in the United States, with 909 individuals screened for O-HBV infection. In addition, it represents the first study of O-HBV prevalence in a large, HIV-positive cohort with a high proportion of men who have sex with men (MSM). Of the four previous studies of O-HBV in the United States, two have been performed in cohorts with injecting drug use (IDU) as the main risk factor for acquisition of HBV infection. The other two studies observed lower percentages of IDU (~20%), and while sexual transmission
of HBV is assumed for the remaining individuals, heterosexual sex versus MSM were not stated.
While the current study examines O-HBV prevalence in a large, US-based, HIV-positive cohort, women, IDU, and Hispanic patients are still underrepresented and should be evaluated in the future.

The exclusion criteria for I were minimal and excluded patients with a previous HBV diagnosis, patients currently on interferon therapy, and patients with non-viral causes of liver disease, such as non-alcoholic steatohepatitis and autoimmune hepatitis. Alternatively, the majority of previous studies examining O-HBV prevalence restricted their patient population to HBsAg-/anti-HBc+ individuals. I represents the second publication from the United States to include patients positive and negative for HBsAg, anti-HBs, and anti-HBc, with the first also published by our group at the University of Cincinnati [102]. I also used a novel experimental design and performed patient testing in the reverse order compared to most investigations. Here, HBV DNA quantification was performed first to ensure that all patients with HBV DNA were identified. Next, HBV infections were stratified based on HBsAg, anti-HBs, and anti-HBc positivity. Most studies have performed serologic testing for HBsAg and/or anti-HBc first, with a minority of samples subsequently tested for HBV DNA. Whether additional O-HBV infections were missed in these previous studies due to the stringent selection criteria or the order of experimental methods is unknown.

I identified C-HBV infection in 3.5% of the 909 HIV-positive individuals tested. While slightly lower than the average of 5% prevalence of C-HBV in the United States [166], the percentage of O-HBV, 1.3%, is comparable to two previous studies in HIV-positive cohorts in the United
States with 1.6% and 2% prevalence. A third study, a multinational study including patients from the United States, did not find any O-HBV infections in 866 HIV-positive individuals [122]. This is likely due to the high percentage of patients on lamivudine (66%) and with undetectable HIV RNA levels (72%), since O-HBV infection appears to be associated with high HIV RNA levels (>10,000 copies/mL) [43]. The fourth study from the United States observed a 9.5% prevalence of O-HBV in HIV-positive patients [43]. While the strict inclusion criteria – HBsAg–/anti-HBc+ individuals – may have resulted in missed O-HBV infections, it also likely resulted in a higher percentage of O-HBV than may have been identified in a broader cohort.

I is the third study worldwide, and the second in the United States to compare C-HBV and O-HBV infections from the same cohort, since most restrict to HBsAg– individuals. While previous studies performed in the United States have associated increased O-HBV prevalence with low CD4 cell counts (< 200 cells/mL) and high HIV RNA levels (>10,000 copies/mL), these studies were performed in comparison to individuals without HBV infection. No significant differences were observed in regards to CD4 and CD8 cell counts and ALT and AST levels between O-HBV infections and HBV-negative individuals, as reported previously [102, 115]. On the other hand, I measured the prevalence of both O-HBV and C-HBV and investigated differences between these two groups in regards to CD4, CD8, ALT, AST, and HBV DNA levels. Importantly, I was the first to show significantly higher ALT and AST levels in C-HBV infections compared to O-HBV infections. It was also the second study worldwide and first in the United States to show significantly higher HBV DNA levels in C-HBV infections compared to O-HBV infections [123], which has been confirmed in a third HIV-positive cohort in South Africa [112].
In addition to the contribution our study has made in determining O-HBV prevalence in a large, US-based, HIV-positive cohort including mostly MSM, I sets an example for future prevalence studies of O-HBV infection. When determining prevalence of O-HBV in any population, HBV DNA should be measured first or at least in all individuals. Although quantitative real-time PCR is expensive when screening large patient populations, it is imperative to obtain an accurate prevalence of O-HBV. In I, the 909 patient sera were first pooled in groups of 10 to detect HBV DNA, and then each patient sample in a positive pool was run individually. This approach can decrease the overall cost of HBV DNA measurement. The importance of HBV DNA quantification also has clinical relevance. While HBsAg and anti-HBc testing is routinely performed, HBV DNA quantification is not. Our study highlights the importance of HBV DNA testing in high-risk groups, since five O-HBV infections were identified in which HBV DNA was the only detectable marker of infection. Identification of O-HBV infection in HIV-positive individuals can aid clinicians in determining the best course of treatment. While the published prevalence study did not include patient follow-up, 14 patients from this cohort – 10 with C-HBV and 4 with O-HBV – had additional samples available for testing. Five patients with C-HBV infection and all 4 with O-HBV infection had undetectable HBV DNA levels upon follow-up (unpublished data, not shown). Among these individuals 5 were concurrently being treated with lamivudine as part of their ART for their HIV, along with 1 patient who received hepatitis B immunization and had undetectable HBV DNA a year later. Consistent with previous studies, all individuals on lamivudine cleared their O-HBV infection [107, 124], stressing the importance of HBV immunization and proper screening for HBV in high-risk populations. In addition, it is important to include medications with anti-HBV activity in HBV/HIV co-infected individuals being treated for HIV.
HBV Diversity

The majority of HBV diversity studies have focused on the prevalence of and differences between various HBV genotypes and subgenotypes within a given country or geographic region. While these types of investigations are important, since treatment efficacy and development of cirrhosis and hepatocellular carcinoma (HCC) can vary depending on HBV genotype [86, 94], they are highly prevalent in the current literature [167-169]. On the other hand, few studies have evaluated HBV quasispecies diversity [170, 171], the population of viral variants that exist within an infected individual, especially in HBV/HIV co-infected individuals. Genetic distance (GD), Shannon entropy, and nonsynonymous-synonymous mutation (dN-dS) are measurements utilized to quantify quasispecies diversity. GD measures the average nucleotide divergence between the viral variants within an individual for a given region of HBV, while Shannon entropy \( S_n = -\sum (p_i \ln p_i) / \ln N \) measures the frequency of each nucleotide sequence among the total number of variants in a given patient. Alternatively, dN-dS values provide information regarding immune selection pressures acting upon a viral genomic region within a patient. Nonsynonymous mutations are nucleotide mutations that also cause an amino acid change within the translated protein sequence, while synonymous mutations change a nucleotide, but do not alter the protein. When more nonsynonymous mutations are present in a specific viral region than synonymous mutations (dN-dS > 0), it indicates that immune selection pressures are acting upon this region, potentially resulting in viral mutation and adaptation. In contrast, when more synonymous mutations than nonsynonymous mutations exist (dN-dS < 0), it is determined that immune selection pressures are not consistently acting against that viral genomic region within a particular patient.
It is important to evaluate quasispecies diversity, since it can have implications for viral mutation, fitness, and adaptability. Previous studies of HBV diversity have not included intrapatient analysis of GD, entropy, and dN-dS calculations at the same time, nor have they been performed in HBV/HIV co-infected individuals. Therefore, we sought to assess HBV diversity in patients from the UCIDC identified in I.

S Gene Diversity in O-HBV

In III, the PreSurface (PreS) and Surface (S) regions of HBV were successfully PCR-amplified from 23 C-HBV infections and 6 O-HBV infections from I, along with 4 additional C-HBV infections from the Hepatology Research Group sample repository at the University of Cincinnati. Due to the overlapping nature of the HBV open reading frames (ORFs), the PreS and S region PCRs also amplify a corresponding region of the polymerase – Pol(PS) and Pol(S), respectively. GD, entropy, and dN-dS calculations were performed for each region, for each patient, and median values were compared among regions of the HBV genome, as well as between O-HBV and C-HBV infections. Several interesting observations were made when comparing HBV genomic regions in C-HBV infections alone. First, significantly higher entropy was observed in PreS compared to S, as well as in Pol(PS) compared to Pol(S) (both p=0.001; see III, Figure 2B). These differences indicate that the PreS and corresponding spacer region of the polymerase are tolerant of mutations and even insertions or deletions, as previously observed for the polymerase spacer [172]. In addition, significantly higher dN-dS values were observed in Pol(PS) compared to PreS (p=0.001; see III, Figure 2C), indicating that PreS mutations may
negatively impact HBsAg structure and function, resulting in non-viable viruses, more often than polymerase spacer mutations negative impact HBV replication.

While the significant observations in C-HBV infections only appeared as trends between O-HBV and C-HBV infections, this was likely due to the small number of O-HBV patients in the study. Overall, O-HBV infections and C-HBV infections were similar in regards to GD and entropy. When comparing dN-dS, significant differences in median values were not observed between O-HBV and C-HBV infections. Interestingly, dN-dS values were greater than zero for 3 regions in O-HBV infections – PreS, S, and Pol(PS) – but only for 1 region in C-HBV infections – Pol(PS). This indicates that immune selection pressures are acting upon these regions consistently in O-HBV-infected individuals, but not in C-HBV-infected individuals. This could suggest that these regions are more conserved in C-HBV infections, while in O-HBV infections, increased mutation may come at a cost to the virus in the form of decreased HBsAg expression and/or decreased HBV replication. The effects of these immune selection pressures on viral mutation are discussed in detail below in the section on Mechanisms of O-HBV Infection.

**X Gene Diversity**

In IV, the PreS, S, and X regions were successfully amplified by PCR from 15 C-HBV infections and 2 O-HBV infections. Again, due to the overlapping HBV ORFs, the X region PCR also amplifies a corresponding region of the polymerase (Pol(X)). At least 10 cloned X gene variants were sequenced per patient. PreS, S, Pol(PS), and Pol(S) data for GD, entropy, and dN-dS calculations for the 17 patients with X gene amplification were included from III. The X and
Pol(X) regions had the highest GD and entropy values, indicating that these nucleotide regions are highly variable, yet maintain their function, suggesting that mutation has a neutral or advantageous effect. The S and Pol(S) regions had the lowest genetic distance and entropy values, indicating that these regions are more conserved, as observed previously (III). While dN-dS values were less than zero, indicating that immune selection pressures were not consistently acting against the X and Pol(X) regions and that X is only highly variable at the nucleotide level compared to the PreS, S, and corresponding Pol regions, it is interesting to note that 2 patients had individual dN-dS values greater than zero. These two patients were identified as genotype A+G dual infections by phylogenetic analysis of the X region, with viral variants that did not group with either genotype A or G reference sequences (see IV, Figure 1B). Through recombinant analysis, it was determined that 10 of 30 viral variants for one patient and 4 of 31 viral variants for the other patient were indeed recombinant A+G viruses with breakpoints within the X gene (the region where the recombination events occurred and genotypes change from A $\rightarrow$ G or G $\rightarrow$ A; see IV, Figure 5).

The X gene is of significant interest due to its ability to regulate various cellular processes, allow viral replication, and may contribute towards development of HCC [75, 79-81]. Given these important roles, it is surprising that intrapatient X gene variability has not been investigated previously. IV provides the first assessment of HBV X gene diversity in HIV-positive patients, and found that the X gene is significantly more variable than the S gene at the nucleotide level. While a similar level of variability was not observed at the amino acid level, indicating that host immune pressures are not consistently acting on the X region, it does not mean that immune selection pressures never target this part of the HBV genome. The two patients in IV with
identified recombination, had dN-dS values greater than zero, indicating that immune selection pressures were consistently acting upon X. These pressures may exist in these patients to select for recombinant A+G variants within these individuals that confer some benefit to the virus, whether increased replication, drug resistance, or altered regulation of cellular processes.

Dual infection with multiple HBV genotypes is frequently identified in HBV prevalence studies worldwide [173-175]. In the United States, genotypes A+G are the most common dual HBV infection, and genotype G single infections are rarely identified [176-178]. Geographic regions where multiple HBV genotypes are circulating allow high-risk individuals to become exposed to and potentially infected with multiple genotypes at once, or through multiple exposures. Infection of the same cell with two HBV genotypes creates an environment where recombination events could occur. To date, HBV recombination has been observed between most HBV genotypes [90, 179-186]. In addition, ‘hot spots’ of HBV recombination have been identified previously and include areas within the PreS, S, and Core regions, but not within X. While IV was not the first study to identify recombination within the X gene [185], it appears that X gene recombination may occur more frequently than originally thought. Additionally, IV was the first study to identify HBV X gene recombination in HBV/HIV co-infected individuals.

It is possible that the decreased competence of the immune system in HIV-infected individuals could result in a higher proportion of dual HBV infections [187]. A higher proportion of dual HBV infections in HIV-positive individuals could allow more recombination events to occur in this patient population. Regardless of the region in which HBV recombination events occur, the effects they may have on the course of HBV and HIV disease within co-infected patients are
undetermined. While the effects of recombination are infrequently investigated in the HBV field, this is not the case in the HIV field. For example, recombination events within the HIV reverse transcriptase have resulted in increased resistance to zidovudine, dual resistance to zidovudine and lamivudine, and dual resistance to an experimental protease inhibitor and zidovudine [188-190].

The specific recombination events between HBV genotype A and G viruses in IV are intriguing for several reasons. First, presence of HBV genotype G has been evaluated as a determinant of liver fibrosis in HIV co-infected patients [191]. Second, genotype G viruses appear to have decreased replicative fitness and have been predicted to require dual infection with another genotype to persist in chronic HBV infections. Recombination with a genotype A virus, for example, may then increase replication or pathogenic potential of the recombinant A+G viruses compared to a genotype G virus. Additional effects on cellular control, replication efficacy, and pathogenesis may result from X gene recombination as well, since the HBx protein has numerous functions within infected cells [74, 79-81]. The 5’ region of X contains the HBV enhancer sequences, responsible for increasing HBV replication, which are upregulated by the HBx protein. Additional binding regions are scattered throughout HBx (Figure 1).
Figure 1. Predicted Regions of the HBx Protein. Within HBx are several predicted binding regions for HBx to transactivate itself (X Binding Region – AA 21-50), to bind hepatitis B virus X associated protein-1 (XAP-1 Binding Region – AA 61-91), and to bind tumor protein 53 (p53 Binding Region – AA 101-154). In addition, one Regulatory Domain has been defined (AA 1-50), along with two Transactivation Domains (AA 60-76 and 110-139).

While differences in function of the HBx proteins encoded by the various genotypes of HBV have not been adequately investigated, if recombination between two genotypes occurs, in vivo activity may be increased, with unknown consequences. Again, few examples exist within the HBV literature, but as an example from the HIV field, several recombination events resulted in selection of a specific V3 loop sequence motif for viral variants in a patient with rapid progression to AIDS [192]. While recombination may have accelerated HIV pathogenesis, changes within the V3 loop of the HIV envelope can alter co-receptor usage, infectivity, and viral neutralization [193-196]. In light of the current data, the X gene should remain a focus of HBV diversity studies, effects of recombination should be investigated in vitro, and the roles of the HBx protein, especially in development of HCC, should continue to be elucidated.
III was the first study to assess intrapatient HBV diversity in O-HBV-infected patients, and one of the few to assess HBV diversity in HBV/HIV co-infected patients. While the sample size was small, with only 27 C-HBV-infected individuals and 6 O-HBV-infected individuals, statistically significant results were obtained. Although differences between C-HBV and O-HBV could not be determined for the X region due to the subset of patients examined in IV, two C-HBV-infected individuals were identified with recombination within X. For future studies, the number of patients should be increased to obtain more reliable results, and additional study groups should be included to assess C-HBV and O-HBV variability in HBV monoinfected patients. Additionally, the field is moving towards analysis of full-length HBV sequences [197, 198], rather than evaluation of specific regions. Full-length sequences provide the opportunity to assess overall viral variability and would enable identification of additional sites of HBV recombination that may exist in dual HBV infections and would be missed by analysis of specific HBV genomic regions.

Mechanisms of Occult Hepatitis B Virus Infection

The definition of O-HBV infection is low-level persistence of HBV DNA in the serum despite a lack of detectable HBsAg. Since O-HBV infection was first described, studies have investigated the potential mechanisms by which it could arise. To date, several mechanisms have been proposed including: 1) persistence of HBV infection in extrahepatic cell types, 2) interference of HBV replication by other viruses, 3) inhibition of HBsAg detection if contained within immune complexes with anti-HBs, 4) altered host immune responses to HBV, and 5) altered HBsAg expression as a result of HBV mutation(s).
1) Several reports have identified tissue/cell types outside the liver that are susceptible to HBV infection, including bone marrow, colon, kidney, spleen, as well as, peripheral blood mononuclear cells (PBMCs) [84]. On the other hand, few studies have successfully shown that these cell types are capable of supporting active HBV replication after infection. To date, the only extrahepatic cell type that appears to harbor active HBV replication are PBMCs, as indicated by detection of the HBV DNA form encoding for the HBV mRNAs – covalently closed circular HBV DNA (cccDNA) [70]. While the immune response, with or without assistance from antiviral therapies, may be able to clear HBV infection in the liver, some argue that it may persist at low levels within PBMCs [199]. Whether this low-level replication could explain the detectable levels of HBV DNA in the blood, despite a negative ELISA for HBsAg, is unclear. One study investigated persistence of HBV DNA after liver transplantation, where 2 of 101 patients experienced HBsAg+ reactivation, but did not focus on how extrahepatic sites of HBV replication could explain O-HBV infection [199]. Therefore, extrahepatic replication of HBV is likely not applicable for most O-HBV-infected individuals.

2) Due to shared transmission routes, HBV is often found in co-infection with HIV and HCV. Additionally, higher prevalence rates of O-HBV have been identified in HIV and/or HCV co-infected patients [100, 200]. It is already known that the HBx protein can increase activity of the HIV LTR, thereby increasing HIV replication overall; however, the direct effects of HIV on HBV have not been identified to date. There may be interactions between these viruses that could impact HBV replication and the possible development of O-HBV infection. In contrast, more is known in regards to the impact that HCV has on the course
of HBV infection. Both the structural HCV core and nonstructural-2 (NS2) proteins have been implicated in HBV inhibition. The HCV core protein has been shown to decrease HBV replication through suppression of the HBV enhancer, HBx protein, and HBV polymerase [146, 148, 149]. In addition to the decrease in expression of HBV transcripts, secretion of HBsAg and HBeAg were also inhibited 2- to 4-fold. Although HCV NS2 may be involved in inhibition of apoptosis [201] and phosphorylation of HCV NS5A [202], its specific functions have not been fully elucidated. NS2 has also been shown to decrease HBsAg and HBeAg secretion \textit{in vitro} and partially inhibit transcription of the HBV pregenomic RNA required for the formation of infectious virions [147]. Given the impact of HCV on HBV, it is plausible that HCV co-infection may impact the development of O-HBV in some patients. Unfortunately, few patients within our cohort are also co-infected with HCV and, therefore, cannot be adequately addressed in the current project.

3) As described in the Introduction, during the HBV life cycle, HBsAg is produced in excess and can be secreted from infected cells in the form of subviral particles. Once these subviral particles enter the bloodstream they elicit host immune responses. A result of the immune response is the production of anti-HBs, capable of binding to HBsAg. Once bound, the antigen and antibody form circulating immune complexes. One study identified 9 patients with detectable HBsAg, which after acid dissociation of immune complexes, increased to 22 patients positive for HBsAg [203]. Therefore, limited HBsAg detection due to its containment within circulating immune complexes represents a possible explanation for O-HBV. Two studies have subsequently evaluated the presence of circulating immune complexes during O-HBV infection. In the first, dissociation of complexes was performed
in HBsAg– patients, with HBV DNA being detectable after dissociation [204]. The second study performed density gradient centrifugation and immunoprecipitation of HBsAg with a PreS1 antibody and failed to identify the presence of circulating immune complexes in patients with O-HBV [150]. Given these recent data, it appears that circulating immune complexes do not play a significant role in O-HBV infection.

4) Clearance of acute HBV infection is mediated by a strong cell-mediated immune response, as mentioned in the Introduction. On the other hand, development of chronic HBV is characterized by weak cell-mediated and humoral immune responses against HBV [205, 206]. O-HBV appears to be a persistent form of HBV infection, and would likely mimic the weak immune responses observed during C-HBV infection. Since stronger immune responses result in HBV clearance, if O-HBV-infected individuals elicit stronger immune responses compared to C-HBV-infected individuals, this could partially explain both decreased HBsAg and decreased HBV DNA levels in the serum. The observed immune response to O-HBV may be increased compared to C-HBV infections, but would still be significantly impaired compared to immune responses resulting in successful clearance of HBV infection. Studies investigating host immune responses in O-HBV-infected patients are lacking; therefore, one aim of this dissertation was to assess immune responses in our patient cohort.

5) Since the mutation rate of HBV is higher than most DNA viruses [85], it allows the virus to consistently change. The resulting mutations within any HBV genomic region may contribute towards the hallmarks of O-HBV infection – decreased HBV replication and a
lack of detectable HBsAg. Therefore, mutations of HBV DNA represent the most likely scenario for the development of O-HBV infection and are the main focus of this dissertation.

**Immunology**

To date, two studies investigating immune responses in O-HBV-infected individuals have been published; one directly characterizing cell-mediated immune responses [105] and our study (II) indirectly assessing immune responses based on regulatory, apoptotic, and fibrogenic markers. Cytokines and immune markers were measured in this study, since O-HBV infection was identified retrospectively in the UCIDC cohort and stored sera were the only samples available for analysis. The hallmarks of a strong cell-mediated immune response are high levels of interleukin-2 (IL-2) and interferon gamma (IFNγ) [6, 206]. These cytokines, along with interferon-gamma-inducible protein 10 (IP-10) are indicative of a Th1 response, while IL-4 and IL-13 would represent a Th2, or antibody-mediated immune response. In addition, IL-10 is a key indicator of viral persistence, where higher levels suggest development of C-HBV infection [207]. In regards to apoptosis, cytotoxic CD8 cells (CTLs), responsible for cytolysis of HBV-infected hepatocytes, mainly induce apoptosis through the Fas system [208]. Soluble Fas (sFas) is capable of binding to the Fas ligand (FasL) on CTLs to inhibit Fas-mediated apoptosis. Alternatively, soluble FasL (sFasL) is capable of inhibiting Fas-mediated apoptosis by binding Fas on hepatocytes. Increased sFas or sFasL compared to healthy controls indicates inhibition of apoptosis. IL-8 is a cytokine induced by viral infection that can enhance viral replication and has been indicated as a marker for the degree of liver fibrosis [209, 210]. Finally, development of
liver fibrosis in patients with C-HBV infection is regulated by TGF-β1 [23]. TGF-β1 has been shown to control cell growth, proliferation, and differentiation, and may also contribute to development of HCC [24]. Therefore, levels of IL-2, IL-4, IL-8, IL-10, IL-13, IFNγ, IP-10, sFas, sFasL, and TGF-β1 were measured in 25 C-HBV infections and 12 O-HBV infections.

**II** represents the first study investigating serum cytokine expression in O-HBV-infected patients. Serum levels of IL-2, IL-4, IL-13, and IFNγ were below detection limits of the multiplex assay for the majority of C-HBV and O-HBV infections. These levels indicate weak immune responses against both C-HBV and O-HBV and imply that these are persistent viral infections. Along the same lines, persistence of O-HBV is supported by similar levels of IL-10 between C-HBV and O-HBV infections. Although not statistically significant, median levels of TGF-β1 were higher in O-HBV infections compared to C-HBV infections, potentially indicating increased fibrosis in O-HBV. One significant difference was observed; sFas levels in O-HBV infections were significantly lower than in C-HBV infections (p=0.01; see **II**, Figure 2). While sFas levels in O-HBV were still higher than in healthy controls, supporting persistent infection, it appears there may be less inhibition of apoptosis in O-HBV compared to C-HBV, which may result in increased pathogenesis.

Since no previous reports had investigated serum cytokine levels in O-HBV infections, it is interesting to note that these data support O-HBV as a persistent infection. Additionally, while increased sFas levels had been previously reported in C-HBV infections [211], they also appear to be increased in O-HBV infections compared to healthy controls. Interestingly, sFas levels were increased in patients with HCC compared to cirrhosis, presumably due to the higher levels
of apoptotic inhibition [212]. Although not statistically significant, our data would also support that O-HBV infection may be associated with increased fibrosis. These results would suggest decreased apoptotic inhibition in O-HBV infection compared to C-HBV infection and, therefore, decreased HCC. If lower sFas levels truly correlate with cirrhosis rather than HCC, the observed association between O-HBV and the development of HCC does not fit with the current data [142, 143]. While this study was performed in HIV-positive patients, all patients were HBV/HIV co-infected and observed differences were not solely the result of HIV infection.

In the previously published study investigating cell-mediated immune responses in O-HBV, 18 liver biopsies from HBsAg+/HBV DNA patients with HCV co-infection were expanded ex vivo to assess T cell function [105]. 11 patients were anti-HBc−, and 7 patients were anti-HBc+, with anti-HBc− patients producing very low levels of IFN and little evidence of T cell expansion. T cells from anti-HBc+ patients, on the other hand, appeared to produce sufficient T cell responses typical of protective memory and may be capable of controlling O-HBV infection. In patients negative for anti-HBc, the dysfunctional CD4 response likely results in an inability to prime CD8 cells. This scenario would likely result in decreased hepatocyte death due to a lack of CTL response and potentially leads to persistence of O-HBV infection.

The results from each of these studies require confirmation in additional cohorts, ideally those in which immune responses can be examined in HBV, HIV, and HCV monoinfected patients and co-infected patients. A larger, prospective cohort is also necessary to effectively evaluate changes in cytokine profiles and cell-mediated immune responses over time. While limited by small patient numbers and cytokine levels below detection limits, our study provides insight into
Mutations of the Hepatitis B Virus Genome

Mutations of the HBV genome remain a probable mechanism for the development of O-HBV infection, resulting in decreased HBV replication and a lack of detectable HBsAg. Viral mutations within any HBV ORF could impact these outcomes. As discussed above (IV) the HBx protein has various functions within HBV-infected cells [74, 79-81]. X gene mutation leading to a change in HBx function as a regulator of cellular processes or HBV replication could 1) alter translation and processing of HBsAg and subviral particles, 2) result in decreased transcription of S mRNAs and therefore decreased HBsAg, and 3) decreased serum HBV DNA levels due to decreased virion production. Along similar lines, mutations within the HBV enhancer I and/or II regions or the basal core promoter (BCP) could directly affect production of pgRNA [213, 214]. Decreased pgRNA production affects viral replication, translation of the viral polymerase (Pol), as well as assembly of infectious virions [215]. While this would mainly impact HBV DNA levels in the serum and may not directly impact HBsAg production, it may inhibit secretion of subviral particles due to decreased virion production. Pol mutations have the ability to directly decrease the replication efficacy of HBV [216]. In addition, nucleotide mutations within Pol have the potential to cause HBsAg amino acid mutations due to the overlapping nature of the ORFs. This has been observed previously, although not always in the context of O-HBV infection, where Pol treatment resistance mutations arise that introduce stop codons into S and prematurely truncate HBsAg [217, 218]. Finally, the most direct effect on HBsAg expression
would likely originate through mutation of the S ORF. Mutations within the S1 and S2 promoters can directly decrease transcription of the PS1 and PS2/S mRNAs, respectively [219]. Introduction of a stop codon into the S gene would truncate HBsAg at any point, but other nucleotide mutations causing a change in the amino acid sequence could alter the structure or processing of HBsAg. Changes in HBsAg structure could result in a lack of HBsAg in several ways, including: 1) detection by antibodies used in commercial assays, 2) protein misfolding leading to HBsAg degradation, induction of the unfolded protein response, or retention of HBsAg and viral particles within infected hepatocytes, and 3) altered formation and/or secretion of subviral particles [220-223].

Since mutations within PreS, S, and Pol may impact HBV replication and HBsAg expression, a mutational analysis was performed in III. Phylogenetic analysis of the PreS and S regions from 27 C-HBV infections and 6 O-HBV infections identified genotypes A (n=19), D (n=3), and G (n=5) in C-HBV infections and genotypes A (n=1), E (n=1), and G (n=3) in O-HBV infections. One O-HBV infection appeared to be a dual HBV infection with genotypes A+G, with genotype A and G viruses in the PreS region and only genotype A viruses in the S region. Additionally, 3 of the 5 genotype G C-HBV infections were later determined to be genotype A+G dual HBV infections upon additional analysis of the X region (IV). Sequence analysis to identify mutations associated with O-HBV infection was performed by comparing O-HBV PreS, S, and Pol amino acid sequences to genotype-matched C-HBV sequences from the same cohort and GenBank reference sequences used in the phylogenetic analysis. Sequence analysis revealed a number of mutations associated with O-HBV infection, listed in III Tables 2 and 3. These included 2 genotype A and 15 genotype G mutations in PreS1, along with 3 genotype A and 2 genotype G
mutations in PreS2. In S, 4 genotype A, 4 genotype E, and 6 genotype G mutations were identified, of which 1 genotype A and 3 genotype E mutations had been identified as associated with O-HBV infection in previously published studies [78, 93, 101, 152]. Additional analysis of the Pol region identified 4 genotype A and 13 genotype G mutations in the Pol spacer region which overlaps with PreS, as well as 3 genotype A, 2 genotype E, and 3 genotype G mutations in the Pol reverse transcriptase (RT) region which overlaps with S. Previous analyses of mutations associated with O-HBV infection have focused on the more prevalent HBV genotypes A, B, C, and D (see Introduction, Table 5), while only one study has investigated genotype E [159], and none have included genotype G in O-HBV-infected patients. This is likely the reason why so many new mutations were identified in association with O-HBV infection in genotypes E and G in III. Further analysis of O-HBV sequences compared to C-HBV sequences by viral epidemiology signature pattern analysis (VESPA) identified mutations significantly associated with O-HBV. These included M103I, K122R, and G145A in a genotype A O-HBV infection, along with the W153C mutation in the Pol RT region as a result of the same nucleotide substitution that causes G145A in S. With the exception of Pol RT W153C, all mutations had been previously published [78, 93].

Although several studies have been published investigating mutations associated with O-HBV infection, only one has been performed in an HIV-positive patients (n=2) [159]. In III, we assessed mutations associated with O-HBV in 6 patients co-infected with HIV for the first time in the United States. It is surprising that so few studies have been performed in HIV (n=2) or HCV (n=2) co-infected patients, since prevalence studies have indicated that O-HBV is found more often in these patient populations. It is also important to note how few studies have
identified mutations associated with O-HBV by comparing O-HBV sequences to those amplified from C-HBV-infected patients from the same cohort. Before III, only 4 studies out of 21 compared O-HBV and C-HBV infections – 3 in India and 1 in Italy. In addition to comparing O-HBV and C-HBV infections to identify mutations, III utilized a rigorous genotype-matched method. Although the S region contains less viral variability compared to other regions of the HBV genome, it still contains inherent variability between genotypes at the amino acid level and requires a genotype-matched analysis to identify mutations instead of natural intergenotypic variation. Genotype-matched mutational analysis was not performed in any of the 4 previous studies comparing O-HBV and C-HBV sequences.

In addition to the mutations associated with O-HBV identified in III, one O-HBV-infected patient harbored both genotype A and G sequences. Before this study, only one O-HBV-infected individual with dual HBV infection had been previously identified, although that individual was infected with genotypes A+D [160]. Our A+G dual infection contained two PreS1 mutations and 3 corresponding polymerase mutations identified during VESPA. Several factors appeared to play a role in development of O-HBV in the previously identified A+D dual infection. Both genotype A and D viruses harbored mutations within the BCP region, while genotype A viruses were replication-competent and genotype D viruses had decreased replication. The antigenic determinant region of genotype A viruses was heavily mutated and HBsAg was weakly detected in vitro, while genotype D viruses could be detected by commercial assays, but only intracellularly, indicating a secretion defect. This could be attributed to a S1 promoter mutation resulting in increased PS1 mRNA, while PS2/S mRNA levels were decreased for an unknown reason [160]. Increased PS1 mRNA can result in increased levels of LHBsAg and when
combined with decreased levels of MHBsAg and SHBsAg can result in arrest of HBsAg and infectious virion secretion [220, 223]. The chances of identifying a dual HBV infection without detectable HBsAg – dual O-HBV infection – would be exceptionally rare if due to viral mutation alone and would have to arise from infection with viral variants from multiple HBV genotypes harboring mutations that affect HBV replication and HBsAg expression. Although only 2 cases have been described to date, this phenomenon may occur more frequently than expected, likely indicating that development of O-HBV infection is a multi-factorial process. Given the speculation that genotype G viruses may have decreased replication efficacy, future analyses should include assessment of full-length sequences from our A+G dual O-HBV infection to completely assess viral mutations and potential recombination within this individual. Additionally, full-length genome analysis should be included for the other genotype G O-HBV infections to confirm that these are genotype G single infections. While 3 specific S mutations identified as significantly associated with O-HBV infection by VESPA were chosen for further analysis, additional mutations in PreS1 in genotypes A and G, along with S mutations in genotype E were also of interest due to their prevalence in 40% or more of clones sequenced and may also be of interest to study in future endeavors.

**Functional Analysis of Mutations Associated with O-HBV Infection**

Three significant S mutations – M103I, K122R, and G145A – associated with O-HBV infection, were chosen to evaluate their functional effects *in vitro*. These mutations were chosen due to their significance in the VESPA analysis, and because they were identified in a genotype A-infected patient, the most prevalent HBV genotype in the United States [87-89]. These 3
mutations lie within the antigenic determinant loops of HBsAg, and may affect HBsAg synthesis, folding, and/or secretion from hepatocytes (Figure 2).

**Figure 2. Predicted Secondary Structure of Genotype A SHBsAg.** Red circles represent the positions within the secondary structure where genotype A mutations M103I, K122R, and G145A reside (adapted from [160]). These mutations were previously identified as associated with O-HBV infection and lie within the antigenic determinant loops of HBsAg.

It was hypothesized that these mutations, alone or in combination, may affect protein folding and processing resulting in retention of HBsAg and infectious HBV virions within hepatocytes *in vivo*, explaining the lack of detectable HBsAg in the serum.
To assess the effects of M103I, K122R, and G145A alone and in combination, they were introduced into S gene sequences from two genotype-matched C-HBV infections. Patients were matched by HBV genotype A, age, race, and HCV serostatus. Once S gene sequences were amplified from the matched C-HBV-infected patients, they were ligated into a commercial pCMV-HA mammalian expression vector, which is under the control of the powerful cytomegalovirus immediate-early (CMV-IE) promoter and adds an influenza hemagglutinin (HA) tag to the 5’ end of the translated protein. Site-directed mutagenesis was then performed to introduce M103I, K122R, and G145A into the C-HBV S gene sequence (for a list of HBsAg expression vectors see V, Table 2). After transfection of individual HBsAg expression vectors into both Huh7 and HepG2 hepatocyte cell lines, cell culture supernatants and cell lysates were collected at days 3, 5, and 7 post-transfection to measure extracellular and intracellular HBsAg levels, respectively, by ELISA. The effects that M103I, K122R, and G145A may have in these in vitro transfection experiments could be interpreted in multiple ways and are explained in Table 1.

Table 1. Potential Outcomes for Functional Analysis.

<table>
<thead>
<tr>
<th>Serum HBsAg</th>
<th>Extracellular HBsAg</th>
<th>Intracellular HBsAg</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic</td>
<td>+++</td>
<td>High levels</td>
<td>Moderate levels</td>
</tr>
<tr>
<td>Occult 1</td>
<td>–</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Occult 2</td>
<td>–</td>
<td>Decreased</td>
<td>Similar/ Increased</td>
</tr>
<tr>
<td>Occult 3</td>
<td>–</td>
<td>Decreased</td>
<td>Similar/ Increased</td>
</tr>
<tr>
<td>Chronic (false negative)</td>
<td>–</td>
<td>High</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

a Secretion defect would be determined through subcellular localization within the Golgi compartments; b ER retention would be determined through subcellular localization within the endoplasmic reticulum (ER).
Overall, the G145A mutation alone significantly decreased both intracellular and extracellular levels of HBsAg across all time points (see V, Figure 1), although M103I+K122R+G145A had the most significant impact on HBsAg expression (see V, Figure 2). Since the combination of M103I+K122R+G145A represents the dominant HBV variant within the characterized O-HBV infection, these in vitro results would indicate that the small amount of HBsAg secreted could result in a lack of detection by commercial assays after dilution in the blood. Even though the most dramatic changes in intracellular and extracellular HBsAg arose with the presence of the G145A, M103I+G145A, K122R+G145A, and M103I+K122R+G145A, the combination of M103I+K122R also decreased HBsAg levels. Therefore, this study provides one mutation that could directly impact HBsAg levels in the serum along with 4 combinations of mutations with similar effects, potentially explaining a specific case of O-HBV infection. Comparison of HBsAg levels in cell lysates versus cell culture supernatants was also performed for each HBsAg expression vector. Intracellular retention of HBsAg was observed when the G145A mutation was present alone or in combination (see V, Table 3). Although this may be an artifact of the low overall levels of extracellular and intracellular HBsAg, it remains an interesting point to be addressed in future studies.

Early studies that identified individuals with HBsAg–/HBV DNA+ infections attributed this phenomenon, now known as O-HBV infection, to diagnostic errors in ELISA kits used for testing and to the ‘window phase’ during HBV clearance. The ‘window phase’ occurs when HBsAg levels decrease below detectable levels before anti-HBs increases to detectable levels and HBV DNA remains detectable at low levels. While this could explain a minority of HBsAg–/HBV DNA+ cases, additional O-HBV infections have resulted via other scenarios. To date, 12
studies have included in vitro functional analysis of mutations associated with O-HBV infection. Of these, 10 identified mutations or combinations of mutations that at least partially explain O-HBV in specific patients (Table 2).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Region</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>A2798C</td>
<td>Polymerase Replication Defect</td>
<td>[158]</td>
</tr>
<tr>
<td>G458A</td>
<td>Surface</td>
<td>Decreased PS2/S mRNA Levels</td>
<td>[159]</td>
</tr>
<tr>
<td>AA</td>
<td>I110M</td>
<td>Surface Impaired HBsAg Secretion</td>
<td>[163]</td>
</tr>
<tr>
<td>G119E</td>
<td>Surface</td>
<td>Impaired HBsAg Secretion</td>
<td>[163]</td>
</tr>
<tr>
<td>R169P</td>
<td>Surface</td>
<td>Impaired HBsAg Secretion</td>
<td>[163]</td>
</tr>
<tr>
<td>P142A</td>
<td>Surface</td>
<td>Decreased HBsAg Expression</td>
<td>[101]</td>
</tr>
<tr>
<td>D144V</td>
<td>Surface</td>
<td>Decreased HBsAg Expression</td>
<td>[101]</td>
</tr>
</tbody>
</table>

Three identified viral variants from O-HBV-infected patients with replication defects based on a single polymerase mutation [158] or multiple mutations throughout the HBV genome [157, 161]. Two additional studies observed decreased HBsAg production due to decreases in PS2/S mRNA transcripts based on a single nucleotide mutation [159] or multiple amino acid mutations [38]. Alternatively, a single mutation within S [163] or multiple mutations within S [160] or the S promoters [103, 165] resulted in impaired HBsAg secretion in four studies. Finally, one study identified two separate S gene mutations which decreased HBsAg expression alone or in combination [101]. This study represents the most similar result to the findings observed in V.

On the other hand, we characterized three HBsAg mutations – M103I, K122R, and G145A – which had not been previously assessed in vitro. Unlike several previous studies, we chose to characterize specific HBsAg mutations in the context of the S gene alone to determine their effects without the influence of outside factors related to HBV replication. Therefore, extracellular and intracellular HBsAg levels were measured over time to observe changes in protein synthesis and secretion. Time-courses have not been presented previously during in vitro
characterization of O-HBV infection, which is surprising since HBsAg expression may change over time. Finally, since in vitro cell culture systems may not fully represent HBV infection in vivo, two hepatocyte cell lines (Huh7 and HepG2) were utilized in V to identify consistent trends related to HBsAg mutations.

For the mutations assessed in V, it appears that O-HBV infection did not result from a diagnostic error since HBsAg was consistently detectable with each HBsAg mutation. This finding is consistent with a previous report investigating G145R along with hypothesized mutations at HBsAg position 145, where G145A was detectable in all kits tested [37]. The G145R mutation is one of the most commonly identified HBsAg mutations due to its initial association with vaccine escape in the 1990s [224]. This mutation has been identified in O-HBV-infected patients [137, 152-154, 156] and, therefore, represents a diagnostic concern. For this reason, commercial companies have repeatedly ensured that diagnostic kits are capable of identifying mutations at position 145 [225]; however, O-HBV infections harboring the G145R mutation still appear. Interestingly, the G145R mutation was reported to result in defective HBsAg secretion [163], although this study was not performed after identification of specific mutations associated with O-HBV infections. Although the focus of V is on the G145A mutation, since it produced the most robust decrease in HBsAg, both M103I and K122R have been identified in O-HBV-infected individuals previously [78, 93, 153] and had an impact on decreased HBsAg expression in vitro. This is possibly due to their positions within the major hydrophilic loops of HBsAg (amino acids 98 – 156) where the secondary structure of the protein could be affected. Either of these mutations could affect protein folding; nonetheless, the combination of M103I+K122R+G145A had the most drastic impact on HBsAg expression.
Although it could be concluded that HBsAg synthesis is significantly reduced, it is not clear in this system whether synthesis is decreased or degradation is increased. Since the natural S gene promoters are not included in the *in vitro* system and expression of all HBsAg variants is driven by the same strong CMV-IE promoter, there should be little to no variation in mRNA expression; therefore, this question cannot be addressed in the current *in vitro* system. Some may argue that if very little HBsAg was synthesized *in vitro* with M103I+K122R+G145A, as a consequence, infectious virions would not be formed due to the lack of available HBsAg. It is important to note that if there is a synthesis defect, additional mutations may reside outside the region of S examined in this study that could partially compensate for the effects of G145A. One mutation that was capable of partially rescuing a G145R mutation was M133I, although it compensated for an HBsAg secretion defect [163]. Alternatively, even though M103I, K122R, and G145A were identified in all clones sequenced for the genotype A O-HBV-infected patient, minor viral variants may still exist at a low frequency that lack these mutations. Methods such as ultra-deep pyrosequencing could address this possibility in future studies. Future investigations of these mutations associated with O-HBV infection are necessary in a full-length, replication-competent *in vitro* system. These studies will aid in differentiating between a defect in HBsAg synthesis versus accelerated HBsAg degradation due to M103I+K122R+G145A, and will also assess the impact of the W153C polymerase mutation, corresponding to G145A, on overall HBV replication.
Additional Viral Diversity Studies

Understanding the various impacts of viral variability is important in any field within virology. Variability can be associated with alterations in treatment response, adaptation to antiviral therapy, and changes in response to immune pressure that can result in evasion of the host’s immune system. It is also important to understand the associations between viral diversity and various host factors, such as age, race, and immunocompetence, as well as viral factors, such as viral load. Associations between these factors are of increasing importance in the setting of viral co-infections, especially with HBV, HCV, and HIV. Each of these viral infections is of interest to the Hepatology Research Group at the University of Cincinnati, and several additional studies of viral diversity are briefly described here.

HCV Diversity

At least 170 million individuals are infected with hepatitis C virus, an RNA virus belonging to the Flaviviridae family of viruses. Its genome is translated into a large polyprotein which is then cleaved into the various structural (nucleocapsid and envelope) and nonstructural (NS1, 2, 3, 4A, 4B, 5A, and 5B) proteins. The majority of HCV diversity studies have focused on variability within the structural genes, especially the envelope since it is most often recognized by the host’s immune system and can change rapidly to avoid clearance. Understanding HCV diversity is increasingly important as diversity is a predictor of both liver disease progression and treatment outcome [226-229]. Of increasing importance with the development of HCV-specific antivirals is the diversity of the NS5B gene, encoding the viral RNA-dependent RNA polymerase.
Therefore, the NS5B region was examined in 29 HCV-positive women, 25 of whom were also co-infected with HIV. NS5B variability was lower than previously observed for the HVR1 region of the envelope. This finding was not unexpected given that NS5B is conserved due to the important role of the viral polymerase in the viral life cycle. On the other hand, there was more variability within NS5B compared to previous data from the 5' untranslated region (UTR). This finding is not surprising, since the UTR is extremely well conserved due to the important RNA secondary structure required for HCV replication, making mutation in this region highly detrimental.

In addition, while many amino acids were identified as under negative selection by the fixed effects likelihood (FEL) and random effects likelihood (REL) methods, two amino acid positions within NS5B, 2807 and 2818, were indicated as under positive selection by REL. While no significant associations were observed between NS5B genetic distance or entropy and HCV RNA level, CD4 cell count, ALT, AST, or HIV viral load, dN-dS trended towards a positive correlation with CD4 cell count. Moreover, there was a significant negative correlation between dN-dS and HCV RNA levels. This indicates that mutations within NS5B may hinder HCV replication. The current analysis of NS5B variability is of great significance in HIV-positive patients where increased HCV RNA levels have been observed in HIV/HCV co-infections. Since HIV co-infection can result in decreased efficacy of HCV treatment, HIV may also affect NS5B variability and potentially impact HCV replication, although further studies are necessary.

Treatment resistance mutations are also of great concern during administration of medications with virus-specific activities, as observed with several reverse transcriptase inhibitors used to treat HIV and HBV. Further investigation of the NS5B region is becoming increasingly
important due to the rapidly expanding number of specifically targeted antiviral therapies against HCV (STAT-C), including protease inhibitors against NS3, the HCV protease, as well as nucleoside and non-nucleoside inhibitors against NS5B. Spontaneous clearance rates for HCV are significantly lower in adults than for HBV and treatment efficacy with pegylated interferon alfa and ribavirin, non-specific antiviral medications, is highly dependent on HCV genotype. For instance, treatment of genotype 2 or 3 HCV infections can result in a sustained viral response (SVR) for up to 90% of patients, while only 65% of patients infected with genotype 1. SVR percentages are even lower in HIV/HCV co-infected patients with 62% for genotypes 2 and 3 and only 29% for genotype 1. Therefore, STAT-C medications provide a promising new avenue for HCV therapy, especially in HIV/HCV co-infected patients. Of the three experimental non-nucleoside inhibitors – GS-9190, A-837093, and HCV-796 – resistance mutations C316Y and C316N have been described [235, 236]. In addition, the mutation S282T was described against an experimental nucleoside inhibitor, NM283 [237]. It is unknown if resistance mutations against STAT-C medications exist as natural variants within HCV-infected individuals. Therefore, 79 STAT-C-naïve women with HCV infections of varying genotypes, mostly genotype 1a, were assessed for presence of STAT-C resistance mutations (VI). The C316Y mutation was identified in 3 separate blood draws collected from an HCV monoinfected, treatment naïve woman over a 4-year period. While this resistance mutation was identified in a single patient, its presence as a naturally occurring HCV variant should not be ignored. This variant may be circulating in HCV-infected individuals in the United States and should be included in screening for HCV resistance prior to initiation of STAT-C.
HIV Diversity

An estimated 40 million individuals are infected with HIV, many of whom are co-infected with HBV and/or HCV. With liver disease becoming a significant cause of morbidity and mortality in HIV-positive patients, potential interactions between these viruses is increasingly important. While it appears that HBV and HCV can replicate within extrahepatic cell types [70, 238], less is known about the potential for HIV to infect liver cell types. In the past few years, several reports have been published indicating that HIV can interact with and infect several cell types within the liver [239], most importantly hepatocytes [68, 240-242], the primary site of HBV and HCV infection. Consequently, investigations into HIV variability within the liver are required. To date, only one study has been published that examined variability in HIV proviral DNA amplified from the liver; therefore, we investigated differences between HIV variants amplified from liver biopsies or postmortem tissue compared to plasma (VIII). HIV RNA was detectable in 9 liver samples, 7 biopsies (from HIV/HCV co-infected patients) and 2 postmortem liver samples (1 HIV/HCV co-infection and 1 HBV/HIV co-infection). HIV gag (nucleocapsid) was amplified from 7 patients, while HIV env (envelope) was amplified from 6 patients. Nucleotide sequences for gag were different between the plasma and liver for all 7 patients, while amino acid sequences for gag were different for 5 of 7 patients. All nucleotide and amino acid sequences for env were different between plasma and liver for all 6 patients. Additional tests for compartmentalization (Mantel’s test and Slatkin-Maddison test) indicated compartmentalization was present between the plasma and liver for 4 patients in the gag region and 5 patients in the env region. This implies that tissue-specific selection pressures exist between the plasma and liver. In addition, several specific amino acids were identified that may distinguish variants
capable of infecting and replicating within different tissues. These differences in HIV variants capable of infecting the liver may have additional implications for our understanding of HIV-mediated liver damage and/or interactions with HBV and HCV in the liver.
General Summary and Concluding Remarks

In summary, this dissertation investigated several aspects of occult hepatitis B virus infection in HIV-positive individuals. First, the prevalence of occult hepatitis B virus was determined in a prospective HIV cohort at the University of Cincinnati Infectious Diseases Center. Second, markers of host immune responses were compared between chronic and occult HBV-infected individuals from this cohort. Third, HBV diversity was assessed in these HIV-positive individuals. Fourth, a plethora of mutations associated with occult HBV were identified within this population. Finally, 3 genotype A mutations within the surface gene of HBV were characterized in an *in vitro* system to assess HBsAg expression. Several conclusions were derived from these studies:

1) O-HBV is prevalent in HIV-positive individuals from the UCIDC, albeit at low levels. O-HBV-infected patients have significantly decreased levels of HBV DNA in the serum compared to C-HBV. C-HBV infections also have significantly higher ALT and AST levels compared to O-HBV infections and HBV-negative patients.

2) Host immune responses appear to be similar between O-HBV and C-HBV infections in HIV-positive patients, indicating that both are persistent forms of HBV infection. In addition, O-HBV infections may have increased levels of apoptosis compared to C-HBV infections, and could lead to increased pathogenesis in these patients. Overall, the immune system likely plays a part in the development and maintenance of persistent O-HBV infection.
3) Viral diversity varies significantly between HBV genomic regions in C-HBV-infected individuals and should be considered when performing mutational analyses. Additionally, increased immune selection pressures appear to act against the PreS and S regions in O-HBV-infected individuals compared to C-HBV-infected individuals, and may result in selection of specific viral mutations.

4) HBV recombination can occur between genotypes A and G within the X region of HBV/HIV co-infected patients. The effects of these recombination events on the natural progression of HBV and/or HIV in these patients are unknown, but may include increased pathogenesis.

5) Several mutations of the HBV genome were associated with O-HBV infection compared to C-HBV infection. After rigorous analysis of these mutations, several were identified in genotypes A, E, and G, which likely impact HBsAg expression.

6) The G145A mutation associated with O-HBV infection appears to directly affect synthesis of HBsAg. Combination of G145A with the M103I and K122R mutations results in barely detectable levels of HBsAg in both cell culture supernatants and cell lysates in vitro in two hepatocyte derived cell lines, Huh7 and HepG2. The presence of these HBsAg mutations and resulting decrease in HBsAg secreted from hepatocytes at least partially explains the lack of HBsAg detection in a genotype A, O-HBV-infected individual.
Taken together, the findings from this dissertation have significantly impacted the field of occult hepatitis B virus infection. The largest O-HBV prevalence study in the US was performed, confirming decreased HBV DNA levels compared to C-HBV infection. Additionally, the first study investigating cytokine and immunologic marker expression was performed in O-HBV-infected individuals. X gene recombination was identified in C-HBV-infected patients with A+G dual infection for the first time in the US. The first rigorous investigation of HBV diversity was performed in HIV-positive individuals and included assessment of O-HBV diversity and identified mutations associated with O-HBV infection. Through further analysis of 3 specific mutations, it appears that they may account for the lack of detectable HBsAg in an O-HBV-infected patient. This represents only the second study worldwide to investigate O-HBV based on single S gene mutations directly resulting in decreased extracellular and intracellular HBsAg levels in vitro.
Future Directions

Since hepatitis B virus was identified in 1970, significant advances have been made regarding HBV diagnosis, treatment, and vaccination, as well as understanding the viral life cycle, host immune response, and pathogenesis. Despite these advances, there are still several questions that remain within the broad field of HBV, and more specifically in regards to occult HBV infection. While this dissertation focuses on future directions for occult HBV research, significant effort should be continued to improve the HBV vaccine to protect against known vaccine escape mutants and identify new medications to combat treatment resistant viruses. In addition, the current trend of HBV research to elucidate the role of the HBx protein in the development of hepatocellular carcinoma should continue. Advances in our understanding of HBV will continue to improve the clinical diagnosis and management of HBV infection and liver disease resulting from chronic HBV infection.

The field of occult HBV research, compared to the field of HBV infection as a whole, is relatively new. To date, several prevalence studies have been performed in various populations worldwide using varying methods, but little is known about the biology and pathogenesis of O-HBV infection. Before additional large-scale studies investigating O-HBV prevalence and future prospective studies are performed, standardization of experimental design, inclusion and exclusion criteria, and ELISA kits used for detection of HBsAg, anti-HBs, and anti-HBc are necessary. HBV DNA levels should be measured in all patients, followed by stratification by detectable serologic markers. Patients with HIV and/or HCV co-infections should not be
excluded and risk factors for infection should be included in patient information. Standardizing these variables will help researchers to effectively compare results among various populations.

Development of O-HBV infection is likely a multifactorial process, with mutation of the HBV genome, co-infection with HCV and/or HIV, and the host immune response likely playing important roles. Once additional information regarding development of O-HBV infection is collected, a classification system will be necessary to categorize distinct types of O-HBV. These categories should be based on:

1) Reasons for decreased HBV DNA
   a. HBV mutation, including region identified, resulting in:
      i. Decreased activity of the viral polymerase
      ii. Impaired virion secretion
   b. HIV and/or HCV co-infection
   c. Increased immune response

2) Reasons for lack of detectable HBsAg
   a. Diagnostic error
   b. “Window period” between infection and clearance
   c. HBV mutation, including region identified, resulting in:
      i. Decreased HBsAg synthesis
      ii. Increased HBsAg degradation
      iii. HBsAg retention in the ER
      iv. Impaired HBsAg secretion
   d. Decreased PS1 and/or PS2/S mRNA synthesis
3) Serologic marker status
   a. Detectable HBsAg in liver?
   b. Immune status, in regards to detectable anti-HBs or anti-HBc

Prospective *in vivo* studies are lacking and are necessary to elucidate: 1) the clinical impact of O-HBV infection, 2) potential changes in O-HBV status over time, 3) the effects of general antiviral therapy and/or HBV-specific medications on the development and clinical outcome of O-HBV infection, 4) host immune responses against O-HBV compared to acute and chronic HBV infections, and 5) differences between O-HBV infections with and without HIV and/or HCV co-infection. Ideally to achieve these goals, a large, multinational, prospective study would be designed to include underrepresented minorities, patients infected with all HBV genotypes, and HIV+, HCV+, and HIV+/HCV+ co-infection groups, to track patients with acute, chronic, and occult HBV infection over the span of 30-40 years to effectively assess development of liver disease. Unfortunately, this approach is not practical for many reasons; therefore, many smaller studies should be performed in established prospective cohorts in various countries across the globe to address the following questions:

1) Does O-HBV infection result in increased development and increased severity of cirrhosis and hepatocellular carcinoma?

While O-HBV infection is associated with increased development of cirrhosis and HCC, these studies have been small in nature, and no concrete mechanisms have been elucidated to date. Many debate whether O-HBV infection truly plays a role in these pathogenic processes or whether these apparent associations are merely coincidental. Additionally, severity of liver disease and development of cirrhosis and HCC may correlate with specific
factors during O-HBV infection, such as increased HBV DNA levels, presence of anti-HBs and/or anti-HBc, and specific viral mutations that may have contributed to development of O-HBV infection.

2) **How might O-HBV infection change over time?**

Whether serologic markers of O-HBV infection may change over time is unknown. One study defined patients that had at least one serum sample without detectable HBsAg as having O-HBV-infection [100], but whether patients with fluctuating HBsAg positivity are in the ‘window period’ before viral clearance is undetermined. Anti-HBs and anti-HBc status may also change over time during chronic HBV infection; therefore, it is necessary to elucidate whether this is also possible during O-HBV infection.

Prospective studies will also provide valuable information regarding HBV variability over time. They will help to determine if specific viral mutations associated with O-HBV infection are capable of persisting over time. Additionally, they could determine how prevalent dual HBV infections are and whether their frequency is increased in the presence of HIV co-infection as indicated in a preliminary study [187].

Finally, whether HBsAg is detectable in the liver compared to the serum provides an additional layer of complexity in defining O-HBV infections, and further investigation is required to differentiate between potential categories of O-HBV infection.

3) **Are the mutations identified in these studies that may partially explain O-HBV infection identified in other populations?**
Several mutations have been identified in association with O-HBV infection. The majority of these mutations have not been examined in functional analyses. However, of those that have, few have been assessed in more than one study. Mutations identified in association with O-HBV should be examined in other populations with higher prevalence rates of HBV and O-HBV. In addition, comparison of O-HBV variants present in patients with various risk factors, for example IDU versus high-risk sexual practices, should be performed and may be influenced by HCV and/or HIV co-infection.

4) Is antiviral therapy with pegylated interferon alfa or HBV-targeted medications effective in O-HBV clearance?

While it is recommended that high-risk patients are vaccinated against HBV [27], current HBV treatment guidelines do not address potential treatment of O-HBV-infected individuals. In an age where HAART-naïve patients are becoming increasingly rare, patients treated for HIV with medications that are effective against HBV should be monitored closely for their effects on O-HBV in future prospective studies in HIV-positive individuals. Preliminary studies from our group and others [107, 124] indicate that in HBV/HIV co-infected individuals, patients compliant with HIV therapy including medications with anti-HBV activity reduce HBV DNA levels to below detectable limits. Therefore, it is necessary to confirm these preliminary findings in individuals with O-HBV infection.

5) How are host cell-mediated, adaptive, and humoral immune responses in O-HBV infection similar or different from those observed in acute HBV and chronic HBV infections?
Only 2 studies have been performed investigating immune responses in O-HBV-infected patients. Both studies had significant limitations, with small sample sizes, analysis of one type of immune response or restriction to surrogate markers of immune responses, and restriction to one sample type, liver or serum. Prospective analysis of adaptive, cell-mediated, and humoral host immune responses, utilizing serum, PBMCs, and liver biopsies, will be required to effectively characterize responses during O-HBV compared to those present during acute or chronic HBV infection. Additionally, prospective analysis of immune responses will help to determine whether host immune factors play a role in development or maintenance of O-HBV infection.

6) *Is the course of O-HBV infection different in the presence/absence of HIV and/or HCV co-infection?*

To date, studies have not included enough patients with O-HBV or those with HIV and/or HCV co-infections to compare differences between patients co-infected with O-HBV/HIV versus O-HBV/HCV. While previously debated, it is clear that HIV is capable of infecting and replicating in various liver cell types [239, 241], introducing a site for direct virus-virus interactions. Data from prospective studies of O-HBV *in vivo*, in addition to corresponding *in vitro* studies discussed below, may elucidate the interactions between all three of these viruses.

In addition to the broad topics that require further investigation in the field of O-HBV infection, several future directions arose directly from the studies presented in this dissertation, as briefly mentioned throughout the discussion.
1) **What are the effects of recombination, in the X gene and elsewhere in the HBV genome, on the course of HBV and HIV in co-infected patients?**

As mentioned in the discussion of IV, X gene recombination is rarely observed. Its identification in two HBV/HIV co-infected patients requires further investigation. Analysis of full-length HBV sequences should be performed to identify additional sites of HBV recombination throughout the viral genome, and *in vitro* experiments should be performed to assess the impact of HIV proteins or infection in hepatocyte cell lines on replication of recombinant HBV variants.

2) **What effects do mutations associated with O-HBV infection in the S region have in a full-length replication-competent system and could other mutations in the BCP, Pol, Enhancer, or X regions impact development of O-HBV infection?**

The G145A mutation in S also results in a W153C mutation in Pol. The effects of W153C on HBV replication are unknown and require a full-length, replication-competent *in vitro* system. Additional mutations within the BCP, Pol, Enhancer, or X regions, have not been fully assessed in the current set of O-HBV-infected individuals. Analysis of full-length viruses could identify additional mutations for functional analysis in future studies.

The HBx protein has many functions during HBV infection, as discussed earlier, including modulation of proteasomal activities [243]. Our data from V indicate that specific S gene mutations result in either decreased HBsAg synthesis or increased HBsAg degradation. If degradation is increased in our current *in vitro* system, it is unclear whether the HBx protein could inhibit degradation and lead to an HBsAg secretion defect. This possibility
should be explored by co-transfecting an expression plasmid encoding HBx into the current system, or in the form of a full-length HBV expression system. Finally, mutations that appear to have an impact on HBsAg expression and/or viral replication in a full-length system should be evaluated in primary hepatocytes to confirm results obtained from experiments in Huh7 and HepG2 cell lines.

3) **What effects do HIV and/or HCV proteins, as well as HIV and/or HCV infection have on HBsAg expression and HBV DNA replication in vitro?**

*In vitro* studies provide an ideal system to elucidate direct and indirect virus-virus interactions. Our current system (V) investigates the effects of HBV mutations on HBsAg expression, but could also be utilized to determine the effects of HIV and/or HCV on HBsAg expression. Future full-length, replication-competent systems to investigate the effects of HBV mutations on both HBsAg expression and HBV replication can also be used to determine the effects of HIV and/or HCV on HBV replication. The various experiments possible are depicted in Figure 1.
Figure 1. Proposed *in vitro* Experiments to Determine Effects of HIV and HCV Viruses and Proteins on HBsAg Expression and HBV Replication. Plasmids encoding for HIV proteins (p24, gp120, or Tat) or HCV proteins (core, NS2) could be co-transfected with HBsAg expression vectors into Huh7 and HepG2 cells and primary hepatocytes, or co-transfected with full-length replication-competent HBV genomes to determine effects on intracellular HBsAg, extracellular HBsAg, and HBV replication. HIV gp120 has proviral effects on HCV replication [244] and induces IFN-stimulated genes [245], although its effects on HBV replication have yet to be investigated. Alternatively, the major structural HIV protein, p24, could be compared with results from gp120. In addition, potential effects of the HIV Tat transactivator protein on HBV are unknown. HCV core and NS2 proteins have been shown to decrease HBV DNA replication [146-149] but their effects, if any, on HBsAg are unknown. Alternatively, infectious HIV and/or HCV virus could infect cells transfected with HBsAg expression vectors or whole HBV genomes.
While these studies provide potential next steps for the field of O-HBV infection research, they are by no means an exhaustive list. Future investigation into O-HBV infection and various aspects of HBV infection in general will undoubtedly provide important insight into these infectious disease processes and lead to improved clinical management.
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The Prevalence and Significance of Occult Hepatitis B Virus in a Prospective Cohort of HIV-Infected Patients

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Background: Occult hepatitis B virus (HBV) is defined as low-level HBV DNA without hepatitis B surface antigen (HBsAg). Prevalence estimates vary widely. We determined the prevalence of occult HBV at the University of Cincinnati Infectious Diseases Center (IDC).

Methods: Patients in the IDC HIV database (n = 3867) were randomly selected using a 25% sampling fraction. Samples were pooled for HBV nucleic acid extraction. Pools were tested for HBV DNA by a real-time polymerase chain reaction (PCR) assay to coamplify core/surface protein regions. The PCR assay was run on all individual samples from each DNA+ pool. DNA+ samples were tested for HBV serologic markers.

Results: A total of 909 patients without known HBV were selected. The mean CD4 count was 384 cells/mm³. Forty-three patients were HBV DNA+. Twelve of 43 were DNA+/HBsAg for HBV serologic markers.

Conclusions: Forty-three percent of those with HBV were not previously identified as HBV+, indicating the need for ongoing screening in high-risk populations. Occult HBV may occur in persons with all negative serologic markers, representing a challenge for identification.

Key Words: hepatitis, hepatitis B virus/HIV coinfection, HIV, occult hepatitis B virus

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patients seen in the HIV clinic from 1988 to the present, a total of 3867 patients. All patients have provided informed consent under an institutional review board (IRB)-approved protocol for entry into this bank and database.

Patients with an available serum sample were included in the study. Exclusion criteria included previously documented HBV infection, interferon-based treatment at the time of serology, and nonviral liver disease (eg, known autoimmune hepatitis, nonalcoholic steatohepatitis). Prior vaccination for HBV was not an exclusion criterion, because patients with occult HBV infection may include those who did not respond to HBV vaccination.16

Patients with serum samples from 1988 through 2004 were randomly selected for HBV DNA testing. Patient information extracted from the database for this study included age, race, gender, risk factor (eg, IDU, men who have sex with men [MSM], health care worker, transfusion, hemophilia) if available, CD4+ and CD8+ cell counts, any ART, hepatitis C virus (HCV) status on the basis of an enzyme-linked immunosorbent assay (ELISA), detectable/undetectable HIV viral load, and HBV serostatus if known (including any record of prior HBV vaccination).

**Laboratory Methods**

**Sample Collection**

Samples consisted of serum or plasma separated from ethylenediaminetetraacetic acid (EDTA) anticoagulated blood. Samples were collected, processed within 4 hours of venipuncture, and stored at −70°C until testing.

**Hepatitis B Virus DNA Extraction**

Initially, using the 909 total patient samples, 91 sample pools were created using 100-μL aliquots of each of 10 samples. HBV nucleic acid extraction was performed on the pools using the Qiagen QIAamp UltraSens Virus kit (Valencia, CA), which is designed to concentrate viral DNA from serum or plasma for increased sensitivity. Briefly, 1-mL sample pools were mixed with a lysis buffer to sediment nucleic acids into a pellet using low-speed centrifugation. After removal of the supernatant, preheated resuspension buffer and proteinase K were added to dissolve the pellet, which was then heated for 10 minutes at 40°C. The lysate was then loaded onto a QIAamp spin column. HBV DNA was eluted with 60 μL of low-salt buffer and stored at 4°C. Each isolation process included a known positive control.

**Hepatitis B Virus DNA Amplification**

Pooled DNA samples were tested for HBV DNA using a real-time PCR assay to simultaneously coamplify 2 separate regions of the HBV genome. The primers Chen forward (5′-AGT GTG GAT TCG CAC TCC T-3′) and Chen reverse (5′-GAG TTC TCC TTC TAG GGG ACC TG-3′) amplified a 119-base pair (bp) fragment of HBV core (positions 2269–2387).17 The primers surface forward (5′-GGA GTT CTC TCA ATT TTC TAG GG-3′) and surface reverse (5′-CAA ATG GCA GTA GAA ACC TGA GC-3′) were developed in-house to amplify a 432-bp fragment of the surface protein (positions 261–692). PCR assays were performed using both primer sets, and a sample was considered positive for HBV DNA if a product was found in either region. Ten microliters of eluted DNA was used in a 50-μL reaction with the Stratagene (Cedar Creek, TX) Brilliant SYBR Green QPCR Master Mix kit and 150 nM of each of the 4 primers. Amplification was performed in triplicate on a Stratagene Mx3000P Real-Time PCR system with the following thermal cycling profile: an initial 10 minutes at 95°C for 1 cycle; 65 cycles of 30 seconds at 95°C, 30 seconds at 56°C, and 40 seconds at 72°C; and then a final 5-minute extension at 72°C. A dissociation profile was generated at the end of each run plotting the fluorescence negative derivative against the melting temperature to confirm the correct sizes of the PCR products generated.

If any product was amplified in the 10-specimen sample pool, the DNA isolation and subsequent real-time PCR assays were repeated on the 10 individual specimens to determine the positive sample(s). Each amplification process included a known positive control from a previous PCR run and a no-template control (PCR reaction mix and water).

**Hepatitis B Virus DNA Quantification**

HBV DNA viral titers were determined by running in triplicate a standard panel (OptiQuant HBV Viral DNA Panel; Acrometrix, Benicia, CA) consisting of 7 samples with HBV DNA concentrations ranging from 0 to 20,000,000 IU/mL. This panel was calibrated against the World Health Organization (WHO) International Standard for HBV DNA for use in assessing and standardizing nucleic acid test procedures. A linear relation between the sample threshold cycle (cT) and the log DNA standard concentration was observed across the range of the panel members ($r = 0.95$). The detection limit of the real-time HBV DNA assay was determined to be 100 IU/mL.

**Hepatitis B Virus Serology**

Serologic testing was performed in triplicate on those individual specimens that were HBV DNA+. Results were obtained using standard commercially available ELISA test kits for anti-HBc, anti-HBs, and HBsAg (BioChain, Hayward, CA). Some patients had prior serologic test results recorded in the database. If retest results did not correlate with prior results, or if results were close to the limit of detection, the samples were tested again. Most (2 of 3) test results were considered accurate.

**Sample Size and Statistical Analysis**

Sample survey techniques were implemented to facilitate DNA testing. For sample size determination within the full 3867-person database, we conservatively assumed an occult HBV prevalence of 1.5%. To detect this proportion of occult patients in a whole sample with a variance of 0.08% (50% of the prevalence) with 95% confidence, a 25% sampling fraction was sufficient, corresponding to 967 patients.

Differences in continuous outcomes between patient groups were determined by the Student $t$ test, the Wilcoxon rank-sum test, analysis of variance with the Scheffé adjustment for multiple comparisons, or the Kruskal-Wallis test. Normality was assessed by the Shapiro-Wilk test, and data were transformed if necessary. Dichotomous outcomes were assessed by the Pearson or Fisher exact test as appropriate. In all cases, 2-tailed $P$ values $\leq 0.05$ were considered statistically significant.

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RESULTS

Patient Population

Using sample survey techniques as described, a 25% sampling fraction was used to calculate a required sample size of 967 patients from the entire sample database for testing. Thus, every fourth patient was randomly selected from the database to yield the required number of samples for testing. Fifty-eight patients were documented to have chronic HBV; these individuals were excluded from further analysis, yielding 909 patients for further analysis. The mean patient age was 35 ± 8.7 years, 77% of patients were male, and 50% were white. Six hundred eighty-eight patients had a recorded HIV viral load; of those, 62% had a detectable HIV viral load. The mean CD4+ count was 385 ± 305 cells/mm³. Twenty-three percent (210 of 909) of patients were on ART. Of these, 128 were on ART with activity against HBV (lamivudine, adefovir, tenofovir, or combinations including these treatments). Serum samples were typically obtained from individuals on entry to the IDC, explaining, in part, the lower level of use of ART.

Hepatitis B Virus DNA

Using a pooled testing approach, 32 sample pools of 100 were positive for HBV DNA. Each individual in the positive pools was then tested separately. Forty-three individual patients (4.7%) were HBV DNA+. Twelve of these 43 patients (27.9% [1.3% of the cohort]) were HBsAg-negative, indicating occult HBV infection. Serologic marker patterns for all HBV DNA+ patients are shown in Table 1. Although the typical marker pattern of anti-HBc alone was seen in 2 patients, 5 patients were negative for all serologic markers, and 4 patients were positive for anti-HBc and anti-HBs. Analysis of demographic and/or clinical differences between the 4 serology groups for the occult patients was not feasible because of the sample size. In the 3 main groups of patients (HBV+, HBsAg+, and occult), gender proportion was the only significant between-group difference, with HBsAg+ patients being 100% male. The most prevalent risk factor for HBV infection was homosexual sex. Of the patients on ART with anti-HBV activity, 4 were chronic HBsAg+. None were occult. The demographic and clinical characteristics of these patient groups are summarized in Tables 2A and 2B. There was a significant difference in HBV DNA titers between occult and HBsAg+ patients (3.71 ± 1.00 vs. 6.09 ± 2.23 log IU/mL; \( P < 0.001 \), t test with Satterthwaite correction), as shown in Figure 1. Interestingly, 1 patient was repeatedly and strongly HBsAg+ yet HBV DNA−. The PCR assay was performed in triplicate and manually on this subject with reproducibly negative results, raising the possibility that sequence mutations precluded amplification with our primer sets. This patient was not included as occult or HBV+ in analysis. Only 24 patients had a record of prior vaccination; none were chronic HBsAg+, but 2 of these were occult HBV+. One patient was anti-HBs− and anti-HBc−, and the other was anti-HBs only. Both had low-level replication (4.29 and 3.11 log IU/mL, respectively).

Liver Enzyme Elevations

Overall, 121 patients had alanine aminotransferase (ALT) elevations greater than 2 times the upper limit of normal (ULN). Of these, 109 were HBV DNA−, 10 were HBsAg+, and 2 had occult HBV infections. Mean levels of ALT and aspartate aminotransferase (AST) for the HBV+ group were 34.5 ± 30 U/L. For the 2 antigen-positive and occult HBV+ groups, mean ALT and AST levels were 64 ± 30 and 33 ± 22 U/L, respectively. Because neither ALT nor AST was normally distributed, values were log-transformed for further analysis. The differences in transformed ALT and AST between the HBsAg+ patients and the other 2 groups were statistically significant (\( P < 0.001 \), analysis of variance [ANOVA] with the Scheffe adjustment) as shown in Figure 2. ALT and AST did not differ between occult versus HBV+ patients, however. Of those 121 patients with elevated ALT, 99 had information available on ART regimens. Seventy-two (73%) of the 99 patients were not on any ART. Eight-four of the 121 patients had HCV ELISA data available, and 20 (24%) were positive by ELISA. Two occult patients had an ALT >2 times the ULN. One was HCV-coinfected and was not on ART; the second was neither HCV+ nor on ART. Of the 31 HBsAg+ patients, 10 had an ALT >2 times the ULN, 2 of whom were on ART; 18 had HCV ELISA records; and all were negative. Log ALT and log AST correlated positively and significantly with the log HBV viral titer (\( r = 0.30, P = 0.05; r = 0.33, P = 0.04 \), respectively).

CD4 and CD8 Cell Counts

Overall, the mean CD4+ count was 385 ± 305 cells/mm³. Although counts were highest in the HBV+ group and lowest in the occult group (shown in Table 2), this trend was not statistically significant (\( P = 0.20 \), ANOVA with log-transformed CD4+ cell count). Similarly, CD8+ counts were lowest in the occult group but not statistically different from the other 2 groups. These trends remained after removing patients on ART from the analysis. CD4+ and CD8+ cells counts were not significantly associated with ALT, AST, or HBV viral titer.

DISCUSSION

Occult HBV is a controversial phenomenon, with conflicting data on its prevalence and clinical significance. In the current analysis, 12 occult patients were detected from a total of 909 subjects, representing a prevalence of 1.3% (95% confidence interval [CI]: 0.6% to 1.9% for the entire cohort of 3867 patients), with a range of serologic markers. This is in contrast to prior studies. For example, an analysis of a random sample of 240 treatment-naive subjects from the Adult AIDS Clinical Trials Group (AACTG) detected a 16% prevalence of anti-HBc alone,\(^9\) of which 10% had detectable HBV DNA. Another recent analysis detected occult HBV infection in 4% of 93 anti–HBc-alone patients in an HIV-positive

<table>
<thead>
<tr>
<th>TABLE 1. Serologic Markers of Patients With Positive HBV DNA by Real-Time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HBc− Anti-HBc+ Anti-HBs− Anti-HBs+ Anti-HBc− Anti-HBc+ Total</td>
</tr>
<tr>
<td>HBsAg+</td>
</tr>
<tr>
<td>HBsAg−</td>
</tr>
</tbody>
</table>

\( \text{Copyright © Lippincott Williams & Wilkins} \)
A study of the French Aquitaine cohort detected HBV DNA in only 1 of 160 HIV-positive anti–HBc-alone individuals, however. It has been postulated that occult HBV strains harbor mutations that prevent generation of excess surface antigen, and some studies have found such sequence variations. This does not adequately address the higher prevalence of occult HBV seen in patients with HIV, however. Rather than identifying those with the anti–HBc-alone marker pattern, we identified those with HBV DNA positivity first and then performed serologic testing. This allowed us to detect 5 subjects harboring occult infection with no serologic evidence of infection, who were negative for HBsAg, anti-HBc, and anti-HBs. In these 5 patients, HBV replication was low (4.3 log IU/mL), 1 patient was on ART, another had elevated ALT (63 U/L), a third had a detectable HIV viral load, and CD4+ counts ranged from 106 to 559 cells/mm3. There were no distinguishing factors that would differentiate these patients from the other occult patients. This phenomenon has not been previously described to our knowledge, likely because it is rare to test for HBV DNA before serologic status.

To address the theoretic possibility of PCR contamination, we retested each of these 5 serologically negative patients separately and in triplicate by real-time PCR assay. As with the first round, PCR products were again detected in the core or surface region (or both) of all 5 samples. In a separate qualitative assay, we amplified the core and presurface regions of the genome from each sample (data not shown). The retesting of serologically negative individuals by real-time PCR assay and amplification using a qualitative PCR assay strongly suggest that our findings are not attributable to contamination.

The finding of 5 “all negative” patients raises the question of clinical relevance. Because occult HBV has been shown to have hepatopathogenic potential, it may eventually prove necessary to identify and monitor these patients. If occult infection is possible with all-negative serologic marker patterns and there are no other clinical signs or symptoms of infection, identification of these patients is not possible unless a sensitive PCR assay is performed, which is an impractical option for most clinical settings. These patients may remain unidentified for years until symptoms develop, and even then, elevated ALT/AST levels may be misattributed to ART-related hepatotoxicity if no other evidence of viral hepatitis is present. Indeed, a recent prospective study detected a significantly

### Table 2A. Demographic and Clinical Characteristics of HBV−, HBsAg+/HBV DNA+, and Occult Patients

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Gender*</th>
<th>Race†</th>
<th>CD4 Count (cells/mm³)</th>
<th>CDS Count (cells/mm³)</th>
<th>% HCV+</th>
<th>% Detectable HIV</th>
<th>Any ART</th>
<th>HBV Viral Titer§ (log IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV− (n = 865)</td>
<td>35.4 ± 8.76</td>
<td>80.9%</td>
<td>339 (149–567)</td>
<td>751 (501–1097)</td>
<td>13.5%</td>
<td>86.6%</td>
<td>20%</td>
<td>N/A</td>
</tr>
<tr>
<td>HBsAg+ and DNA+ (n = 31)</td>
<td>35.9 ± 7.95</td>
<td>100%</td>
<td>298 (148–416)</td>
<td>865 (611–1332)</td>
<td>0%</td>
<td>92.3%</td>
<td>18.75%</td>
<td>6.09 ± 2.23</td>
</tr>
<tr>
<td>Occult (HBsAg− HBV DNA+) (n = 12)</td>
<td>33.4 ± 6.32</td>
<td>75%</td>
<td>167 (118–490)</td>
<td>539 (354–788)</td>
<td>14.3%</td>
<td>100%</td>
<td>25%</td>
<td>3.71 ± 1.00</td>
</tr>
</tbody>
</table>

*P = 0.02 for difference in gender proportions between groups.
†Racial categories: White (W), African American (AA), Hispanic (H), and Other (O).
‡Numbers of samples available for each analysis are reported in cells (n) if different from total n in each category.
§P = 0.001 for difference between HBV DNA+ groups.
N/A indicates not available; SD, standard deviation.

HCV− cohort. A study of the French Aquitaine cohort detected HBV DNA in only 1 of 160 HIV-positive anti–HBC-alone individuals, however. It has been postulated that occult HBV strains harbor mutations that prevent generation of excess surface antigen, and some studies have found such sequence variations. This does not adequately address the higher prevalence of occult HBV seen in patients with HIV, however. Rather than identifying those with the anti–HBC-alone marker pattern, we identified those with HBV DNA positivity first and then performed serologic testing. This allowed us to detect 5 subjects harboring occult infection with no serologic evidence of infection, who were negative for HBsAg, anti-HBc, and anti-HBs. In these 5 patients, HBV replication was low (<4.3 log IU/mL), 1 patient was on ART, another had elevated ALT (63 U/L), a third had a detectable HIV viral load, and CD4+ counts ranged from 106 to 559 cells/mm³. There were no distinguishing factors that would differentiate these patients from the other occult patients. This phenomenon has not been previously described to our knowledge, likely because it is rare to test for HBV DNA before serologic status. To address the theoretic possibility of PCR contamination, we retested each of these 5 serologically negative patients separately and in triplicate by real-time PCR assay. As with the first round, PCR products were again detected in the core or surface region (or both) of all 5 samples. In a separate qualitative assay, we amplified the core and presurface regions of the genome from each sample (data not shown). The retesting of serologically negative individuals by real-time PCR assay and amplification using a qualitative PCR assay strongly suggest that our findings are not attributable to contamination.

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### Table 2B. Proportions of HBV+ HIV-Infected Patients With Risk Factors for HBV Acquisition

<table>
<thead>
<tr>
<th>Any Drug Use</th>
<th>Past IDU</th>
<th>Alcohol Use</th>
<th>Marijuana Use</th>
<th>Tobacco Use</th>
<th>Crack Cocaine</th>
<th>Other Drug Use</th>
<th>Homosexual Sex</th>
<th>Commercial Sex</th>
<th>Health Care Worker</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg+ and DNA+ n/N (%)</td>
<td>11/26 (42%)</td>
<td>7/24 (29%)</td>
<td>3/20 (15%)</td>
<td>10/21 (48%)</td>
<td>2/3 (0.87%)</td>
<td>17/22 (77%)</td>
<td>0/23 (0%)</td>
<td>1/23 (0.43%)</td>
<td></td>
</tr>
<tr>
<td>Occult (HBsAg− HBV DNA+) n/N (%)</td>
<td>2/12 (1.7%)</td>
<td>0/11 (0%)</td>
<td>3/8 (38%)</td>
<td>3/8 (38%)</td>
<td>0/9 (0%)</td>
<td>6/11 (55%)</td>
<td>0/11 (0%)</td>
<td>2/11 (18%)</td>
<td></td>
</tr>
</tbody>
</table>

Between-group differences in risk factor proportions (drug use, sexual exposure, and health care worker) were not statistically significant (P = 0.20).
higher rate of liver enzyme elevations in HIV-positive patients with occult HBV than in those without HBV DNA.\(^2\)

Occult HBV has been implicated in HBV transmission through blood donation\(^9\)\(^,\)\(^24\) and liver transplantation;\(^25\) in HBV reactivation;\(^22\)\(^,\)\(^26\) and in significant liver pathologic findings, including hepatocellular carcinoma (HCC).\(^11\)\(^,\)\(^13\) In vitro and in vivo studies have shown that the hepatitis B X gene (HBx) proteins bind to p53, a tumor suppressor gene, modifying its function.\(^27\)\(^–\)\(^30\) Although many activities of HBx are proapoptotic,\(^31\)\(^,\)\(^32\) mutations in the HBx encoding sequences from HCC cells can inhibit this activity.\(^29\)\(^,\)\(^33\) It is unclear if occult HBV patients have an altered prevalence of HBx mutations compared with other chronic HBV patients or if coinfection with HIV could mediate this effect. Virologic characterization of occult HBV and longitudinal studies of infected patients are required to understand the clinical implications of occult infection.

Several limitations to this study should be noted. The cross-sectional nature of the study does not permit conclusions regarding the clinical significance of occult HBV, although 1 occult HBV patient with ALT \(>2\) times the ULN had no other risk factors for liver enzyme elevations, suggesting that adverse clinical findings may occur in at least some cases. The single-center nature of this analysis could limit its generalizability to settings with significantly different demographic characteristics. We were unable to conduct separate testing for IgM and IgG antibodies. It is possible, therefore, that some of the occult patients actually represent a “window” phase of infection, in which the patient has been acutely infected and lost core antibody but has not yet developed anti-HBs. Our prior analysis in the AIDS Clinical Trials Group (ACTG) did include IgM testing, however, and no patient in a window phase was identified.\(^4\) Thus, this possibility seems unlikely. Finally, the number of occult patients limits power for in-depth analysis. Expanding the characterization of this cohort should facilitate further comparisons and contrasts.

Questions and controversy surrounding occult HBV remain. Until clinical occult HBV is better characterized by prospective studies, it would be impractical to recommend DNA testing on serologically negative patients. Conversely, patients with HIV who are at risk for HBV (occult or otherwise) should be screened for surface antigen and core antibody, and screening should be repeated every 2 to 3 years if negative. In the event of unexplained liver enzyme abnormalities and negative serology, occult HBV may be suspected and DNA testing may be warranted, even in the case of negative serology. It is also important to emphasize the low vaccination rate in this patient population and to note that 2 patients with prior vaccine histories were actually HBV DNA\(^-\). Patients with HIV and a history of prior vaccination should be repeatedly screened for anti-HBs titers. It is critical to determine if progression to end-stage liver disease (ESLD) or HCC with occult HBV is hastened by HIV coinfection, as is the case with HCV. We anticipate that occult infection is likely to present an ongoing challenge in the management of HBV until such studies are completed in the years to come.

REFERENCES


II
Cytokine expression during chronic versus occult hepatitis B virus infection in HIV co-infected individuals

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Occult hepatitis B virus
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**A B S T R A C T**

Chronic hepatitis B virus infection is characterized by persistent detectable levels of hepatitis B surface antigen (HBsAg) and HBV DNA in the serum. In contrast, HBsAg is not detectable during occult HBV infection, despite the presence of HBV DNA. An altered host immune response could play a role in the development of occult HBV infection; however, potential differences in immune responses among chronic and occult HBV-infected patients have not been evaluated in vivo. In the current study, we evaluated serum levels of regulatory, apoptotic, and fibrotic/anti-fibrotic cytokines/markers as indicators of immune responses in 25 chronic and 12 occult HBV-infected patients. More than half of the patients in both chronic and occult HBV infection groups had IL-2, IL-4, IL-13, and IFN-γ levels below detectable limits. In contrast, most patients had detectable levels of IL-8, IL-10, IP-10, sFas, sFasL, and TGF-β1. Of these, only sFas was significantly different between the two groups, with lower levels observed during occult compared to chronic HBV infection. As a surrogate marker of apoptotic inhibition, decreased sFas during occult HBV infection suggests that apoptosis occurs at different rates in occult compared to chronic HBV infection and may contribute to persistence of occult HBV infection.

**1. Introduction**

Globally, 350 million people are infected with chronic HBV with approximately 330,000 new cases per year in the United States alone. HBV is a non-cytopathic virus; therefore, liver damage—leading to fibrosis, cirrhosis and hepatocellular carcinoma (HCC)—is considered to be the direct result of the immune response to viral infection [1,2]. Due to their shared routes of transmission, HIV is common among persons with HBV. Moreover, liver disease has become a major cause of morbidity and mortality in HBV/HIV co-infected individuals due to prolonged survival with advances in antiretroviral therapy (ART) [3].

Numerous cytokines are involved in cell-mediated and humoral immune responses, as well as antiviral activity, viral clearance, apoptosis, and fibrogenesis. In acute HBV infection, a strong cell-mediated Th1 response, characterized by IL-2 and IFN-γ, is mounted against HBV and is involved in viral clearance [1,4] and immunopathogenesis of liver disease [1,5]. IFN-γ is also a major contributor of viral clearance by activating macrophages, as well as rendering neighboring uninfected cells resistant to infection [6]. In addition, in some cases, an increase in IL-10 production has been linked to persistence of chronic HBV and hepatitis C virus (HCV) infections [7]. On the other hand, a strong humoral response is characterized by increased IL-4. Chronic HBV infection develops in individuals that do not mount sufficient immune responses to clear acute HBV infection, which occurs in less than 1% of infected immunocompetent adults [8] and in about 90% of infected neonates [5]. Both cell-mediated and humoral immune responses against HBV are weak in chronically infected individuals compared to individuals with acute HBV infection [4,9].

Several factors could be involved in the development of chronic HBV infection [1], including: (1) CD8+ T-cell exhaustion or partial tolerance [10]; (2) decreased CD4+ T-cell responsiveness through dendritic cell (DC) impairment [11,12] or increased PD-1 expression [2,13]; (3) impairment of innate immune responses through decreased natural killer (NK) cell function [14]; and (4) increased number of regulatory T-cells leading to enhanced suppression of HBV-specific T-cell responses and decreased viral clearance [8,15,16], similar to that observed in HCV infection [17].

While several cytokines possess antiviral activities, other cytokines have been implicated in viral persistence and immunopathogenesis of the disease. For example, it has been demonstrated that apoptosis of HBV-infected hepatocytes occurs primarily through...
the Fas-mediated pathway; soluble Fas (sFas) and soluble Fas ligand (sFasL) are inhibitors of this apoptotic pathway [18], allowing for the persistence of viral infection. While they are involved in mediating liver cell injury, their presence is increased during chronic HBV infection and correlates with persistence of chronic HCV infection [19], as well as with development of HCC [20]. In addition, IL-8 has been examined as a marker of liver damage due to its ability to inhibit the antiviral activity of IFN-α [21,22]. Finally, TGF-β1 is a major regulator of liver fibrosis. It is involved in the control of multiple cellular processes, such as growth, proliferation and differentiation, as well as control of the immune system, down regulating the Th1 response [23], and has been implicated in the development of HCC [24].

While chronic HBV infection is diagnosed by the detection of HBsAg and HBV DNA in the serum, occult HBV infection is defined by the lack of detectable circulating HBsAg in the presence of low levels of HBV replication [25]. One potential mechanism that may explain the disparity between these two distinct forms of HBV infection is an altered host immunologic response [26]. A stronger immune response during occult HBV infection relative to chronic HBV infection could partially explain the decreased HBV DNA levels and lack of detectable HBsAg in these patients. Since this mechanism has not been investigated in vivo to date, we measured the serum cytokine levels of several apoptotic, fibrotic/antifibrotic and immunologic response markers to test the hypothesis that their expression levels differ between chronic and occult HBV infections.

2. Materials and methods

2.1. Patient population

Nine hundred and nine HIV-positive individuals from the University of Cincinnati Infectious Disease Center (UC IDC) were previously evaluated in a retrospective study to determine HBV status [25]. HBV DNA was quantified using real-time PCR performed in triplicate and compared to a standard panel to determine viral titer (lower limit of detection [LLD] of 100 IU/mL). Serologic markers of HBV infection (HBsAg, anti-HBc and anti-HBs) were subsequently detected by ELISA (BioChain, Hayward, CA) for patients positive for HBV DNA. Thirty-one chronic (HBsAg+/HBV DNA+) and 12 occult (HBsAg+/HBV DNA−) HBV infections were identified. HCV status was evaluated using standard clinical enzyme-linked immunosorbent assays (ELISA). As samples were collected from 1989 to 2004, HIV RNA levels were not assessed for all individuals. When available, HIV RNA levels were determined by either qualitative or quantitative reverse transcriptase polymerase chain reactions (rtPCR).

2.2. Multiplex immunoassay

In the current retrospective analysis, serum samples were evaluated, as peripheral blood mononuclear cells (PBMCs) were not routinely collected. Serum samples were available for 25 persons with chronic HBV infection and all 12 persons with occult HBV infection.

LINCOplex multiplex immunoassays (Linco Research, St. Charles, MO) were used to determine cytokine levels from patient serum in duplicate. LINCOplex human cytokine/chemokine panel—7plex required 25 μL of serum to measure levels of IL-2, IL-4, IL-8, IL-10, IL-13, IFN-γ, and IP-10 (LLD of 16 pg/mL). The LINCOplex human serum sepsis/apoptosis panel—2plex required 25 μL of 1:10 diluted serum to measure levels of sFas and sFasL. (LLD of 48 pg/mL). Finally, the LINCOplex TGF-β1—1plex kit required 50 μL of 1:50 diluted serum to measure TGF-β1 (LLD of 320 pg/mL). Average values were calculated for each patient and compared to a standard curve to obtain the concentration for each cytokine.

2.3. Statistical analysis

Wilcoxon rank-sum tests were performed to compare demographics for chronic and occult HBV infected patients. Fisher’s exact test was performed to compare serum cytokine detection rates between chronic and occult HBV infected patients. Additional statistical analysis was performed on cytokines for which at least 50% of the patients had detectable levels (IL-8, IL-10, IP-10, sFas, sFasL, and TGF-β1). Observed cytokine concentrations were log-transformed to approximate a normal distribution and were evaluated using Wilcoxon rank-sum tests. P values <0.05 were considered statistically significant for all Fisher’s exact and Wilcoxon rank-sum tests. Spearman rank tests were utilized to evaluate correlations between each cytokine, as well as between each cytokine and age, gender, race, and HBV DNA titer; however, HIV RNA level was not included due to the limited number of subjects with available data. The Bonferroni correction for multiple comparisons was utilized; p values <0.0014 between cytokines and <0.00125 between cytokines and age, gender, race, and HBV DNA titer, were considered statistically significant.

3. Results

3.1. Characterization of patient population

Patient demographics for the 25 patients with chronic HBV infection and the 12 with occult HBV infection are listed in Table 1. The median age for chronic HBV patients was 34.7 years and 32.9 years for occult HBV patients. Fifty-six percent of chronic HBV patients were African-American and 44% were Caucasian, while 42% of occult HBV patients were African-American and 50% were Caucasian. One hundred percent of chronic HBV patients and 75% of occult HBV patients were male. Median alanine aminotransferase (ALT) levels were 49.0 U/L in chronic HBV patients and 26.0 U/L in occult HBV patients. One occult HBV patient was also co-infected with HCV. All patients were previously diagnosed serologically with HIV infection. At the time of serum sample collection, HIV RNA was detectable by either a qualitative or a quantitative assay in 17 of 19 (84%) chronic HBV patients tested and in 7 of 7 (100%) occult HBV patients tested. Among those individuals with quantitative HIV viral loads, the median HIV RNA level was 6.3 × 10^4 copies/mL for chronic HBV patients and 1.7 × 10^6 copies/mL for occult HBV patients. Four chronic HBV patients (16%), as well as 3 occult HBV patients (25%), were receiving antiretroviral therapy at the time of sample collection. Overall, 3 chronic HBV patients (12%) were taking at least one medication that inhibits both HIV and HBV. The median CD4+ cell count in chronic HBV infections was 167 cells/mL compared to 306 cells/mL in chronic HBV infections, although this difference was not significantly different. The median HBV DNA level was significantly lower among persons with occult HBV infection, 8.2 × 10^3 IU/mL, compared to 8.0 × 10^6 IU/mL among persons with chronic HBV infection (p = 0.019).

3.2. Serum cytokine detection rates

Cytokine expression levels were below the LLD in greater than 50% of samples for both the chronic and occult HBV patient groups for IL-2, IL-4, IL-13, and IFN-γ (Fig. 1). IL-2 was detectable in 4% of chronic HBV patients, while it was below the detectable limit for all 12 occult HBV patients. IL-4 was detectable in 32% of chronic HBV patients and 42% of occult HBV patients, while both IL-13...
and IFN-γ were detectable in 40% of chronic HBV patients and 8% of occult HBV patients. Due to the limited data available for analysis, these cytokines were excluded from further statistical evaluation. However, it is interesting to note that undetectable levels in chronic and occult HBV-infected patients were decreased compared to previously reported serum levels in healthy (HIV negative, HBV negative, HCV negative) individuals [20,27–33]. Previously published serum cytokine levels in healthy individuals, listed in Table 2, were all within the detectable ranges of the multiplex immunoassays. IL-8 and IL-10, on the other hand, were detectable in 72% and 84% of chronic HBV patients, as well as in 67% and 58% of occult HBV patients, respectively. Both sFas and sFasL had 100% detection rates in chronic HBV patients and 100% and 92% detection, respectively, in occult HBV patients. Finally, 76% of chronic HBV patients had detectable TGF-β1, as well as 83% of occult HBV patients. There were no statistically significant differences between the detection rates in chronic versus occult HBV patients for any cytokine measured.

### 3.3. Serum cytokine expression levels

Decreased levels of IL-8, IL-10, IP-10, sFas and sFasL were observed in occult HBV patients compared to chronic HBV patients (Table 3). In contrast, increased levels of TGF-β1 were observed in occult HBV infection compared to chronic HBV infection (Table 3). For both occult and chronic HBV infections, sFas levels were increased compared to previously published levels in uninfected individuals (600–1500 pg/mL) [20,30–32]. However, a statistically significant decrease in sFas levels was observed among occult HBV patients compared to chronic HBV patients (geometric mean = 7089.0 pg/mL versus 10242.3 pg/mL; p = 0.01) (Table 3 and Fig. 2). Additional analysis did not identify any statistically significant correlations between cytokines or between individual cytokines and age, gender, race, and HBV DNA level (data not shown).

### 4. Discussion

Occult HBV infection is frequently identified via retrospective testing, and long-term clinical follow-up of patients with occult HBV infection has yet to be adequately explored. Similarly, immunologic evaluation of occult HBV infection is absent from the available literature. To our knowledge, our study represents the first evaluation of serum cytokine expression profiles during occult HBV infection. The cytokines evaluated—IL-2, IL-4, IL-8, IL-10, IL-13, IFN-γ, IP-10, sFas, sFasL, and TGF-β1—are involved in promoting antiviral activity and viral clearance, inhibition of antiviral activity, the development of viral persistence, apoptotic inhibition, and/or fibrogenesis.

Detection rates in this study were below 50% for 4 of 10 cytokines measured (IL-2, IL-4, IL-13, and IFN-γ) in both chronic and occult HBV infection groups (Fig. 1) and has been observed previously in the setting of chronic HBV infection for IL-2, IL-4, and IFN-γ [34]. While positive responses cannot be evaluated for these cytokines, cytokine levels below detectable limits may indicate that either these cytokines do not play a role in occult HBV infection or that occult HBV infection occurs in the absence of detectable antiviral activity.
infection, or that their absence is critically important for viral persistence, since published normal levels would be detectable. This may suggest that in occult HBV infection, both Th1 (indicated by IL-2 and IFN-γ) and Th2 responses (indicated by IL-4) are weak, similar to what has been reported in chronic HBV infection [4,9]. While HIV could also contribute to overall cytokine expression in our population [35], all individuals were HIV co-infected. Moreover, it is unlikely that antiretroviral therapy significantly impacts these results as only a small number of individuals were receiving ART in either HBV patient group.

Consistent with a previous report, IL-10 levels appeared slightly lower in individuals with chronic HBV infection compared to normal controls [36], although the differences were not statistically significant. Increased IP-10 levels compared to normal controls was also observed in all HBV/HIV co-infected individuals in this study. Increased IP-10 have been identified previously in chronic HBV infection [37], as well as in productive HIV infection, where IP-10 synthesis was induced [38,39].

Although our study was limited by the low detection rates for certain cytokines and the relatively small number of samples evaluated, sFas levels were significantly decreased during occult HBV infection (p = 0.01) (Table 3 and Fig. 2). As sFas inhibits Fas-mediated apoptosis of HBV-infected hepatocytes, decreased sFas levels suggest less apoptotic inhibition, although apoptosis may still occur through alternative pathways [18]. During chronic HBV infection, increased sFas levels have been observed previously [40]. Similarly, in this study, sFas levels were significantly increased in patients with chronic HBV infection, compared to sFas levels previ-}

### Table 2

Normal serum cytokine levels. Average serum cytokine levels from healthy individuals as previously published (in pg/mL).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Normal level (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>28.1</td>
</tr>
<tr>
<td>IL-4</td>
<td>144.6</td>
</tr>
<tr>
<td>IL-8</td>
<td>59.9</td>
</tr>
<tr>
<td>IL-10</td>
<td>40.9</td>
</tr>
<tr>
<td>IL-13</td>
<td>19.6</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>19.8</td>
</tr>
<tr>
<td>IP-10</td>
<td>82.6</td>
</tr>
<tr>
<td>sFas</td>
<td>1049.0</td>
</tr>
<tr>
<td>sFasl</td>
<td>130.0</td>
</tr>
<tr>
<td>TGF-β</td>
<td>3542.0</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Normal level (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2a</td>
<td>28.1</td>
</tr>
<tr>
<td>IL-4a</td>
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</tr>
<tr>
<td>IL-8a</td>
<td>59.9</td>
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<td>IL-10a</td>
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<tr>
<td>IFN-γa</td>
<td>19.8</td>
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<tr>
<td>IP-10b</td>
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</tr>
<tr>
<td>sFasc</td>
<td>1049.0</td>
</tr>
<tr>
<td>sFasl</td>
<td>130.0</td>
</tr>
<tr>
<td>TGF-βe</td>
<td>3542.0</td>
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</tbody>
</table>

α Refs. [27,28].
β Refs. [28,29].
γ Refs. [20,30–32].
d Ref. [32].
e Ref. [33].

### Table 3

Measured serum cytokine levels. Number of patients and percentages in parentheses are given for cytokines with detectable levels above 50% in occult and chronic HBV patients. Geometric mean values in pg/mL (with 95% confidence intervals in parentheses) are given for each cytokine during occult and chronic HBV infections. p Values were determined using Wilcoxon rank-sum tests. The comparison for sFas was statistically significant (<0.05) and is indicated in bold.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Infection status</th>
<th>Detection</th>
<th>Geometric mean (95% CI)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>Occult</td>
<td>8 (67%)</td>
<td>13.7 pg/mL (6.1–30.4)</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>Chronic</td>
<td>18 (72%)</td>
<td>39.9 pg/mL (23.9–66.4)</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>Occult</td>
<td>7 (58%)</td>
<td>26.0 pg/mL (12.2–55.6)</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>Chronic</td>
<td>21 (84%)</td>
<td>49.6 pg/mL (29.1–84.6)</td>
<td></td>
</tr>
<tr>
<td>IP-10</td>
<td>Occult</td>
<td>12 (100%)</td>
<td>381.7 pg/mL (247.5–558.8)</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Chronic</td>
<td>25 (100%)</td>
<td>515.8 pg/mL (430.7–617.8)</td>
<td></td>
</tr>
<tr>
<td>sFas</td>
<td>Occult</td>
<td>12 (100%)</td>
<td>7089.0 pg/mL (5644.1–8903.8)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Chronic</td>
<td>25 (100%)</td>
<td>10242.3 pg/mL (9054.4–11586.0)</td>
<td></td>
</tr>
<tr>
<td>sFasl</td>
<td>Occult</td>
<td>11 (92%)</td>
<td>126.3 pg/mL (85.6–186.4)</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Chronic</td>
<td>25 (100%)</td>
<td>168.9 pg/mL (130.6–218.4)</td>
<td></td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Occult</td>
<td>10 (83%)</td>
<td>10300 pg/mL (2900–36800)</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>Chronic</td>
<td>19 (76%)</td>
<td>4400 pg/mL (1200–16900)</td>
<td></td>
</tr>
</tbody>
</table>
in an oral presentation at the HIV & Liver Disease 2008 Conference in Jackson Hole, WY, September 25–27, 2008 and as a poster (#811) at the 16th Conference on Retroviruses and Opportunistic Infections in Montréal, Québec, Canada, February 8–12, 2009. We would also like to thank Alyssa Sproles for performing the LINCOpex multiplex assays.

References


III
Genomic variability associated with the presence of occult hepatitis B virus in HIV co-infected individuals

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SUMMARY. Occult hepatitis B virus (O-HBV) infection is characterized by the presence of HBV DNA without detectable hepatitis B surface antigen (HBV DNA+/HBsAg−) in the serum. Although O-HBV is more prevalent during HBV/HIV co-infection, analysis of HBV mutations in co-infected patients is limited. In this preliminary study, HBV PreSurface (PreS) and surface (S) regions were amplified from 33 HIV-positive patient serum samples—27 chronic HBV (C-HBV) and six O-HBV infections. HBV genotype was determined by phylogenetic analysis, while quasispecies diversity was quantified for the PreS, S and overlapping polymerase regions. C-HBV infections harboured genotypes A, D and G, compared to A, E, G and one mixed A/G infection for O-HBV. Interestingly, nonsynonymous–synonymous mutation values indicated positive immune selection in three regions for O-HBV vs one for C-HBV. Sequence analysis further identified new O-HBV mutations, in addition to several previously reported mutations within the HBsAg antigenic determinant. Several of these O-HBV mutations likely contribute to the lack of detectable HBsAg in O-HBV infection by interfering with detection in serologic assays, altering antigen secretion and/or decreasing replicative fitness.

Keywords: diversity, HBV polymerase, HBV/HIV co-infection, hepatitis B surface antigen, occult hepatitis B virus.

INTRODUCTION

There are 350 million chronic carriers of the hepatitis B virus (C-HBV) worldwide. C-HBV infection is characterized by detectable hepatitis B surface antigen (HBsAg) in the serum [1]. Occult HBV infection (O-HBV), in contrast, is defined as low level HBV replication without detectable circulating HBsAg [2]. Antibodies against HBV core protein (anti-HBc) had been considered the sole serological marker of O-HBV infection [3]; however, serologically negative individuals have been described with HBV DNA as the only detectable marker of infection [4].

Occult HBV infection is transmissible through blood transfusion from human to human [5] and human to chimpanzee [6]. In addition, C-HBV infection developed after a liver transplant from an O-HBV-infected donor [7]. Retrospective studies have also identified O-HBV infection in 16–68% of tumours in patients with hepatocellular carcinoma (HCC) [8,9]. Similarly, O-HBV infection was associated with development of cirrhosis and HCC [10]. Nonetheless, the clinical consequences of prolonged O-HBV infection remain unclear.

HIV co-infection is common because of shared blood-borne transmission routes. While advances in antiretroviral therapy (ART) have prolonged AIDS-free survival in HIV/HBV co-infected patients, liver disease has emerged as a leading cause of morbidity and mortality [11]. In HIV-positive cohorts, the prevalence of O-HBV infection is highly variable: Nunez et al. [12] did not identify any patients with detectable HBV DNA among 85 HIV+/HBsAg−/anti-HBc+ injection drug users (IDUs); while Hofer et al. [13] detected serum HBV DNA in 51 of 57 patients (89.5%) who were solely anti-HBc+, O-HBV infections have also been identified in hepatitis C virus (HCV)-positive IDUs [14] and liver biopsies from patients with normal liver biochemistry and without prior liver disease [15].

Abbreviations: ART, antiretroviral therapy; C-HBV, chronic carriers of the hepatitis B virus; dN–dS, nonsynonymous–synonymous; HBc, HBV core protein; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; IDU, injection drug user; LLD, lower limit of detection; O-HBV, occult hepatitis B virus; ORF, open reading frame; PreS, PreSurface; RT, reverse transcriptase; SSA, signature sequence analysis; S, surface.

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It is unclear why HBsAg is undetectable during O-HBV infection, although several hypotheses exist. One possibility is that HBsAg is not produced or is expressed at levels below detectable limits of current diagnostic assays. Alternatively, HBsAg could be produced but not secreted from infected hepatocytes. Importantly, altered expression of HBsAg likely results from HBV mutation(s) [16]. Mutations in either the surface open reading frame (ORF), containing the PreS and surface (S) regions, or their corresponding spacer and reverse transcriptase (RT) regions of the overlapping polymerase ORF could directly affect HBsAg production. To date, a limited number of studies have investigated O-HBV mutations, in relatively few individuals [17–24]. Here, we describe a detailed evaluation of HBV genomic sequences from chronic (HBsAg+) and occult (HBsAg−) HBV-infected individuals in the same cohort and assess the presence of HBsAg mutations associated with O-HBV infection.

MATERIALS AND METHODS

Patient population

This prospective HIV-positive cohort was previously described in an analysis of O-HBV infection [4]. HBV DNA levels were determined by real-time PCR [lower limit of detection (LLD) = 67 copies/mL or 100 IU/mL] and HBV serologic markers, HBsAg, anti-HBc and anti-HBs, were evaluated by ELISA (LLD = 0.5 ng/mL, 2 NCU/mL and 10 mIU/mL, respectively) (Biochain, Hayward, CA, USA). HCV serostatus was determined by ELISA, measuring antibodies against HCV. As samples were collected between 1989 and 2004, HIV RNA levels were not available for all individuals. ALT levels above 92 U/L (two times the upper limit of local normal levels [25]) were considered elevated. Here, 23 C-HBV and six O-HBV serum samples were utilized, along with four HIV-positive, C-HBV samples from our serum repository.

PCR amplification

Hepatitis B virus DNA extracted from 200 to 400 μL of serum was used in separate nested PCRs for the PreS (549 bp) and S (339 bp) regions using primers [21,26] and amplification conditions listed in Supplemental Table S1. DNA amplification was performed using the GeneAmp PCR reagent kit with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) according to manufacturer’s instructions. Five microliters of HBV DNA initially served as template, while 1 μL of first round product served as template for the second round. A previously amplified HBV-positive patient serum sample served as a positive control, while HBV DNA-negative serum samples and a reaction without template served as negative controls.

Cloning and sequencing

PCR products were electrophoresed, purified and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). Ten clones per region per individual were sequenced bidirectionally. All sequences have been submitted to GenBank under accession numbers EU769235–EU769292.

Phylogenetic analysis

Sequence alignments were created for the PreS, S and their corresponding polymerase spacer [Pol(PS)] and RT [Pol(S)] regions, respectively, using the neighbour-joining method in CLUSTAL X [27]. Published references [28] and additional GenBank sequences were chosen at random to achieve five per genotype (Supplemental Table S2). For phylogenetic trees, the statistical robustness and reliability of the branching order were assessed using bootstrap analysis with 100 replicates. Calculations for genetic distance and non-synonymous–synonymous (dN–dS) mutation values [29] were performed using MEGA 3.1 [30]. Shannon entropy ($S_n = - \sum p_i \ln p_i$) was calculated, where $p_i$ was the frequency of each distinct nucleotide sequence and $N$ was the total number of sequences analysed.

Mutational analysis

GenBank was searched for complete HBV genomes. One thousand nine hundred and ninety-three sequences were inspected, and all non-HBV, primate, recombinant and O-HBV genomes were excluded. The remaining full-length HBV sequences for genotypes A ($n = 143$), E ($n = 58$) and G ($n = 23$) were included in the mutational analysis. GenBank references, as well as C-HBV sequences generated in this study, were compared to O-HBV sequences to identify distinct amino acid mutations in each genomic region that characterize O-HBV infections. Analyses were performed in a genotype-matched manner because of distinct biological and clinical differences among HBV genotypes [31]. The PreS and S regions analysed encode for a region that characterize O-HBV infections. Analyses were performed in a genotype-matched manner because of distinct biological and clinical differences among HBV genotypes [31]. The PreS and S regions analysed encode for a 58–156 of the polymerase RT region (all numbered according to [32]). Mutations were considered to be associated with O-HBV infection if they were (i) identified in ≥1 O-HBV clonal sequence and (ii) not identified in any genotype-matched C-HBV sequences from this study or GenBank references analysed. Identified mutations were then compared to published mutations associated with O-HBV infection [17–24]. Mutation prevalence was also determined in all GenBank and C-HBV sequences after stratification by HBV genotype. To further identify signature amino acids, O-HBV sequences were compared to genotype-matched C-HBV sequences from this...
study using viral epidemiology signature pattern analysis [33] at a threshold of 0.5.

**Statistical analysis**

Wilcoxon rank-sum tests were performed on patient demographics, and P-values < 0.05 were considered significant. For quasispecies parameters (genetic distance, entropy and dN–dS), values of each dependent variable were rank-ordered, and two-factor analysis of variance (ANOVA) tests were performed. Infection status was a between-subjects factor with two levels (occult or chronic), while Region was a within-subjects factor with four levels [PreS, S, Pol(S), Pol(PS)]. For each outcome, 12 planned pairwise comparisons of interest were defined and utilized a t-type procedure where the mean difference in ranks for the conditions being compared was divided by the appropriate ANOVA error term (between-region comparisons using the within-subject error term and between-infection comparisons using the between-subject error term). A critical threshold for significance of P = 0.0042 (P = 0.05/12) was used to control for the overall Type I error rate at alpha = 0.05.

**RESULTS**

**HBV region amplification**

PreS was amplified and cloned from 19 of 27 (70.4%) C-HBV and 4 of 12 (33.3%) O-HBV infections. S was amplified and cloned from 25 of 27 (92.6%) C-HBV and 6 of 12 (50%) O-HBV infections. There were no statistically significant differences in patient demographics between C- and O-HBV-infected patients (Table 1), although median HBV DNA level was higher in C-HBV infections – 9.0 × 10⁶ IU/mL – than in...

<table>
<thead>
<tr>
<th>HBV infection</th>
<th>Chronic (n=27)</th>
<th>Occult (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age*</td>
<td>35.8 years (21.2–58)</td>
<td>35.4 years (26.7–42.2)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>African-American</td>
<td>14 (51.9%)</td>
<td>3 (50.0%)</td>
</tr>
<tr>
<td>Caucasian</td>
<td>12 (44.4%)</td>
<td>2 (33.3%)</td>
</tr>
<tr>
<td>Other</td>
<td>1 (3.7%)</td>
<td>1 (16.7%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>27 (100%)</td>
<td>5 (83.3%)</td>
</tr>
<tr>
<td>Female</td>
<td>0</td>
<td>1 (16.7%)</td>
</tr>
<tr>
<td>ALT*</td>
<td>56.0 U/L (17–140)</td>
<td>27.0 U/L (23–87)</td>
</tr>
<tr>
<td>HCV serostatus†</td>
<td>0</td>
<td>1 (25%)</td>
</tr>
<tr>
<td>HIV† Detectable</td>
<td>21/23 (91%)</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>Viral load*</td>
<td>6.0 × 10⁴ copies/mL (7.2 × 10²–2.0 × 10⁵)</td>
<td>1.7 × 10⁵ copies/mL (1.0 × 10⁴–3.7 × 10⁵)</td>
</tr>
<tr>
<td>ART On therapy</td>
<td>6 (22.2%)</td>
<td>1 (16.7%)</td>
</tr>
<tr>
<td>With HBV activity</td>
<td>7 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>CD4⁺ count*</td>
<td>330.5 cells/mL (7–665)</td>
<td>141.0 cells/mL (6–559)</td>
</tr>
<tr>
<td>HBV DNA*</td>
<td>9.0 × 10⁶ IU/mL (2.1 × 10⁵–7.6 × 10⁸)</td>
<td>1.4 × 10⁴ IU/mL (1.1 × 10³–7.6 × 10⁵)</td>
</tr>
</tbody>
</table>

Local normal ALT levels were 5–46 U/L [25]: ALT levels are considered elevated when >92 U/L (two times the upper limit of normal). HCV serostatus was evaluated using anti-HCV ELISA. ART, antiretroviral therapy. *Median values are given with the range in parentheses. †Information was not available for all individuals (the number of patients with available information is listed under the heading); therefore, the median ALT and CD4⁺ count and percent detectable HCV and HIV are given for the individuals tested. ‡One additional individual is on HBV mono-therapy but not ART.
O-HBV infections – $1.4 \times 10^4$ IU/mL ($P < 0.05$), as reported previously [4].

**Identification of HBV genotypes**

Consensus nucleotide sequences were generated and aligned with reference sequences. In the PreS region, genotypes A ($n = 15$), D ($n = 1$) and G ($n = 2$) were identified in C-HBV subjects; genotypes A ($n = 1$) and G ($n = 2$) were identified in O-HBV subjects (Fig. 1a). Additionally, one mixed infection with genotypes A and G was identified (Occult 2). In S, genotypes A ($n = 17$), D ($n = 3$) and G ($n = 5$) were identified in C-HBV subjects, and genotypes A ($n = 2$), E ($n = 1$) and G ($n = 3$) were identified in O-HBV subjects, in agreement with PreS (Fig. 1b), although only genotype A was present for the mixed O-HBV infection.

**Evaluation of quasispecies diversity**

Clonal alignments were performed to assess quasispecies diversity. In PreS, all patients showed significant quasispecies diversity ($\geq 2$ distinct viral variants) (Supplemental Fig. S1). In S, while all patients with O-HBV showed significant quasispecies diversity, 5 of 27 C-HBV infections did not (Supplemental Fig. S2). No significant differences were found between C- and O-HBV infections with respect to genetic distance (Fig. 2a), entropy (Fig. 2b) or dN–dS (Fig. 2c), although dN–dS values were >0 – indicating positive selection – in three regions for O-HBV infections – PreS, S and Pol(PS) – compared to only Pol(PS) in C-HBV infections.

Among C-HBV patients, dN–dS values were significantly higher in Pol(PS) compared to PreS ($P = 0.001$). A significant increase in entropy was also noted in PreS and Pol(PS) compared to S and Pol(S), respectively (both, $P = 0.001$) (Fig. 2b).

**Identification of occult hepatitis B virus sequence mutations**

Genotype-matched sequence analysis was performed, identifying several novel mutations associated with O-HBV infection: five genotype A and 17 genotype G mutations in PreS, with three genotype A, one genotype E and six genotype G mutations, in addition to several previously published
mutations in S (Table 2). Several S mutations identified reside within the antigenic determinant region of HBsAg (amino acids 100–165), suggesting that they may impact HBsAg detection.

Additional mutations associated with O-HBV were identified in the corresponding polymerase ORF (Table 3). In the spacer region, four genotype A and 13 genotype G mutations were identified, with three mutations for both genotypes A and G and two for genotype E in the RT region.

Prevalence of mutations associated with occult hepatitis B virus infection across genotypes
Occult hepatitis B virus mutation prevalence was also determined amongst distinct genotypes. Genotype A, E and G GenBank sequences and C-HBV sequences from this study were searched for all identified O-HBV mutations. Several O-HBV mutations were identified at low frequencies in C-HBV sequences of different genotypes for the PreS1 (Fig. 3a), PreS2 (Fig. 3b), S (Fig. 3c) and polymerase spacer (Fig. 3d) regions. No RT mutations were identified in any other sequences.

Signature sequence analysis
Signature sequence analysis (SSA) was used to identify mutations more commonly found in O-HBV compared to C-HBV. SSA identified two PreS1 mutations in Occult 2 (genotype A), along with three corresponding polymerase spacer mutations, previously excluded because of low frequency in C-HBV sequences. In Occult 4 (genotype G), 50% of clones harboured a deletion of the first three N-terminal amino acids of PreS1. Another 40% of clones...
contained five mutations – M1I, G2R, L3I, S4P and W5R – below the 50% threshold for SSA. In the corresponding polymerase spacer, the first seven N-terminal amino acids were deleted in 50% of clones, and five mutations – G5Q, A6D, F7S, L8stop and G10S – were present in another 40%.

In S, SSA identified three mutations in Occult 3 (genotype A) – M103I, K122R and G145A (bold, Table 2), present in all clones, although M103I and K122R were found at low levels in C-HBV sequences. In the corresponding polymerase RT region, two mutations – V112I and W153C – were identified in all clones, but only W153C was absent from chronic genotype A sequences (bold, Table 3). This mutation coincides with the G145A mutation in the S region; while other mutations have been reported at RT position 153 [22,34], none were a tryptophan-to-cysteine mutation.

**Table 2** PreSurface (PreS) and surface (S) mutations. S open reading frame mutations identified in Occult hepatitis B virus-infected individuals

<table>
<thead>
<tr>
<th>Surface region</th>
<th>HBV genotype</th>
<th>Newly identified</th>
<th>Previously identified</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PreS1 A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>N98Y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>M11</td>
<td>D41N</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G2R</td>
<td>P88L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L3I</td>
<td>A89T</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S4P</td>
<td>P93L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>W5R</td>
<td>R102G</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S16P</td>
<td>T105I</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F23S</td>
<td>P106S</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D26Y</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PreS2 A</td>
<td>S28G</td>
<td></td>
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<tr>
<td></td>
<td>H41Q</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>D51G</td>
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<td></td>
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<tr>
<td>G</td>
<td>L20P</td>
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<td></td>
<td>T37A</td>
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<tr>
<td>Surface A</td>
<td>Y72H</td>
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<td></td>
<td>I82T</td>
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</tr>
<tr>
<td></td>
<td>A128T</td>
<td></td>
<td></td>
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<tr>
<td>E</td>
<td>Y100F</td>
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<td></td>
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<tr>
<td>G</td>
<td>S55P</td>
<td></td>
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<tr>
<td></td>
<td>P62L</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>F80S</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>I86V</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>L95W</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S136P</td>
<td></td>
<td></td>
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</tbody>
</table>

Mutations also identified in the signature sequence analysis are indicated in bold. *Mutations that reside within the antigenic determinant loops of hepatitis B surface antigen (AA 100–165).

DISCUSSION

Potential virological differences between C-HBV and O-HBV have not been well defined. It is clear that mutations in the S and Pol ORFs have the potential to (i) alter protein secretion from hepatocytes, (ii) alter protein structure, thereby inhibiting antibody binding in commercial HBsAg detection assays and/or (iii) decrease the overall replication efficacy of the virus. In this preliminary study, we examined genotype distribution and quasispecies diversity within HIV-positive patients previously identified with C-HBV or O-HBV infection [4]. Similar to previous reports, genotype A was most common [35–37]; however, genotype G (24%) also exists within this cohort. While genotype G mono-infection has been reported previously [38], it is most commonly found in mixed infections with genotype A [37,39], of which we identified one in an O-HBV-infected individual. To our knowledge, only one mixed infection has been previously reported in the setting of O-HBV infection [20]. These data suggest that genotype G mono-infection may be more common in the United States than previously reported and further support the existence of mixed O-HBV infections.

Although HBV has a lower mutation rate than RNA viruses, it is higher than most DNA viruses. To date, few
studies have examined intrapatient diversity of HBV \cite{40,41}, and none have included patients with O-HBV infection. Sequencing 10 clones has previously been demonstrated to effectively evaluate quasispecies diversity in HCV \cite{42}; therefore, this number should also be sufficient for HBV given its lower mutation rate. Although our sample size is relatively small, dN–dS values were greater than zero for three of four genomic regions analysed. This indicates that positive immune selection pressures are acting against these regions in O-HBV infection, potentially resulting in mutations that may adversely affect the production and/or detection of HBsAg. No significant differences in genetic distance, Shannon entropy or dN–dS were observed between C-HBV and O-HBV infections. When comparing regions within C-HBV infections, the significantly lower dN–dS for PreS vs Pol(PS) \((P = 0.001)\) indicates increased synonymous mutation within PreS and highlights its intrinsic ability to tolerate mutations \cite{43}, although the spacer region is also highly tolerant of mutations/deletions. Significantly, higher entropies were also observed in PreS and Pol(PS) compared to S and Pol(S), respectively \((P = 0.001)\), in C-HBV infections, again suggesting that functional constraints are less stringent for the PreS and spacer regions.

It is important to emphasize that mutations associated with O-HBV are frequently identified without a robust comparison with C-HBV infection of the same cohort, making it difficult to exclude natural polymorphisms and/or genotype-specific differences. To more effectively characterize virological differences between C- vs O-HBV, we performed genotype-matched sequence analysis of the PreS, S and polymerase spacer and RT regions to identify O-HBV mutations absent in C-HBV. Multiple mutations were
identified, including several not previously described, although many were only found in one variant. New O-HBV mutations were particularly common for genotypes E and G, as previous investigations have focused primarily on the most common HBV genotypes (A–D). Because of the paucity of sequence data for these genotypes, a portion of these mutations could represent naturally polymorphic sites.

It is known that mutations within the S ORF affect the antigenicity [16,22] and detection of HBsAg [24,44,45]. Three O-HBV-infected individuals harboured mutations with direct importance to virus replication. Two sets of mutations in or near the antigenic determinant of HBsAg were of interest: Occult 3 (genotype A) – M103I, K122R and G145A – and Occult 6 (genotype E) – F85C, Y100S and G145R. While K122R is considered a polymorphism defining the d/y sub-serotypes, its effects in combination with the other two are unknown. The positions of these mutations – either alone or in combination – could alter the secondary structure of HBsAg and impair its detection. In particular, G145R resulted in undetectable HBsAg levels using three of four commercial assays [45]. RT mutations at position 153 – W153C and R153Q – result from the same nucleotide substitution as G145A and G145R in the S ORF. While G145A and G145R have been previously reported [18,21,22], these specific RT153 mutations have not been described. Although neither patient was receiving ART at the time, W153Q has been indicated in lamivudine resistance and was found to decrease replicative fitness in vitro along with additional mutations [34], although single RT mutations are also capable of decreasing HBV replication [46].

Most viral sequences for Occult 4 contained a defective or absent large HBsAg start codon. Although the M1I start codon mutation was also identified in 0.7% of genotype A GenBank reference sequences, these contain another potential start codon at position 12, where genotype G does not. Therefore, genotype G infections may not be able to overcome the consequences of this mutation. Defects in LHBsAg can lead to elimination of PreS1 synthesis or accumulation of large HBsAg (LHBsAg) within the endoplasmic reticulum of hepatocytes. Such mutations have been shown to arrest virion and subviral particle secretion [47,48], and could result in a lack of detectable HBsAg in the serum. Collectively, these data suggest that mutations at distinct amino acid positions in HBsAg or deletions at the N-terminus of the large HBsAg could play a role in persistence of O-HBV infection. Future studies are necessary to characterize additional genomic regions, and a functional analysis is ongoing to evaluate the effects of mutations identified here on HBsAg expression, retention and detection in vitro.

ACKNOWLEDGEMENTS

This work was supported by Bristol-Myers Squibb and by a K24 award from NIDDK (DK070528) to KES. Preliminary data were presented at the 15th Conference on Retroviruses and Opportunistic Infections in Boston, MA, February 3–6, 2008.

REFERENCES

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Phylogenetic tree for the PreS region of HBV. Nucleotide sequences from the first 10 clones sequenced per individual for both chronic (○) and occult (▲) HBV infections are shown. All patients showed quasispecies variation consisting of at least 2 distinct viral variants. One mixed infection (Occult 2) was identified consisting of genotypes A and G.

Fig. S2 Phylogenetic tree for the S region of HBV. Nucleotide sequences from the first 10 clones sequenced per individual for both chronic (○) and occult (▲) HBV infections are shown. All but five patients (Chronics 4, 8, 9, 12 and 19) showed quasispecies variation consisting of at least 2 distinct viral variants.

Table S1 PCR Primer Information.* Nucleotide start position according to GenBank reference X97848. Amplification conditions: PreS – initial denaturation step of 2 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 8 min; S initial denaturation step of 5 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 s and extension at 72°C for 40 s, followed by a final extension at 72°C for 5 min.

Table S2 Phylogenetic Analysis References. Accession numbers are listed for the 5 GenBank sequences chosen as references for each HBV genotype.

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### Supplemental Table 1: PCR Primer Information.

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**PCR Primer Information.** * Nucleotide start position according to GenBank reference X97848.

Amplification conditions: PreS – initial denaturation step of 2 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 8 min; S – initial denaturation step of 5 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 40 sec, followed by a final extension at 72°C for 5 min.
**Supplemental Table 2**

**Supplemental Table 2: Phylogenetic Analysis References**

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**Phylogenetic Analysis References.** Accession numbers are listed for the 5 GenBank sequences chosen as references for each HBV genotype.
Phylogenetic tree for the PreS region of HBV. Nucleotide sequences from the first 10 clones sequenced per individual for both chronic (○) and occult (△) HBV infections are shown. All patients showed quasispecies variation consisting of at least 2 distinct viral variants. One mixed infection (Occult 2) was identified consisting of genotypes A and G.
**Phylogenetic tree for the S region of HBV.** Nucleotide sequences from the first 10 clones sequenced per individual for both chronic (○) and occult (▵) HBV infections are shown. All but five patients (Chronics 4, 8, 9, 12 and 19) showed quasispecies variation consisting of at least 2 distinct viral variants.
IV
Hepatitis B Virus (HBV) X Gene Diversity and Evidence of Recombination in HBV/HIV Co-Infected Persons

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The high frequency of mutation during hepatitis B virus (HBV) infection has resulted in 8 genotypes (A–H) with varying effects on disease severity and treatment efficacy. However, analysis of intrapatient HBV diversity is limited, especially during HIV co-infection. Therefore, a preliminary study was performed to analyze HBV X gene diversity in 17 HBV/HIV co-infected individuals. Phylogenetic analysis revealed HBV genotype A in 13 individuals (76.5%) or genotype E in 1 individual (5.9%). Additionally, 3 individuals were dually infected with HBV genotypes A and G (17.6%). Overall, higher genetic distance and entropy were observed in the X region and overlapping polymerase (Pol(X)) regions when compared to the PreS, S, and overlapping polymerase (Pol(PS) and Pol(S)) regions analyzed in the same patients as part of a previous study. In addition, multiple viral variants from 2 individuals with dual HBV infection did not group with either genotype A or G by phylogenetic analysis, indicating possible recombination. SimPlot bootscan analysis confirmed recombination breakpoints within the X gene in both individuals. Recombination between HBV genotypes may represent an important evolutionary strategy that enhances overall pathogenic potential and/or alters the downstream effects of the HBV X protein. J. Med. Virol. © 2011 Wiley-Liss, Inc.

KEY WORDS: intrapatient diversity; dual HBV infection; HBV/HIV co-infection; hepatitis B virus X protein (HBx); quasispecies; recombination

INTRODUCTION

Hepatitis B virus (HBV) is one of the leading causes of liver cirrhosis, as well as hepatocellular carcinoma (HCC). Currently, at least 350 million individuals are living with chronic HBV infection [Lavanchy, 2004]. Compared to most DNA viruses, HBV has a high mutation rate—approximately $2 \times 10^{-5}$ nucleotide substitutions per site per year [Okamoto et al., 1987], although this is lower than most RNA viruses. Thus far, 8 distinct HBV genotypes (A–H) have been identified, which differ by at least 8% at the nucleotide level [Stuyver et al., 2000]. Dual infection with multiple distinct HBV genotypes is also common [Ramos et al., 2007; Sanchez et al., 2007; Kurbanov et al., 2010], and recombination between HBV genotypes has been observed [Bollyky et al., 1996; Morozov et al., 2000; Cui et al., 2002; Sugauchi et al., 2002; Kato et al., 2002b; Kurbanov et al., 2005; Suwannakarn et al., 2005; Bekondi et al., 2007; Osiowy et al., 2008]. While the overall number of HBV infections due to recombinant viruses is unknown, recombinant variants may be more prevalent than initially thought and are capable of becoming the dominant variants of HBV within a population, as observed in Tibet [Cui et al., 2002]. In the United States, genotype A is common, although all HBV genotypes have been reported [Stuyver et al., 2000; Moriya et al., 2002; Chu et al., 2003]. Evaluating HBV genotypes is clinically relevant, as genotype can impact both treatment efficacy and severity of liver disease [Fung and Lok, 2004; Schaefer, 2005]. Nonetheless, few studies have been performed to date that directly assess intrapatient HBV genetic diversity.

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The severity of disease and the development of cirrhosis and HCC have been linked to the HBV X protein (HBx) [Bouchard and Schneider, 2004; Zhang et al., 2006]. HBx is a 154 amino acid regulatory protein whose functions include direct and indirect modulation of protein degradation, apoptosis, signal transduction, transcription, and cell cycle progression [Bouchard and Schneider, 2004]. HBx has also been implicated in transcriptional activation of nuclear factor-κB, constitutive activation of extracellular signal-related kinases and c-Jun N-terminal kinases, as well as exit from G0 in arrested cells [Mahe et al., 1991; Madden and Slagle, 2001; Nijhara et al., 2001]. Furthermore, HBx is associated with the development of HCC in patients [Zhang et al., 2006], although this association remains controversial [Lee et al., 1990; Kim et al., 1991].

In HBV/HIV co-infected individuals, an increase in HBV replication may be observed [Gilson et al., 1997; Colin et al., 1999]. In contrast, HBx acts as a nuclear co-activator capable of inducing transcriptional activity of the HIV long terminal repeat [Balsano et al., 1993; Gomez-Gonzalo et al., 2001], resulting in increased HIV replication and further impairment of the host immune function. However, few studies have analyzed intrapatient HBV quasispecies diversity in HBV/HIV co-infected individuals, especially in the X region. Therefore, we conducted a preliminary analysis of X gene variability in HBV/HIV-infected individuals.

MATERIALS AND METHODS

Patient Population

We previously determined the prevalence of HBV infection in a cross-sectional analysis of HIV-positive individuals being followed at the University of Cincinnati Infectious Diseases Center [Shire et al., 2007]. HBV DNA levels were quantified in patient serum samples using a real-time PCR assay with a lower limit of detection of 100 IU/ml. Serologic markers of HBV infection—HBsAg, anti-HBc, and anti-HBs—were measured by ELISA [Shire et al., 2007]. In a previous report, we quantified intrapatient HBV quasispecies diversity within the PreS, S, and overlapping polymerase regions—denoted Pol(PS) and Pol(S)—within this population [Martin et al., 2010]. For the current analysis, the X gene was amplified from a convenience sample of 17 individuals with HBV/HIV co-infection.

PCR Amplification and Cloning

Two hundred to 400 μl of patient serum was used to extract HBV DNA, of which 5 μl served as the template for amplification of full-length HBV genomes [Ginther et al., 1995]. One microliter of the full-length PCR product was then utilized as the template for a second round X gene-specific PCR [389 bp; nucleotides (nt) 1294–1683 according to X97848] using primers P198-S ([Uchida et al., 1994]; nt 1294–1316) and CDR1-AS ([Kannangai et al., 2004]; nt 1683–1661). Amplification conditions were as follows: 94°C for 5 min, followed by 40 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, followed by a final extension step at 72°C for 5 min. The GeneAmp PCR Reagent Kit with AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA) was used for amplifications according to the manufacturer’s instructions. HBV DNA-negative serum samples and a reaction without template served as negative controls, while a previously amplified HBV DNA-positive serum sample served as a positive control. All PCR products were gel purified and cloned into the pGEM-T Easy vector (Promega, Madison, WI). A median number of 10 viral variants were sequenced per individual and submitted to GenBank under accession numbers HM484887–HM484967.

Phylogenetic Analysis

Sequence alignments were initially created using the neighbor-joining method in Clustal X [Thompson et al., 1997] for both the X region and the overlapping segment of the polymerase open reading frame (Pol(X)). The statistical robustness and reliability of the branching order were assessed using bootstrap analysis with 1,000 replicates. Additional phylogenetic inference was performed using a Bayesian Markov chain Monte Carlo (MCMC) approach as implemented in the Bayesian Evolutionary Analysis by Sampling Trees (BEAST) v1.5.0 program [Drummond and Rambaut, 2007] under an uncorrelated lognormal relaxed molecular clock and the generalized time reversible (GTR) model with nucleotide site heterogeneity estimated using a gamma distribution. The BEAST MCMC analysis was run for a chain length of 50,000,000 with sampling every 5,000th generation. Results were visualized in Tracer v1.4 to confirm chain convergence, and the effective sample size (ESS) was calculated for each parameter. All ESS values were >500 indicating sufficient sampling. The maximum clade credibility tree was selected from the posterior tree distribution after a 10% burn-in using TreeAnnotator v1.5.0. Posterior probabilities >90% were considered statistically significant.

Shannon entropy was calculated using the following equation: 
\[ S_n = -\sum (p_i \log_2 p_i) / \ln N, \]
where \( p_i \) is the frequency of each distinct nucleotide sequence, and \( N \) is the total number of sequences analyzed per patient. Intrapatient genetic distances (GD) were calculated by pairwise comparison of nucleotide sequences using the Kimura method, while non-synonymous (dN) and synonymous (dS) mutations were calculated via the Nei–Gojobori method [Nei and Gojobori, 1986] as implemented in the MEGA 4 software [Tamura et al., 2007].

Recombinant Analysis

To assess intergenotypic recombination, consensus HBx sequences from each patient were aligned with
non-recombinant GenBank references representing all HBV genotypes. Individual HBx viral variants that were outliers by phylogenetic analysis were further compared to the consensus sequences of non-recombinant variants for that patient. Similarity plots and bootscan analyses were then performed in SimPlot version 3.5.1 [Lole et al., 1999] using the Kimura 2-parameter with a 100 bp window and a 10 bp step. GenBank reference X75657 (genotype E) was utilized as an outlier. Additionally, principal coordinate analysis [Higgins, 1992] was performed to assess patterns in sequence data using the PCOORD program, accessible at http://www.hiv.lanl.gov/content/sequence/PCOORD/PCOORD.html.

**Statistical Analysis**

For quasispecies parameters (GD, entropy, and dN–dS), each dependent variable was rank-ordered, and analysis of variance (ANOVA) tests were performed between regions—including PreS, S, Pol(S), Pol(PS), and Pol(X)—with the Tukey correction for multiple comparisons. Spearman’s correlations between quasispecies parameters and age, race, alanine aminotransferase (ALT), aspartate aminotransferase (AST), HBV DNA, and CD4 cell count were performed. P-values <0.05 were considered significant.

**RESULTS**

**Patient Population Characteristics**

For the HBV/HIV co-infected individuals in this study, 15 were HBsAg+/HBV DNA+, 2 were HBsAg−/HBV DNA+. All were male; 65% were African-American and 35% were Caucasian, with a median age of 36.1 years (range = 21.2–51.3 years). HBV DNA levels ranged from $2.8 \times 10^2$ to $7.6 \times 10^8$ IU/ml with a median of $1.5 \times 10^7$ IU/ml. The median ALT and AST were 52 U/L (17–114) and 54 U/L (24–78), respectively. The median CD4 cell count was 265 cells/ml (7–665), and the median HIV RNA level was $5.8 \times 10^4$ copies/ml ($7.2 \times 10^2$–$2 \times 10^5$).

**HBV Genotypes**

The HBV genotypes determined by analysis of the X gene were consistent with those previously identified based on analysis of the PreS and S regions [Martin et al., 2010] and included genotypes A (n = 13, 76.5%) and E (n = 1, 5.9%). Additionally, 3 individuals (17.6%) were dually infected with HBV genotypes A and G (Fig. 1A). Importantly, all 3 A+G dual infections (HBsAg+/HBV DNA+) were previously characterized as genotype G single infections based on analysis of the PreS and S regions [Martin...
et al., 2010]. In addition, several HBx variants—10 from patient 241571 and 4 from patient 243541—appeared as outliers when compared to the consensus A or consensus G sequences for that particular individual (underlined, Fig. 1B). Interestingly, these viral variants clustered with different genotypes when the alternate open reading frames (ORFs) were analyzed. For instance, 8 of 10 outlier variants for patient 241571 and 1 of 4 outlier variants for patient 243541 clustered with genotype G reference sequences in the Pol ORF (5’ end) and with genotype A reference sequences for the X ORF (3’ end). The remaining 2 outlier variants for patient 241571 and 3 outlier variants for patient 243541 clustered with genotype A reference sequences for the Pol ORF (5’ end) but with genotype G reference sequences for the X ORF (3’ end; data not shown).

**Quasispecies Diversity**

Overall, intrapatient median GD (Fig. 2A) and entropy (Fig. 2B) values were slightly higher for the X and Pol(X) regions compared to those previously reported for the PreS, S, and the corresponding Pol(PS) and Pol(S) regions. Median GD was similar between X and PreS (0.0035 vs. 0.0019; $P = 0.128$) and approached significance when comparing X and S (0.0035 vs. 0; $P = 0.009$). Median GD for Pol(X) was 0.0032 compared to 0.0019 for the Pol(PS) region ($P = 0.134$) and also approached significance when compared to 0 for the Pol(S) region ($P = 0.011$). For the X region, median entropy was 0.4088 compared to 0.4084 for the PreS region ($P = 0.987$) and 0.1412 for the S region ($P = 0.026$). For Pol(X), entropy was significantly higher compared to Pol(S) (0.5931 vs. 0.1412; $P = 0.002$), but not when compared to the Pol(PS) region (0.5931 vs. 0.4084; $P = 0.441$).

Median dN–dS values above 0 were not observed for either the X or Pol(X) regions, indicating that positive immune selection pressures were not consistently acting upon this region in the majority of patients (Fig. 2C). Although dN–dS values for X were less than 0 for all patients, it is interesting to note that positive dN–dS values were observed for the genotype A variants of patient 241571 and for the genotype G variants of patient 243541, indicating that positive immune selection pressure is present in those individuals with putative recombinant viruses. No significant associations were observed between the various indicators of quasispecies diversity and CD4 cell count, HIV viral load, or HBV DNA level.

**Recombinant Analysis**

For patients with potential X gene recombination, separate viral DNA extractions, PCRs, and cloning were performed to confirm the presence of recombination. Additional variants sequenced were included in the recombinant analysis to further explore intergenotypic recombination. HBV nucleotide sequences for all viral variants from patients 241571 and 243541 were

Fig. 2. Dot plots for (A) genetic distance, (B) Shannon entropy, and (C) dN–dS values. ● represents individual patient values, while ⬨ represents median values for each region. Box and whisker plots indicate upper and lower quartiles, as well as outliers. The solid line in (C) represents a dN–dS value of 0.
compared to GenBank genotype references using SimPlot. For patient 241571, 7 viral variants belonged to genotype A (23.3%), 13 viral variants belonged to genotype G (43.3%), and 10 viral variants showed evidence of A/G recombination (33.3%). For patient 243541, 15 viral variants belonged to genotype A (48.4%), 12 viral variants belonged to genotype G (38.7%), and 4 viral variants showed evidence of A/G recombination (12.9%).

BootScan analyses indicated that 8 of 10 recombinant variants from patient 241571 clustered with genotype G at the 5' end of the amplified region but clustered with genotype A at the 3' end. The breakpoints for three viral variants were located at nucleotide 1397 (according to X97848) in the overlapping polymerase and X ORFs, while the other 5 had breakpoints corresponding to nucleotides 1497–1587 (representative variant shown in Fig. 3).
indicated that the remaining 2 variants for patient 241571 displayed the opposite recombination pattern with the 5' end of the amplified region clustering with genotype A, but clustering with genotype G at the 3' end with breakpoints at nucleotides 1407–1417 (all breakpoints shown in Fig. 5). Similarly, recombination analysis for patient 243541 indicated that 3 viral variants clustered with genotype A at the 5' end, but with genotype G at the 3' end. Breakpoints were observed at nucleotides 1417, 1547, and 1577 within the Pol/X overlap region (representative variant shown in Fig. 4). The remaining variant from patient 243541 displayed recombination with the 5' end clustering with genotype G, the 3' end clustering with genotype A, and a breakpoint at nucleotide 1527 (all breakpoints shown in Fig. 5). Phylogenetic trees

![Fig. 4. Recombinant analysis for recombinant variant 8 from patient 243541. A representative SimPlot bootscan using the Kimura 2-parameter, with 500 replicates, a window of 100 bp, and a 10 bp step, was performed with GenBank genotype references (A), as well as patient-specific consensus sequences for genotypes A and G (B).](image-url)
constructed for the 5' end or the 3' end of each recombinant sequence provided clear evidence of relatedness between the recombinant variants and the consensus sequence of the genotype indicated by BootScan analysis (data not shown).

Using principal coordinate analysis, the 10 recombinant variants for patient 241571 clustered into two distinct groups between genotype A and genotype G reference sequences. One group of 2 recombinants was located adjacent to genotype G, while the other group consisting of 8 recombinants was located adjacent to genotype A (Supplemental Figure 1A). This distribution highlights the variability in breakpoints observed by BootScan analysis. In contrast, the remaining non-recombinant viral variants from patient 241571 grouped closely with reference sequences for either genotype A (n = 7) or G (n = 13). For patient 243541, PCOORD analysis demonstrated that the 4 recombinant variants clustered as one large group consisting of 8 recombinants was located adjacent to genotype A and the genotype G reference sequences. The remaining viral variants from patient 243541 grouped closely with references for either genotype A (n = 15) or G (n = 12; Supplemental Figure 1B).

**DISCUSSION**

To date, most HBV studies have focused mainly on genotypic diversity within a given population [Amini-Bavil-Olyaee et al., 2006; Datta et al., 2008] and limited data are available on X gene variability despite a growing number of studies suggesting its importance in the HBV life cycle and regulation of several cellular processes [Mahé et al., 1991; Madden and Slagle, 2001; Nijhara et al., 2001; Zhang et al., 2006]. However, the current preliminary study aimed to investigate intrapatient HBV diversity within the X and overlapping polymerase genes. Overall, the X and Pol(X) regions tended to have higher GD and entropy values. Significantly higher entropy values were observed in the Pol(X) region when compared to the Pol(S) (P = 0.002) region, previously analyzed in the same patients [Martin et al., 2010].

This analysis also provided the opportunity to investigate intergenotypic recombination. Of the 17 individuals included in this study, three dual HBV infections with genotypes A+G (17.6%) were detected. HBV genotype G single infections are rare in the available literature [Alvarado-Esquível et al., 2006; Chudy et al., 2006; Pas et al., 2008], as the vast majority of genotype G infections are found as dual infections with genotypes A, C, or H [Kato et al., 2002a; Suwannakarn et al., 2005; Sanchez et al., 2007]. It has been suggested that genotype G infections may be characterized by impaired replication and require dual infection with a functional HBV strain to maintain chronicity, although replication-competent genotype G variants have been described [Li et al., 2007]. The three dual HBV infections identified in this study had been reported previously as genotype A (n = 2) or G (n = 1) single infections based on analysis of the PreS and S regions. However, this discrepancy is not entirely surprising given that immune selection pressures may act independently on distinct genomic regions and a lack of recombination in one region does not rule out recombination within another region [Allen et al., 2005; Harrington et al., 2007; Streeck et al., 2008].

Identification of dual HBV infection is increasingly common, and recombination has been identified between most HBV genotypes [Simmonds and Midgley, 2005]. Several recombination “hot spots” have been identified within the HBV genome, including PreS1/PreS2 (nt 3150–100), the 3’ end of S (nt 650–830), and both ends of core (nt 1640–1900 and 2330–2450) [Simmonds and Midgley, 2005; Yang et al., 2006]. Osiowy et al. also identified several recombination breakpoints within A+G dually infected individuals, including several within the X gene. Here, we identified two regions within the X gene—nt 1397–1417 and nt 1497–1587—in which HBV recombination occurred. These regions are highly conserved between genotypes A and G and likely facilitate intergenotypic recombination events.

It is reasonable to suggest that decreased immune function, such as during HIV co-infection, may render HBV/HIV co-infected individuals more susceptible to dual HBV infection. Interestingly, a study investigating HBV genotypes identified more dual HBV infections in HBV/HIV co-infected individuals than expected—40% compared to 0.3% prevalence in the US [Shire et al., 2006]. In addition, dual infections
also trended towards an association with increased HIV viral load. While the effects of recombination events in A+G dual infections are currently unknown, it is interesting to note that genotype G has been described as a determinant of liver fibrosis in immunocompromised individuals [Lacombe et al., 2006]. Therefore, in HBV/HIV co-infected individuals, genotype A viruses may further enhance the pathogenic potential of genotype G viruses through intergenotypic recombination. Furthermore, recombination within the X gene could result in additional functions of the HBx regulatory protein with yet unknown consequences on HBV pathogenesis, especially in HBV/HIV co-infected patients. Future studies should investigate the prevalence of dual HBV infection and HBV recombination in HBV mono-infected individuals compared to HBV/HIV co-infected individuals. Full-length HBV genomes should be sequenced to identify genomic regions that favor HBV recombination, and the potential effects these recombination events may have on pathogenesis and disease progression should be elucidated.

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REFERENCES


Principal coordinate analysis (PCOORD) plots for all 30 variants from patient 241571 (A) and all 31 variants from patient 243541 (B). Non-recombinant viral variants are circled and indicated by (O), while recombinant viral variants are circled and indicated by (X). Numbers adjacent to each group represent the number of variants within that grouping. Letters A-H indicate GenBank reference sequences. Two groups of recombinant variants were identified for patient 241571, while all recombinant variants grouped together for patient 243541.
Mutations Associated with Occult Hepatitis B Virus Infection Result in Decreased Surface Antigen Expression In Vitro

Running Title: Occult HBsAg Expression

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Abstract

Occult hepatitis B virus (O-HBV) infection is characterized by the absence of detectable hepatitis B surface antigen (HBsAg) in the serum, despite detectable HBV DNA. Studies investigating the mechanisms underlying the development of O-HBV infection are lacking in the current literature, although viral mutations in the surface region, resulting in decreased HBsAg expression or secretion, represent one potential mechanism. Wild-type HBsAg expression vectors were constructed from genotype-matched chronic HBV sequences. Site-directed mutagenesis was then utilized to introduce three genotype A mutations – M103I, K122R, and G145A – associated with O-HBV infection in vivo, alone and in combination, into the wild-type HBsAg vectors. Transfection of Huh7 and HepG2 cell lines was performed, and cell culture supernatants and cell lysates were collected over 7 days to assess the effects of these mutations on extracellular and intracellular HBsAg levels. The G145A mutation resulted in significantly decreased extracellular and intracellular HBsAg expression in vitro. The most pronounced reduction in HBsAg expression was observed when all 3 mutations were present. The mutations evaluated in vitro in the current study resulted in decreased HBsAg expression and potentially increased hepatic retention and/or decreased hepatic secretion of synthesized HBsAg, which could explain the lack of HBsAg detection that is characteristic of O-HBV infection in vivo.

Keywords:

occult hepatitis B virus
hepatitis B surface antigen (HBsAg)
HBV/HIV co-infection
HBsAg mutants
G145A
Introduction

Greater than 2 billion people have been exposed to hepatitis B virus (HBV) worldwide and more than 350 million individuals are currently living with chronic HBV infection [Lavanchy, 2004]. While chronic HBV (C-HBV) infection is typically diagnosed by detectable serum hepatitis B surface antigen (HBsAg) at least twice over a 6-month period [Lok and McMahon, 2007], occult HBV (O-HBV) infection is characterized by a lack of detectable HBsAg in the serum despite low-level HBV replication [Bodsworth et al., 1991]. O-HBV infection was once identified by the presence of antibodies against HBV core (anti-HBc) alone in the absence of HBsAg [Jilg et al., 1995], although cases have now been reported in which HBV DNA is the only detectable marker of O-HBV infection [Shire et al., 2007].

The clinical relevance of O-HBV has not been thoroughly explored; however, case-control studies have reported that O-HBV infection is associated with increased incidence of hepatocellular carcinoma (HCC) and severity of liver fibrosis [Kannangai et al., 2004; Squadrito et al., 2006; Tamori et al., 2003]. Recent prospective studies have confirmed this connection between O-HBV and HCC [Ikeda et al., 2009; Matsuoka et al., 2008]. In addition, O-HBV is transmissible via blood transfusion in humans and primates, as well as solid organ transplantation [Baginski et al., 1992; Campe et al., 2005; Thiers et al., 1988; Yuen et al., 2011]. In both scenarios, transmission of O-HBV infection may result in either C-HBV or O-HBV in the recipient.

Globally, a number of studies have investigated the prevalence of O-HBV infection and reported rates from 0% – 89.5%, depending on the population, laboratory methods, and inclusion/exclusion criteria [Hofer et al., 1998; Nunez et al., 2002]. In the US, the prevalence of
O-HBV ranges from 0% – 45% with detection most frequently associated with HIV and/or HCV co-infection [Rodríguez-Torres et al., 2007; Torbenson et al., 2004]. Few studies have performed virologic comparisons between C-HBV and O-HBV patients from the same cohort, and functional analysis of mutations associated with O-HBV infection is rarely performed.

The potential mechanisms resulting in O-HBV infection include 1) complexes of HBsAg with antibodies against surface (anti-HBs) that circulate in the serum but are not readily detected by standard HBsAg ELISAs, 2) interference of HBV replication by other viral co-infections, 3) altered host immunologic responses, and/or 4) mutations within the HBV genome that result in altered HBsAg expression [Hu, 2002]. We previously identified multiple mutations associated with O-HBV infection by comparing viral sequences from O-HBV infections with those isolated from genotype-matched C-HBV infections from the same cohort [Martin et al., 2010]. For the current study, the small HBsAg mutations M103I, K122R, and G145A from a genotype A O-HBV infection were evaluated for their effects on HBsAg synthesis and secretion in vitro.
Materials and Methods

**Mutations Associated with O-HBV Infection.** Identification of mutations associated with occult HBV infection was performed as described previously [Martin et al., 2010]. Briefly, sera were evaluated for markers of HBV infection, including real-time PCR for HBV DNA and ELISAs for serologic markers (HBsAg, anti-HBs, and anti-HBc) [Shire et al., 2007]. Our previous analysis of the Pre-Surface, Surface, and Polymerase regions of the HBV genome identified a number of mutations associated with O-HBV infection, which were absent in genotype-matched C-HBV infections from the same cohort and GenBank reference sequences [Martin et al., 2010]. Three mutations within the small HBsAg – M103I, K122R, and G145A – from an individual with a genotype A infection were evaluated in the current study, since genotype A represents the most common HBV genotype in the US [Chu et al., 2003]. The most common amino acid at these three positions among the GenBank reference sequences included in our previous study are listed in Table 1.

**Construction of HBsAg Expression Vectors.** HBV DNA was extracted from 200 uL of serum using the QIAamp UltraSens Virus Extraction Kit (Qiagen, Valencia, CA). The patient with O-HBV infection was matched to 2 C-HBV infected, HIV-positive individuals from the same cohort, based on HBV genotype, age <5 years apart, gender, ethnicity, and HCV serostatus. PCR amplification of the full-length HBV genome was performed using primers and amplification conditions previously described [Günther et al., 1995] and the Picomaxx High Fidelity PCR System (Agilent Technologies, Santa Clara, CA). Second round primers – sHBs-EcoRI-F (5’ CGA ATT CGG GAC CCT GTG ACG AAC) and sHBs-KpnI-R (5’ GGG GTA CCC ATC TCT TTG TTT TGT TAG) – were designed to amplify the entire S region (721 bp, nt 138-859, according to X97848) and contained EcoRI and KpnI sites to facilitate cloning into the
pCMV-HA cloning vector (Clontech, Mountain View, CA), thereby producing small HBsAg proteins containing a 5’ hemagglutinin (HA) tag. Site-directed mutagenesis was performed to introduce the M103I, K122R, and G145A mutations – separately or in combination – into each C-HBV expression vector using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent). The specific HBsAg expression vectors are listed in Table 2.

Transfection and HBsAg Quantification. Huh7 and HepG2 human hepatoma cell lines were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and RPMI-1640 medium with 4% FBS and 4 mM L-glutamine, respectively. Huh7 cells were seeded at a concentration of 5x10^4 cells per well in 24-well plates, while HepG2 cells were seeded at a concentration of 1x10^5 cells per well. 24 hours later, cells were transfected with 2 ug DNA per well with 3 uL FuGENE 6 (Roche Applied Science, Indianapolis, IN). Media were changed after 24 hours, and collected every 48 hours thereafter. Supernatants collected on days 3 and 5 were pooled for the day 5 sample, while supernatants collected on days 3, 5, and 7 were pooled for the day 7 sample, and clarified by centrifugation at 10,000 rpm for 5 minutes. On days 3, 5, and 7, cells were washed three times with phosphate buffered saline (PBS) and lysed with 200 uL lysis buffer – Tris-HCl (50 mmol/L, pH 7.8), NaCl (150 mmol/L), EDTA (1 mmol/L), 0.5% Nonidet P-40 (NP-40), and 1/100th volume of protease inhibitors (cOmplete Mini Protease Inhibitor Cocktail, Roche). Plates were frozen, thawed, and lysates were clarified by centrifugation at 13,000 g for 3 minutes. HBsAg was quantified in supernatants and cell lysates by ELISA (Biochain, Hayward, CA) using recombinant HBsAg (adr subtype) at dilutions of 500 ng/mL – 0.25 ng/mL as standards. The OD_{450} was measured and compared to a standard curve from 0.25 ng/mL – 10 ng/mL. Samples with OD_{450} >10ng/mL were diluted and re-measured. The lower limit of detection for this assay
was 0.5 ng/mL. Total nanograms HBsAg per sample at each time point were corrected for the proper dilution and the ratio of $\text{lysate}_{\text{HBsAg}}:\text{supernatant}_{\text{HBsAg}}$ were calculated for each HBsAg construct.

*Statistical Analysis.* Within each combination of cell line, compartment, wild-type group, and time point, data were analyzed by a one-factor, repeated-measures ANOVA. Type of mutant (with wild-type as the reference level) was the repeated factor, and comparisons of each mutant condition to the wild-type were performed using Dunnett’s procedure for comparing multiple treatments to a common control condition. The maximum family-wise type I error rate for Dunnett’s procedure was set at $\alpha=0.05$. 

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**Results**

*Wild-type HBsAg Expression in Huh7 and HepG2 Cells.* HBsAg levels in the supernatants and lysates of transfected Huh7 and HepG2 cells were assessed for the wild-type (i.e., chronic HBV) expression vectors on days 3, 5, and 7 post-transfection. Overall, HBsAg expression levels were higher in Huh7 cells compared to HepG2 cells (Figures 1A-B compared to 1C-D). No significant differences were observed between wells with regards to viable cell number (Supplemental Figure 1A) or transfection efficiency determined via co-transfection of a β–galactosidase-encoding vector (Supplemental Figure 1B).

Supernatant HBsAg levels in Huh7 cells transfected with the wild-type expression vector increased between day 3 and day 5 post-transfection and decreased slightly by day 7 (Figure 1A). Lysate HBsAg levels in Huh7 cells showed a similar trend with a greater decrease between days 5 and 7 post-transfection (Figure 1B). Similarly, in HepG2 cells, wild-type supernatant HBsAg levels increased between days 3 and 5 post-transfection and plateaued by day 7 (Figure 1C). Wild-type lysate HBsAg levels in HepG2 cells mimicked the trends observed in Huh7 cells as well (Figure 1D). Similar results were observed when the second distinct wild-type expression vector was utilized (Supplemental Figure 2). However, supernatant HBsAg levels were higher and lysate HBsAg levels were lower, indicating that HBsAg secretion is higher with the second C-HBV backbone, presumably due to the 2 amino acid mutations (G164E and S212F) that differentiate these two backbones.

*Effects of Single Point Mutations on HBsAg Expression.* HBsAg expression was also measured in supernatants and lysates of Huh7 and HepG2 cells transfected with vectors containing the M103I, K122R, or G145A mutations. Supernatant levels of HBsAg were similar
between wild-type, M103I, and K122R vectors in both Huh7 and HepG2 cells (Figures 1A and C). In cell lysates, HBsAg levels were slightly lower for expression vectors containing the M103I and K122R mutations compared to the wild-type expression vector (Figures 1B and D). In contrast, the G145A mutation resulted in significantly lower HBsAg levels in both cell supernatants and lysates. These trends were also observed for the second wild-type expression vector (Supplemental Figure 2), although a more pronounced decrease in supernatant HBsAg was observed in Huh7 cells for vectors containing the M103I and K122R mutations alone (Supplemental Figure 2A).

**Effects of Multiple Mutations on HBsAg Expression.** When examining the combined effects of these mutations, M103I+K122R resulted in decreased HBsAg levels in both supernatants (Figures 2A and C) and lysates (Figures 2B and D), but remained well above the ELISA detection limit. In contrast, combinations of mutations that contained G145A resulted in significantly lower HBsAg levels, several of which were below detectable limits in both supernatants and lysates. The most pronounced reductions in HBsAg synthesis were observed when all three mutations (M103I+K122R+G145A) were present (Figure 2 and Supplemental Figure 3).

**Effects of Mutations on HBsAg Secretion.** Ratios of lysate (intracellular) to supernatant (extracellular) HBsAg were calculated to compare HBsAg secretion versus retention in hepatocyte cell lines. At day 3 post-transfection, the wild-type vector resulted in higher intracellular levels of HBsAg in Huh7 cells (ratios >1 in bold, Table 3). Over time, more HBsAg was secreted into cell supernatants, leading to ratios <1 (Table 3). The G145A, M103I+G145A, K122R+G145A, and M103I+K122R+G145A vectors continued to have higher intracellular HBsAg levels at all time points in Huh7 cells, when above the detection limit. Similar results
were observed in HepG2 cells, in which the only constructs with ratios >1 were those containing
the G145A mutation alone or in combination. These findings were not observed with the second
wild-type vectors (Supplemental Table 1), since lysate HBsAg levels were lower overall with
these constructs.
Discussion

To date, the majority of studies on occult HBV infection have focused on its prevalence in various populations [Hofer et al., 1998; Torbenson et al., 2004] and/or the identification of mutations associated with O-HBV [Kao et al., 2002; Weinberger et al., 2000]. In contrast, functional analysis of specific HBV mutations on HBsAg synthesis has only rarely been reported [Hass et al., 2005; Jeantet et al., 2002; Schories et al., 2000]. Thus, to determine whether mutations associated with O-HBV infection may play a role in the lack of detectable HBsAg in these individuals, additional investigation is required. The current study focused on the effects of three previously identified O-HBV mutations in the S region – M103I, K122R, and G145A – from an HIV co-infected patient [Martin et al., 2010]. The current in vitro system evaluates HBsAg protein expression since transcription for all vectors is driven by the CMV-IE promoter with little to no variation in mRNA expression. We found that when HBsAg mutations were combined, HBsAg expression was significantly reduced. Extracellular levels of HBsAg were slightly above detectable levels at times, indicating that the HBsAg secreted from infected hepatocytes would likely be below the limit of detection for commercial assays once diluted in the blood. Although the lack of detectable HBsAg could represent a diagnostic error [El Chaar et al., 2010; Jeantet et al., 2004; Louisirirotchanakul et al., 2010; Mizuochi et al., 2006; Moerman et al., 2004], HBsAg synthesis was consistently detectable with the commercial ELISA kit utilized in the current study even for the G145A mutation, which generally resulted in very low HBsAg levels. In addition, several commercial ELISA kits were assessed for detection of several amino acid changes at position 145, including G145A, although not identified in vivo, which was detectable with all kits tested [Coleman et al., 1999]; thus, a false-negative HBsAg result seems unlikely.
Of the three mutations characterized here, the G145A mutation contributes the most to decreased HBsAg synthesis in vitro. This mutation was previously associated with O-HBV infection [Kao et al., 2002] and has been identified as a potential vaccine escape mutant [Seddigh-Tonekaboni et al., 2000]. Although the genotype of the O-HBV-infected patient was not specified by Kao et al., the study was performed in Taiwan; thus, the genotype is likely B or C. Additional mutations at position 145 of the S region have been previously characterized, although not always in O-HBV-infected patients [Amini-Bavil-Olyaee et al., 2006; Bock et al., 2002; Echevarría and Avellón, 2006]. A G145R mutation has been identified in HBV genotypes B and C previously and has been described as an immune escape mutant [Echevarría and Avellón, 2006], as well as a potential contributor to lamivudine resistance [Amini-Bavil-Olyaee et al., 2006]. These studies consistently indicated that the G145R mutation resulted in decreased HBsAg levels and increased replication in lamivudine resistant mutants, especially those with additional polymerase mutations, such as L526M and M550V [Bock et al., 2002]. Additionally, while the M103I or K122R mutations alone did not significantly affect HBsAg expression, when these two mutations were combined, HBsAg expression was decreased somewhat. Although the K122R mutation results in a serotype change from ‘d’ to ‘y’, both K122R and M103I may have minor effects with respect to protein folding, antigenicity, detection by anti-HBs, and protein expression, due to their location within the antigenic determinant region of HBsAg [Hu, 2002; Jeantet et al., 2004; Moerman et al., 2004; Schories et al., 2000; Weinberger et al., 2000; Wu et al., 2010].

In the current study, not only did the mutations assessed decrease HBsAg synthesis, but the small amount of HBsAg produced was retained within the hepatocytes, as indicated by higher intracellular HBsAg ratios in Table 3. It was originally hypothesized that certain HBsAg
mutations may result in a drastic increase in retention of viral proteins due to misfolding [Bruss and Ganem, 1991; Kalinina et al., 2001; Khan et al., 2004]. However, the low levels of intracellular HBsAg observed indicate that if HBsAg misfolding is occurring, then proper proteasomal degradation is likely still taking place. Nonetheless, future investigations should evaluate the folding and sub-cellular localization of mutant forms of HBsAg.

Previous studies varied substantially in the methodologies utilized to evaluate O-HBV infection. A small number of studies have utilized full-length, replication-competent O-HBV genomes containing one polymerase mutation (terminal protein mutation T165P) [Blum et al., 1991a] or several amino acid mutations throughout the genome [Blum et al., 1991b; Miyagawa et al., 2008] that resulted in defective replication, or overall decreases in HBsAg and HBV DNA levels. Other studies identified a single nucleotide [Hass et al., 2005] or amino acid mutation [Jeantet et al., 2004] that lead to decreased PS2/S mRNA levels, resulting in decreased HBsAg. Additional investigations studied heavily mutated viral sequences within the PS1 and PS2/S promoters [Sengupta et al., 2007] or S region [Jeantet et al., 2002] that lead to altered HBsAg secretion. However, to date, only three studies have focused specifically on individual mutations within the S region and their effects on HBsAg synthesis. One study evaluated the Y100C mutation. Surprisingly, HBsAg levels were higher than wild-type; thus, why HBsAg was not detectable in the serum remains unclear [Mello et al., 2011]. In the second study, HBsAg expression was decreased by 40% with the P142A mutation and by 60% with the D144V mutation; however, when both mutations were included, HBsAg expression was decreased by only 20% [Schories et al., 2000]. Finally, in the third study, the I110M, G119E, and R169P mutations were each capable of impairing virion secretion [Ito et al., 2011].
Several aspects of the current investigation are unique compared to previously published studies. First, the specific mutations assessed in this study – M103I, K122R, and G145A – had not been included in previous functional analyses of HBsAg expression during O-HBV infection and, therefore, extend the small body of previously available research that utilized *in vitro* models to evaluate specific instances of undetectable HBsAg *in vivo*. The current also examined changes in HBsAg expression over 7 days, since HBsAg synthesis and secretion are dynamic processes that may fluctuate over time. Additionally, both Huh7 and HepG2 cell lines were utilized to assess potential differences between these cell lines *in vitro*, since hepatocyte cell lines may not recapitulate infection in humans. Finally, two chronic/wild-type HBV sequences were used as backbones for the constructed HBsAg expression vectors to assess which observed trends were consistent.

In summary, the focus of this study was to differentiate the specific effects of the S gene mutations M103I, K122R, and G145A on HBsAg synthesis. The G145A mutation alone and in combination resulted in significantly decreased extracellular and intracellular HBsAg expression. Interestingly, the G145A mutation, also results in a W153C mutation in the reverse transcriptase region of the HBV polymerase. Previous mutations at this position, although not W153C, have been shown to decrease HBV replication [Bock et al., 2002]. In the future, full-length, replication-competent viruses will be generated to evaluate the simultaneous effects of these and other mutations on HBsAg synthesis and viral replication.
Acknowledgements

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References


HBsAg II assay with the Architect®, AxSym®, and Advia® Centaur HBsAg screening assays. Journal of Medical Virology 82(5):755-762.


Table 1

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<th>C</th>
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Most Common Amino Acids at Positions of Interest by Genotype. Five GenBank references for each HBV genotype (A-H), used previously to identify mutations associated with O-HBV infection [Martin et al., 2010], were screened to identify the most common amino acid present at positions 103, 122, and 145 of the small HBsAg compared to the mutations associated with O-HBV being examined. * indicates that the amino acid position was variable for the given genotype.
**Table 2**

<table>
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<th>Genotype A HBsAg Expression Vectors</th>
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<td>Wild Type 1</td>
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<tr>
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<tr>
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<tr>
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<td>M103I + K122R + G145A</td>
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<td>pCMV-HA alone (negative control)</td>
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</table>

**Genotype A HBsAg Expression Vectors.** Wild-type partial S ORF sequences were derived from 2 genotype A chronic HBV infections and ligated into the pCMV-HA vector. The wild-type vectors served as positive controls and were the backbones into which mutations associated with occult HBV infection were introduced by site-directed mutagenesis. A total of 17 vectors were utilized in these experiments as listed above.
**Figure 1**

Total ng of HBsAg produced by transfection of pCMV-HA alone, wild-type vector 1 alone or with mutations M103I, K122R, or G145A, were calculated for supernatants (A, C) and cell lysates (B, D) from Huh7 (A, B) and HepG2 (C, D) cells in duplicate and compared at days 3 (grey bars), 5 (white bars), and 7 (black bars) post-transfection. * p<0.05; ** p<0.01; † p<0.001; ‡ p<0.0001.
**Figure 2**

**Total ng of HBsAg** produced by transfection of pCMV-HA alone, wild-type vector 1 alone or with mutation combinations M103I+K122R, M103I+G145A, K122R+G145A, or M103I+K122R+G145A, were calculated for supernatants (A, C) and cell lysates (B, D) from Huh7 (A, B) and HepG2 (C, D) cells in duplicate and compared for days 3 (grey bars), 5 (white bars), and 7 (black bars) post-transfection. * p<0.05; ** p<0.01; + p<0.001; ++ p<0.0001.
### Table 3

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<th>Day 7</th>
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**HBsAg<sub>lysate</sub>:HBsAg<sub>supernatant</sub> Ratios.** Ratios comparing lysate HBsAg to supernatant HBsAg (HBsAg<sub>lysate</sub>:HBsAg<sub>supernatant</sub>) were calculated at days 3, 5, and 7 post-transfection for all constructs with the wild-type 1 backbone in both Huh7 and HepG2 cells. **Bold** values indicate constructs with higher intracellular/lysate levels of HBsAg (values >1). UD – indicates undetectable levels of HBsAg (below the lower limit of detection for the ELISA, <0.5 ng/mL) in the lysate and/or supernatant.
Supplementary Methods

*Transfection and HBsAg Quantification.* Cell viability post-transfection was determined by trypan blue staining on day 7. Huh7 and HepG2 cells were seeded in duplicate as described in the methods section. Cells were transfected with 2 ug of wild type vector 1, G145A, M103I+K122R+G145A, or pCMV-HA. Media were changed 24 hours post-transfection and every 48 hours thereafter. After 7 days, cells were washed, trypsinized, and resuspended in fresh media before staining with trypan blue and counting. Mean number of cells were determined for each construct, log-transformed, and compared between constructs (Supplemental Figure 1A).

Additionally, 2 additional wells of Huh7 and HepG2 cells were seeded to determine transfection efficiency. 50 ng of plasmid p0Z-17, encoding for β-galactosidase, was co-transfected along with 2 ug of each construct listed above, while pCMV-HA alone served as a negative control without co-transfection of p0Z-17. Media was changed after 24 hours, and every 48 hours until day 7. On day 7 post-transfection, cells were washed twice with PBS, and fixed for 5 minutes with 2% formaldehyde. Cells were incubated with staining solution (5 mM phosphate ferricyanide, 5 mM phosphate ferrocyanide, 2 mM MgCl₂, and 1 mg/mL X-gal) diluted in PBS, for 2 hours at 37°C. Five fields of view, at 25X power, were counted per well to determine the number of blue cells per well. The average between duplicates was determined and compared between constructs (Supplemental Figure 1B).
**Supplemental Figure 1**

**Viable Cell Counts and Transfection Efficiency.** Huh7 and HepG2 cells were transfected in duplicate with 2 ug wild type vector 1 alone, with mutation G145A, with mutations M103I+K122R+G145A, or pCMV-HA alone. On day 7 post-transfection, viable cell counts were determined by staining with trypan blue (A) and transfection efficiency was determined by staining with X-gal after co-transfection with 50 ng plasmid p0Z-17, encoding for β-galactosidase (B).
**Supplemental Figure 2**

*Total ng of HBsAg* produced by transfection of pCMV-HA alone, wild-type vector 2 alone or with mutations M103I, K122R, or G145A, were calculated for supernatants (A, C) and cell lysates (B, D) from Huh7 (A, B) and HepG2 (C, D) cells in duplicate and compared at days 3 (grey bars), 5 (white bars), and 7 (black bars) post-transfection. * p<0.05; ** p<0.01; + p<0.001; ++ p<0.0001.

![Graphs showing total ng of HBsAg for Huh7 and HepG2 cells](image)
Supplemental Figure 3

**Total ng of HBsAg** produced by transfection of pCMV-HA alone, wild-type vector 2 alone or with mutation combinations M103I+K122R, M103I+G145A, K122R+G145A, or M103I+K122R+G145A, were calculated for supernatants (A, C) and cell lysates (B, D) from Huh7 (A, B) and HepG2 (C, D) cells in duplicate and compared for days 3 (grey bars), 5 (white bars), and 7 (black bars) post-transfection. * p<0.05; ** p<0.01; + p<0.001; ++ p<0.0001.
**Supplemental Table 1**

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<td>Huh7</td>
<td>HepG2</td>
<td>Huh7</td>
</tr>
<tr>
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<td>0.16</td>
<td>0.26</td>
</tr>
<tr>
<td>M103I</td>
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<td>0.16</td>
<td>0.20</td>
</tr>
<tr>
<td>K122R</td>
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<td>0.16</td>
<td>0.28</td>
</tr>
<tr>
<td>G145A</td>
<td>0.34</td>
<td>UD/UD</td>
<td>0.18</td>
</tr>
<tr>
<td>M103I+K122R</td>
<td>0.31</td>
<td>0.37</td>
<td>0.22</td>
</tr>
<tr>
<td>M103I+G145A</td>
<td>0.46</td>
<td>UD/UD</td>
<td>0.31</td>
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<tr>
<td>K122R+G145A</td>
<td>UD/UD</td>
<td>UD/UD</td>
<td>UD/UD</td>
</tr>
<tr>
<td>M103I+K122R+G145A</td>
<td>UD/UD</td>
<td>UD/UD</td>
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<tr>
<td>pCMV-HA</td>
<td>UD/UD</td>
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</table>

**HBsAg\textsubscript{lysate}:HBsAg\textsubscript{supernatant} Ratios.** Ratios comparing lysate HBsAg to supernatant HBsAg (HBsAg\textsubscript{lysate}:HBsAg\textsubscript{supernatant}) were calculated at days 3, 5, and 7 post-transfection for all constructs with the wild-type 2 backbone in both Huh7 and HepG2 cells. **Bold** values indicate constructs with higher intracellular/lysate levels of HBsAg (values >1). UD – indicates undetectable levels of HBsAg (below the lower limit of detection for the ELISA, <0.5 ng/mL) in the lysate and/or supernatant.
VI
Screening for hepatitis C virus non-nucleotide resistance mutations in treatment-naive women

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Objectives: Hepatitis C virus (HCV) non-nucleoside inhibitors (NNIs) target the viral RNA-dependent RNA polymerase encoded by the NS5B gene. Several NNIs share a similar allosteric binding site, and their antiviral efficacy is attenuated by a cysteine-to-tyrosine mutation at amino acid 316 (C316Y). In the current study, we assessed NS5B resistance mutations in treatment-naive individuals from a prospective natural history study of viral infections in women.

Methods: Partial NS5B sequences from HCV-positive women were amplified by RT–PCR. Additionally, subcloning was performed to evaluate intrapatient variability in selected samples.

Results: HCV NS5B genotypes were 45 genotype 1a (57.0%), 11 genotype 1b (13.9%), 5 genotype 2a (6.3%), 3 genotype 2b (3.8%), 9 genotype 3a (11.4%) and 6 genotype 4a (7.6%). One HCV genotype 1a-infected patient was found to have the C316Y mutation (1.3%). Clonal analysis further revealed that all NS5B sequences from this individual—representing three serum samples collected 4 years apart—contained the C316Y mutation. In contrast, the S282T resistance mutation was not found in any samples.

Conclusions: The C316Y polymerase resistance mutation was found in 1.3% of samples from HCV-infected women. The presence of this mutation over time suggests significant replicative fitness of this variant and has implications for development of new specifically targeted antiviral therapies against HCV (STAT-C) targeting this region.

Keywords: HCV, NS5B, C316Y

Introduction

An estimated 170 million people are infected with chronic hepatitis C virus (HCV) worldwide. The viral NS5B gene encodes an RNA-dependent, RNA polymerase that lacks a proofreading mechanism, thus generating extensive viral diversity. This leads to development of viral quasispecies—distinct viral variants within an individual—as well as multiple HCV genotypes at the population level.

Specifically targeted antiviral therapies against HCV (STAT-C) are in active development and represent the newest advances in HCV treatment. STAT-C agents include protease inhibitors, nucleoside inhibitors and non-nucleoside inhibitors (NNIs), as well as less explored targets. A subset of NNIs binds to the palm domain near the polymerase active site. Among these, experimental agents such as GS-9190 and HCV-796 demonstrate antiviral activity in human clinical trials, while A-837093 has shown promise in animal models.¹–³ STAT-C monotherapy effectively reduces viral load shortly after treatment initiation. However, the existence of low level, pre-existing resistance mutations permits the rapid emergence of drug resistance and treatment failure during monotherapy.¹,³,⁴ As originally described by Villano et al.,⁴ the most frequently observed mutation associated with HCV-796 resistance is the cysteine-to-tyrosine signature mutation at position 316 of NS5B (C316Y). C316Y is associated with a 166-fold reduction in susceptibility to HCV-796 in vitro. Additionally, the cysteine-to-asparagine polymorphism (C316N) confers a modest 26-fold reduction in susceptibility and is relatively common in genotype 1b-infected individuals but is not considered a drug resistance mutation.* However, the prevalence of

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the C316Y mutation in untreated individuals has not been ade-
quately explored outside of the clinical trial setting. We analysed
data from the HIV Epidemiologic Research (HER) Study—a pro-
spective natural history study of HIV infection in women con-
ducted from 1993 to 1999—to assess NSSB resistance mutations in
treatment-naive individuals.

Materials and methods

A total of 871 HIV-infected women and 439 demographically
matched, uninfected women were recruited from four US sites into
the HER Study. By study design, approximately half of the women
reported having used injection drugs at least once since 1985, while
the other half reported only sexual risk behaviours. All participants
provided informed consent prior to sample and clinical data collec-
tion. Institutional review boards approved the study at each site and
at the CDC. The overall prevalence of HCV was 56.5%. For the
current analysis, HER Study subjects were included if they were:
(i) HCV antibody positive at baseline; (ii) from the Rhode Island
site; and (iii) had serum samples available for additional analysis.
Both HIV-positive and HIV-negative subjects were included. Of the
327 women enrolled at the Rhode Island study site, 169 were HCV
seropositive and had detectable HCV RNA. A total of 129 of these
samples were available for the current analysis, and NSSB could be
amplified by direct sequencing for 79.

Viral RNA was extracted from serum using the QIAamp Viral
RNA kit (Qiagen). A partial segment of the NSSB gene was ampli-
fied using a One-Step RT-PCR kit (Qiagen) and the primers PR3:
TAT gAY ACC CgC TgY TT gTC TAT gAY ACC CgC TgY TTT gAC
and PR4: gCN gAR TAY CTV gTC gAT gCC TC (nucleotides 8256–8278
relative to the start of H77) and PR4: gCN gAR TAY CTV gTC
TAATgCC TC (nucleotides 8636–8619) using the
GeneAmp PCR kit (Applied Biosystems). Reaction conditions were
30 min at 50°C, 15 min at 95°C, followed by 5 cycles of 30 s at
93°C, 45 s at 60°C and 60 s at 72°C, followed by 35 cycles of 30 s
at 93°C, 45 s at 60°C to 50°C and 60 s at 72°C, followed by a 5 min
final elongation step at 72°C. First-round PCR products were then
amplified by semi-nested PCR with the primers PR3 and PR5: gCT
AgT CAT AgC CTC CgT (nucleotides 8636–8619) using the
GeneAmp PCR kit (Applied Biosystems). Reaction conditions were
2 min at 95°C, followed by 36 cycles of 30 s at 95°C, 45 s at 55°C
and 1 min at 72°C, followed by a final elongation step for 10 min
at 72°C. PCR products were gel purified and sequenced directly
using dye terminator chemistry. To confirm these sequence results,
multiple clones per time point were ligated into the pGEM-T Easy
Vector (Promega) and sequenced for a subset of individuals.

Sequences corresponding to amino acids 227–344 of the poly-
merase were aligned using the neighbour-joining method in
the ClustalX software. The GenBank database references used
to confirm HCV genotype included 1a (AF009606, AY278830,
AF511948), 1b (AJ000009, D10934), 1c (D14853), 2a (AB047639),
2b (AB030907), 2c (D50409), 3a (D28917), 3b (D63821), 4a
(Y11604) and 6a (Y12083). The statistical robustness and reliabil-
ity of the branching order within the phylogenetic tree were confirmed
by bootstrap analysis using 100 replicates. The region of NSSB
amplified included the resistance mutations S282T and C316Y. These
mutations were assessed in consensus, as well as clonal
sequences, when available.

Results

The NSSB region was amplified and sequenced directly from 79
patient serum samples. Demographic and clinical details of this
cohort are provided elsewhere. Briefly, the HCV genotype
distribution included 45 genotype 1a (57.0%), 11 genotype 1b
(13.9%), 5 genotype 2a (6.3%), 3 genotype 2b (3.8%), 9 geno-
type 3a (11.4%) and 6 genotype 4a (7.6%). Amino acid align-
dent did not reveal the presence of the other major resistance
mutation—S282T—or any known compensatory mutations in
the region analysed for any of the 79 women. In contrast, one
individual possessed the C316Y mutation in the consensus
sequence from a serum sample collected on 17 March 1994. This
patient was a 37-year-old, HIV-negative, white female with a
history of injection drug use. Direct sequencing of NSSB
demonstrated that she was infected with HCV genotype 1a.
Clonal analysis of multiple viral variants from the initial time
point, as well as two additional time points collected on 7
September 1994 and 17 August 1998, also revealed the presence
of C316Y as the dominant amino acid at this position (20 of 20
clones).

Discussion

Our understanding of HCV resistance mutations reflects lessons
previously learned from the HIV literature. For instance,
drug-resistant viruses are generally less fit than wild-type
viruses, though high degrees of viral fitness with certain variants
have been observed. Moreover, drug-resistant viruses are often
present at very low frequencies in treatment-naive individuals,
yet rapidly emerge during the course of antiviral therapy. Using
a genotype 1b replicon system, others have demonstrated
that the C316Y mutation confers a 30% reduction in viral fitness
compared with the wild-type in vitro. However, resistance
may be more complicated in vivo, as a chimpanzee infected
with HCV 1b maintained the C316Y mutation, as well as a
glycine-to-aspartic acid mutation at position 554 (G554D), even
after the cessation of therapy. Thus, compensatory mutations
may also play a role in maintaining certain drug resistance
mutations in vivo even after treatment discontinuation. Likely,
HCV RNA levels of naturally occurring NS3/NS5B drug-
resistant isolates from untreated individuals are equivalent to
those of non-resistant/wild-type virus isolates. Thus, these
naturally occurring mutations probably confer a selective
advantage in vivo, although the existence of compensatory
mutations will require further longitudinal analysis in large
population-based studies.

In the current analysis, positions 217–347 of NSSB were
examined, covering known resistance mutations at residues 282
and 316. The incidence of the C316Y mutation was 1 in 79
patients (1.3%). Importantly, we were able to detect the C316Y
mutation by direct sequencing; thus, C316Y represents the domi-
nant amino acid present in the viral quasispecies of this subject.
An additional 149 representative sequences from the HCV
Sequence Database were also reviewed for the presence of
the C316Y mutation; however, no other sequences contained the
C316Y mutation (Table 1). Interestingly, a recent analysis of
507 HCV treatment-naïve patients reported predominant NSSB
resistance mutations in 10 individuals at position 423 and one
individual at position 415 (2.8% combined); however, no
C316Y resistance mutations were reported. C316 is highly
conserved in genotype 1a, but polymorphic in genotype 1b. Others
have noted that the baseline frequency of HCV drug
resistance mutations is 5.0%–8.6%, which is sufficiently high to
justify resistance testing, assuming similar costs and response rates of antiretrovirals against HIV.13

Our finding contributes to emerging evidence suggesting that NNI resistance mutations can be maintained as the dominant sequence over the course of several years in untreated individuals, thereby potentially limiting the use of particular NNIs in a subset of individuals. Further longitudinal evaluation of resistance profiles in treatment-naive individuals may better define the demographic and virological predictors of STAT-C outcome.

Acknowledgements

This work was supported by an NIDA R21 (DA022148) award to J. T. B. and an NIDDK K24 (DK 070528) award to K. E. S. Data collection at Brown University was funded by the CDC cooperative agreement U64/CCU106795.

Transparency declarations

None to declare.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the CDC.

References


VII
Variability of the Polymerase Gene (NS5B) in Hepatitis C Virus-Infected Women

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There are limited data on diversity within the hepatitis C virus polymerase (NS5B). In concordance with its key functional role during the life cycle, NS5B intrapatient variability was low. Moreover, differences between NS5B nonsynonymous (dN) and synonymous (dS) mutation rates (dN − dS) were positively correlated with CD4 cell count, while nonsynonymous mutations were strongly correlated with reduced replication in vivo.

The NS5B protein of hepatitis C virus (HCV) is an RNA-dependent RNA polymerase that lacks a proofreading mechanism, resulting in a population of distinct but closely related viral variants, termed viral quasispecies, within an infected individual (16). The advent of potent NS5B inhibitors mandated a focus on the identification and monitoring of putative NS5B resistance mutations. HCV quasispecies diversity is an important predictor of liver disease progression as well as HCV treatment outcome (7, 13–15, 18). Most of these studies have focused on structural genomic regions; thus, limited data are available regarding nonstructural regions. To address this issue, we assessed serum NS5B variability in chronic HCV infection. (These data were presented at the 4th International Workshop on HIV and Hepatitis Co-Infection held in Madrid, Spain, in June 2008.)

From 1993 to 2000, a prospective natural history study of HIV infection, the HER Study, was conducted with U.S. women (17). Women were included in the current study if they were (i) HCV antibody positive, (ii) from the Rhode Island study site, and (iii) had available serum samples for analysis. All participants provided informed consent prior to sample and clinical data collection. RNA extractions and amplifications of a 384-bp region of NS5B were performed as described previously (4). Gel-purified PCR products were ligated into the pGEM-T Easy vector, and a median of 10 plasmids per sample was sequenced. We and others have shown that analysis of ~10 sequences accurately and reproducibly represents intrapatient quasispecies diversity (1, 20). All alignments were performed using Clustal X and compared to database references to determine the HCV genotype. Intrapatient genetic distances, Shannon entropy, and differences between nonsynonymous (dN) and synonymous (dS) mutation rates (dN − dS) were calculated as described elsewhere (1). Codons under positive or negative selection were detected via the fixed effects likelihood (FEL) and random effects likelihood (REL) methods as implemented in the Datamonkey program (12). P values of <0.05 were considered statistically significant.

A randomly selected subset of genotype 1-infected women that included 25 with HIV/HCV coinfection and 4 with HCV monoinfection were analyzed. Mean aspartate transaminase (AST) and alanine aminotransferase (ALT) levels were 58.5 U/ml and 51.9 U/ml, respectively. For the HIV/HCV-coinfected women, the median CD4 cell count was 358.7 cells/ml, and 23 (92%) had detectable plasma HIV viral loads. Of 22 HIV/HCV-coinfected women with available data, 13 (59%) were receiving antiretroviral therapy at the time of serum collection. Twenty-six women were infected with HCV genotype 1a, and 3 were infected with genotype 1b. Among the partial NS5B consensus sequences generated, 21 of 114 (18.4%) amino acid positions analyzed were variable (Fig. 1). The NS5B motifs A to D were conserved at nearly all amino acid positions, including the GDD active site. S282T and C316Y resistance mutations were not observed in any serum samples, although we have previously reported the persistence of the C316Y mutation in a treatment-naïve HCV-monoinfected woman from the same cohort (4). We further evaluated multiple measures of NS5B intrapatient variability. The median intrapatient genetic distance was 0.90% (range, 0.30% to 1.50%). These values are lower than we reported previously for hypervariable region 1 (HVR1) but higher than genetic distance measures for the S’-untranslated region (1). Median intrapatient entropy was 0.83 (range, 0.53 to 1.00). The median intrapatient dN − dS value for NS5B was −0.011 (range, −0.035 to 0.005), indicating negative or purifying immune selection pressures have preserved this functionally constrained genomic region. While it was not our intent to compare NS5B diversity in the presence/absence of HIV coinfection, we did observe a higher median genetic distance for the HIV/HCV-...
coinfected women than for the HCV-monoinfected women (0.8% versus 0.4%; \( P = 0.002 \)), even with the small population size. Site-specific codon selection was examined in each patient as summarized in Table 1. Using the FEL method, there were no positively selected amino acid sites in NS5B among the 29 women examined; however, there were 14 negatively selected amino acid sites identified in 9 women. Three of these amino acids—2730, 2747, and 2769 of the region analyzed—were identified in more than one woman. Using the REL method, 2 amino acids—2807 and 2818—were found to be under positive selection pressure.

![Sequence logo diagram](http://weblogo.berkeley.edu) showing amino acids 227 through 340 of the HCV NS5B protein from 26 genotype 1a consensus sequences. Numbers shown below the sequence represent the amino acid position in the complete HCV polyprotein. For polymorphic sites (i.e., those with more than one letter shown per position), the height of each letter represents the relative proportion of each amino acid at that position.

**TABLE 1.** Codons under positive or negative selection pressure among 29 HCV-seropositive women with genotype 1 based on the FEL and REL methods

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<th>Codon no. based on REL with #:</th>
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* Codons under positive or negative selection pressure among 29 HCV-seropositive women with genotype 1. Shown are codons with a significance of \( P \leq 0.05 \) (FEL method) or posterior probability of \( \geq 0.99 \) (REL method). Codons detected in more than one individual are underlined.
TABLE 2. Correlation of NS5B intrapatient diversity with clinical variables among 29 HCV-infected women

<table>
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<tr>
<th>Comparison</th>
<th>Spearman correlation coefficient</th>
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<tr>
<td>NS5B genetic distance vs HCV RNA</td>
<td>0.286</td>
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<tr>
<td>NS5B entropy vs HCV RNA</td>
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<td>NS</td>
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<tr>
<td>NS5B (dN - dS) vs HCV RNA</td>
<td>-0.492</td>
<td>0.007</td>
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<tr>
<td>NS5B genetic distance vs CD4 cell count</td>
<td>-0.033</td>
<td>NS</td>
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<tr>
<td>NS5B entropy vs CD4 cell count</td>
<td>0.186</td>
<td>NS</td>
</tr>
<tr>
<td>NS5B (dN - dS) vs CD4 cell count</td>
<td>0.387</td>
<td>0.056</td>
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<tr>
<td>NS5B genetic distance vs ALT</td>
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<tr>
<td>NS5B entropy vs ALT</td>
<td>-0.002</td>
<td>NS</td>
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<tr>
<td>NS5B (dN - dS) vs ALT</td>
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<tr>
<td>NS5B genetic distance vs AST</td>
<td>-0.178</td>
<td>NS</td>
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<tr>
<td>NS5B entropy vs AST</td>
<td>-0.144</td>
<td>NS</td>
</tr>
<tr>
<td>NS5B (dN - dS) vs AST</td>
<td>0.165</td>
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<tr>
<td>NS5B genetic distance vs HIV viral load</td>
<td>0.122</td>
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<tr>
<td>NS5B entropy vs HIV viral load</td>
<td>-0.079</td>
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<tr>
<td>NS5B (dN - dS) vs HIV viral load</td>
<td>-0.142</td>
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</table>

* P values of <0.10 are shown. NS, not significant.

Few data are available for nonstructural genomic regions, despite their potential as therapeutic targets to reduce viral replication. Our data support the concept that viral load is correlated with HCV RNA levels, suggesting that nonsynonymous mutations within NS5B could impact viral replication. In contrast, NS5B variability was not associated with HIV viral load, although the median NS5B genetic distance was higher for HIV/HCV-coinfected women than HCV-monoinfected women. This finding is particularly interesting given that HCV RNA levels are significantly elevated during HIV/HCV co-infection (19, 21) and HIV co-infection is associated with lower HCV treatment response (2, 11). Thus, HIV may influence NS5B variability, although this intriguing possibility requires a longitudinal analysis of NS5B diversity.

Our study has several potential limitations, including the modest population size, analysis of partial NS5B sequences, and the inclusion of relatively few HCV-monoinfected controls. The analysis included only HCV genotype 1, and the relevance of our findings to non-1 genotypes is unknown. While our data may be influenced by PCR-generated mutations, the various measures of quasispecies diversity reported here suggest that NS5B diversity was in excess of the Tag polymerase error rate (3). Future studies aimed at functional characterization of unique NS5B sequences identified in vivo will also provide data on how NS5B variability impacts replicative fitness. In summary, our data demonstrated a link between NS5B viral diversity and viral load that could impact targeted drug therapy. Longitudinal analysis of the cohort with relation to disease and treatment outcome is warranted.

Nucleotide sequence accession numbers. Consensus NS5B sequences have been submitted to GenBank under accession numbers GU131368 to GU131396.

We thank the HER Study staff and participants. The HER Study group consists of Robert S. Klein, Ellie Schoenhau, Julia Arsten, Robert D. Burk, Penelope Demas, and Andrea Howard from Montefiore Medical Center and the Albert Einstein College of Medicine; Paula Schuman, Jack Sobel, Suzanne Omhit, William Brown, Michael Long, Wayne Lancaster, and Jose Vazquez from the Wayne State University School of Medicine; Anne Rompalo, David Vlahov, and David Celentano from the Johns Hopkins University School of Medicine; Charles Carpenter, Kenneth Mayer, Susan Cu-Uvin, Timothy Flanagan, Joseph Hogan, Valerie Stone, Karen Tashima, and Josiah Rich from the Brown University School of Medicine; Ann Duerr, Lytt I. Gardner, Chad Heilig, Scott D. Holmberg, Denise J. Jamieson, Janet S. Moore, Ruby M. Phelps, Dawn K. Smith, and Dora Warren from the Centers for Disease Control and Prevention; and Katherine Davenny from the National Institute on Drug Abuse.

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REFERENCES


VIII
HIV Variability in the Liver and Evidence of Possible Compartmentalization

Jason T. Blackard, Gang Ma, Christina M. Martin, Susan D. Rouster, M. Tarek Shata, and Kenneth E. Sherman

Abstract

There is growing evidence to suggest that HIV may interact with several hepatic cell types; however, evaluation of HIV variability in liver tissue has not been addressed to date. Among 16 HIV-positive individuals examined, nine (56%) had detectable HIV RNA in the liver. The mean CD4 cell count for these nine individuals was 337 cells/mm³ (range: 0–601), while their mean plasma HIV RNA level was 106,974 copies/ml (range: 1200–320,740). Among individuals in this study with detectable HIV in both the plasma and the liver, the consensus gag nucleotide sequences for each tissue type were different for seven of seven (100%) individuals, while amino acid sequences were distinct for five of seven (71%). Consensus envelope (env) nucleotide and amino acid sequences were also distinct in the plasma and liver tissue for six of six (100%) individuals. Statistical evidence of compartmentalization between HIV in the plasma and in the liver was demonstrated, and multiple liver-specific amino acids were identified that may distinguish HIV variants replicating within the liver. These preliminary data demonstrate that HIV is frequently detectable in the liver of HIV-positive persons at various levels of immunosuppression. Possible compartmentalization may reflect tissue-specific selection pressures that drive viral adaptation to the liver microenvironment and may facilitate interactions with other hepatotropic viruses.

Introduction

Hepatic disease is increasingly recognized as a major cause of morbidity and mortality among HIV-positive individuals. Furthermore, HIV coinfection is associated with enhanced hepatitis C virus (HCV) replication, more advanced liver fibrosis and cirrhosis, higher rates of progressive liver disease and death, and decreased HCV treatment response. While liver biopsies represent the gold standard for detecting liver damage, their utilization in individuals with HIV remains infrequent despite high frequencies of both hepatomegaly and liver enzyme abnormalities. Even HIV-positive individuals with no evidence of viral hepatitis coinfection often exhibit mild-to-moderate increases in liver enzyme levels. A recent study also found that HIV RNA levels were positively associated with liver fibrosis in HIV monoinfected persons even after controlling for other confounders, thus supporting the involvement of HIV itself in hepatic disease.

Several lines of evidence suggest that HIV is present in the liver. For instance, HIV RNA and proviral DNA have been detected in liver biopsies from persons with HIV infection. Immunohistochemistry and in situ hybridization studies using liver specimens from HIV-infected individuals have also demonstrated HIV p24 protein and HIV RNA in Kupffer cells, inflammatory mononuclear cells, sinusoidal cells, and hepatocytes. Efficient activation of the HIV long terminal repeat has also been reported in hepatocytes. Importantly, we and others have demonstrated that HIV can infect hepatocyte-derived cell lines, as well as primary hepatocytes, although likely at lower levels than occurs during infection of lymphocytes. Collectively, these data would indicate that several distinct cell types within the liver might be permissive to HIV infection.

A hallmark of RNA viruses is their extreme variability. Within an individual, a population of viral variants termed the viral quasispecies exists. These variants may allow for the rapid, adaptive response of HIV to immunologic selection pressures and/or antiviral therapy. Several studies have demonstrated an association between quasispecies diversity and HIV disease progression. Importantly, HIV variability is not evenly distributed throughout the body, and distinct viral subpopulations may exist in different compartments within an infected individual. For example, the blood and male genital tract may represent distinct HIV compartments as viral diversity and/or the majority sequences are often discordant in the blood compared to the genital tract.
Similarly, HIV compartmentalization may occur in the brain and cerebrospinal fluid, suggesting that viral adaptation is frequently necessary for efficient infection of and replication within a particular cell/tissue type. Currently, there are no published reports on HIV diversity within the liver despite the link between HIV and liver disease. Thus, it is not clear if all variants of HIV present within an individual are equally capable of infecting the liver or if selection of particular HIV variants with tropism for the liver is occurring. Therefore, we investigated the presence of HIV RNA in liver biopsies and addressed whether HIV variability in the liver differed from that in the plasma.

Materials and Methods

Study participants

For this pilot study, a convenience sampling of 12 HIV-infected individuals was randomly selected from those receiving routine clinical care at the University of Cincinnati College of Medicine or those being evaluated for the initiation of antiretroviral therapy (ART). All subjects signed informed consents permitting collection of tissue and blood. Liver tissue and plasma collected at the time of autopsy were available for an additional four individuals through the National Disease Research Interchange.

Reverse transcriptase polymerase chain reaction (RT-PCR) amplification of HIV

Viral RNA was extracted from 140 μl of patient plasma using the QiaAmp Viral RNA kit or from homogenized liver biopsies (typically 1–2 mm in length) using the RNasy Mini kit. HIV RNA was detected by nested RT-PCR for HIV gag and env as described previously. Briefly, to amplify a 485-nucleotide fragment of gag (nucleotides 1237–1721 of the HIV reference HXB2), first round primers were 5′-TGC TGC TCC CAA GAA CCC AAG-3′ and second round primers were 5′-AGY CAA TGG GT-3′. All RT-PCR amplifications included one reaction containing no reverse transcriptase and a separate reaction containing no reverse transcriptase polymerase chain reaction (RT-PCR) amplification of HIV

Compartmentalization analyses

Compartmentalization of viral variants was assessed using Mantel’s test as previously described to explore HIV compartmentalization. Briefly, the Kimura two-parameter (S-M) test for population gene flow as implemented in the BEAST v1.5.0 program under an uncorrelated log-normal relaxed molecular clock using the generalized time reversible (GTR) or Hasegawa, Kishino, and Yano (HKY) model with nucleotide site heterogeneity estimated using a gamma distribution. The MCMC analysis was run for a chain length of 50,000,000 with sampling every 5000th generation. Results were visualized in Tracer v1.4 to confirm chain convergence, and the effective sample size (ESS) was calculated for each parameter. All ESS values were >500 indicating sufficient sampling. The maximum clade credibility tree was selected from the posterior tree distribution after a 10% burn-in using TreeAnnotator v1.5.0. Consensus sequences have been submitted to GenBank under accession numbers HM365303–HM365331.

Intrapatient genetic distances were calculated by pairwise comparison of nucleotide sequences at each time point using the Kimura method of MegAlign (DNASTAR, Inc., Madison, WI). Shannon entropy [\(S_e = -\sum (p_i \ln p_i)/\ln N\)], where \(p_i\) is the frequency of each distinct nucleotide sequence and \(N\) is the total number of sequences analyzed, was also calculated. Entropy values vary from 0 (all sequences are identical) to 1 (all sequences are distinct). Nonsynonymous (dN) and synonymous (dS) mutations were calculated via the Nei–Gojobori method in MEGA. Amino acid translations and manual editing to preserve the open reading frame were performed in MacClade version 4.08. The Viral Epidemiology Signature Pattern Analysis (VESPA) program was used to determine the frequency of each amino acid in liver-derived versus serum-derived viral variants for each individual. Only amino acid signatures above a 70% threshold were considered significant.

Envelope coreceptor utilization was determined using the VESPA program at http://genomics.ces.udel.edu/8080/vespa/v3.html.
HIV VARIABILITY IN THE LIVER

A convenience sample of 16 HIV-positive individuals was utilized for this pilot study (Table 1). The average CD4 cell count was 401 cells/mm³ (range: 0–764 cells/mm³). Plasma HIV viral loads were detectable in 12 of 13 individuals (mean: 68,131 copies/ml; range: 463–320,740 copies/ml) with available data. ART utilization was not an exclusion criteria; however, only two individuals were receiving ART at the time of sample collection. Thirteen of 16 individuals were HCV seropositive, while two individuals were hepatitis B virus (HBV) surface antigen positive. The median ALT and AST values were 58 U/liter and 77 U/liter, respectively.

Using RT-PCR, nine individuals (56.3%) had detectable HIV RNA in the liver. This included eight individuals with HIV gag detected in their livers, and six individuals with HIV env detected in their livers. To further confirm the quality of the extracted RNA, RT-PCR for GAPDH was attempted for 10 individuals, while measures of diversity were compared using the Wilcoxon rank sum test (Statistix 9.0; Analytical Software, Tallahassee, FL).

Results

Patient demographics and HIV detection

A convenience sample of 16 HIV-positive individuals was utilized for this pilot study (Table 1). The average CD4 cell count was 401 cells/mm³ (range: 0–764 cells/mm³). Plasma HIV viral loads were detectable in 12 of 13 individuals (mean: 68,131 copies/ml; range: 463–320,740 copies/ml) with available data. ART utilization was not an exclusion criteria; however, only two individuals were receiving ART at the time of sample collection. Thirteen of 16 individuals were HCV seropositive, while two individuals were hepatitis B virus (HBV) surface antigen positive. The median ALT and AST values were 58 U/liter and 77 U/liter, respectively.

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Intrapatient HIV variability

We performed a phylogenetic analysis of HIV gag and/or env sequences for the nine individuals with detectable HIV RNA in the liver. All were infected with HIV-1 subtype B, and sequences clustered by individual suggesting that there were no epidemiologically linked infections. The consensus gag nucleotide sequences were different for seven of seven (100%) individuals with matched liver biopsies and plasma using the NJ approach (data not shown). Similar analyses and results were obtained using a Bayesian inference approach based on a GTR or HKY substitution model (Fig. 1A). Consensus gag amino acid sequences were distinct for five of seven (71.4%) individuals, while two individuals had synonymous nucleotide changes not affecting the resultant amino acid sequence. Similarly, the consensus env nucleotide sequences were different for six of six (100%) individuals with matched liver biopsies and plasma using both NJ (data not shown) and Bayesian approaches (Fig. 1B). The consensus env amino acid sequences were distinct for all six individuals.

To more accurately represent intrapatient diversity, individual viral variants derived from plasma and liver biopsies were compared. In individuals with available gag sequence

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>HIV viral load (copies/ml)</th>
<th>CD4 cell count (cells/mm³)</th>
<th>HIV treatment at time of biopsy</th>
<th>Year of HIV diagnosis</th>
<th>ALT (U/liter)</th>
<th>AST (U/liter)</th>
<th>HCV serostatus</th>
<th>HBV surface antigen</th>
<th>Risk factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cin 01</td>
<td>56,335</td>
<td>408</td>
<td>No</td>
<td>1995</td>
<td>91</td>
<td>91</td>
<td>Positive</td>
<td>Negative</td>
<td>Sexual</td>
</tr>
<tr>
<td>Cin 02</td>
<td>218,106</td>
<td>423</td>
<td>No</td>
<td>1989</td>
<td>39</td>
<td>62</td>
<td>Positive</td>
<td>Negative</td>
<td>Sexual</td>
</tr>
<tr>
<td>Cin 03</td>
<td>320,740</td>
<td>338</td>
<td>No</td>
<td>2005</td>
<td>53</td>
<td>77</td>
<td>Positive</td>
<td>Negative</td>
<td>Sexual</td>
</tr>
<tr>
<td>Cin 04</td>
<td>98,919</td>
<td>265</td>
<td>No</td>
<td>2006</td>
<td>177</td>
<td>212</td>
<td>Positive</td>
<td>Negative</td>
<td>Sexual</td>
</tr>
<tr>
<td>Cin 05</td>
<td>29,638</td>
<td>346</td>
<td>No</td>
<td>1987</td>
<td>33</td>
<td>185</td>
<td>Positive</td>
<td>Negative; drug use</td>
<td>Sexual; drug use</td>
</tr>
<tr>
<td>1347</td>
<td>17,813</td>
<td>569</td>
<td>No</td>
<td>1988</td>
<td>NA</td>
<td>NA</td>
<td>Positive; Transfusion</td>
<td>Negative; Drug use</td>
<td>HCV; Drug use; Drug use</td>
</tr>
<tr>
<td>1370</td>
<td>5,481</td>
<td>764</td>
<td>No</td>
<td>1997</td>
<td>NA</td>
<td>NA</td>
<td>Positive; Negative; Sexual</td>
<td>Negative; Drug use</td>
<td>HCV; Drug use; Drug use</td>
</tr>
<tr>
<td>1493</td>
<td>6,126</td>
<td>397</td>
<td>No</td>
<td>1990</td>
<td>59</td>
<td>42</td>
<td>Positive; Negative; Sexual</td>
<td>Negative; Drug use</td>
<td>HCV; Drug use; Drug use</td>
</tr>
<tr>
<td>1580</td>
<td>1,200</td>
<td>410</td>
<td>No</td>
<td>2001</td>
<td>NA</td>
<td>NA</td>
<td>Positive; Negative; Sexual</td>
<td>Drug use</td>
<td>Drug use; Drug use</td>
</tr>
<tr>
<td>1627</td>
<td>463</td>
<td>547</td>
<td>NA</td>
<td>1990</td>
<td>58</td>
<td>47</td>
<td>Positive; Drug use</td>
<td>Drug use</td>
<td>Drug use; Drug use</td>
</tr>
<tr>
<td>1724</td>
<td>&lt;48</td>
<td>304</td>
<td>Yes</td>
<td>1999</td>
<td>56</td>
<td>54</td>
<td>Positive; Drug use</td>
<td>Drug use</td>
<td>Drug use; Drug use</td>
</tr>
<tr>
<td>1756</td>
<td>66,570</td>
<td>601</td>
<td>No</td>
<td>1986</td>
<td>154</td>
<td>108</td>
<td>Negative; Sexual; Drug use</td>
<td>Drug use</td>
<td>Drug use; Drug use</td>
</tr>
<tr>
<td>HV52²</td>
<td>64,485</td>
<td>0</td>
<td>No</td>
<td>1992</td>
<td>NA</td>
<td>NA</td>
<td>Negative; Drug use</td>
<td>Drug use</td>
<td>Drug use; Drug use</td>
</tr>
<tr>
<td>HV67²</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>2005</td>
<td>NA</td>
<td>NA</td>
<td>Negative; Drug use</td>
<td>Drug use</td>
<td>Drug use; Drug use</td>
</tr>
<tr>
<td>HV104²</td>
<td>NA</td>
<td>NA</td>
<td>Yes</td>
<td>1988</td>
<td>NA</td>
<td>NA</td>
<td>Positive; Negative; Sexual</td>
<td>Drug use</td>
<td>Drug use; Drug use</td>
</tr>
<tr>
<td>HV106²</td>
<td>NA</td>
<td>238</td>
<td>No</td>
<td>2000</td>
<td>NA</td>
<td>NA</td>
<td>Positive; Drug use</td>
<td>Drug use</td>
<td>Drug use; Drug use</td>
</tr>
</tbody>
</table>

aNormal values for healthy individuals are defined as <19 U/liter for women and <30 U/liter for men.

bDenotes samples collected at autopsy.

PIDs in bold had detectable HIV gag and/or env sequences in liver biopsy. NA, not available; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; HBV, hepatitis B virus.
data, the median intrapatient genetic distance was 1.36% in the plasma (range: 0.80–3.12%) and 1.09% in the liver biopsy tissue (range: 0.00–2.77%) as shown in Supplementary Fig. S1A (Supplementary Data are available online at www.liebertonline.com/aid) [p = not significant (NS)]. Median gag entropy, which represents both the number of distinct variants as well as their frequencies, was 1.00 in the plasma (range: 0.84–1.00) and 0.83 in the liver biopsy tissue (range: 0.00–1.00) as shown in Supplementary Fig. S1C (p = 0.021). As an indicator of positive selection, dN-dS values were calculated for gag but were not greater than 0 for any plasma or liver biopsy tissue analyzed. Median dN-dS values were −0.045 (range: −0.082 to −0.026) and −0.30 (−0.053–0.00) in the plasma and liver biopsy tissue, respectively (Supplementary Fig. S1E; p = NS).

In individuals with available env sequence data, the median intrapatient genetic distance was 2.17% in the plasma (range: 0.00–6.47%) and 0.16% in the liver biopsy tissue (range: 0.00–1.85%) (Supplementary Fig. S1B; p = 0.059). Median env entropy was 0.77 in the plasma (range: 0.18–1.00) and 0.36 in the

FIG. 1. Consensus nucleotide sequences in gag (A) and env (B) from plasma and liver biopsy tissue using a Bayesian inference approach. Relevant posterior probabilities greater than 90% are shown. Relevant bootstrap values >700 out of 1000 from a neighbor-joining approach are shown in parentheses. The reference sequences HXB2 (accession number K03455) and NL4-3 (M19921) are also included.
HIV VARIABILITY IN THE LIVER

liver biopsy tissue (range: 0.00–0.84) (Supplementary Fig. S1D; \( p = \text{NS} \)). Values for \( dN/dS \) were greater than 0 for one plasma sample (1580) and three liver biopsies (Cin05, 1580, and 1756). Median \( dN/dS \) values were \(-0.0032\) (range: \(-0.0250–0.0234\)) and \(0.0003\) \((-0.0205–0.0147\)) in plasma and liver biopsy samples, respectively (Supplementary Fig. S1F; \( p = \text{NS} \)).

Coreceptor utilization

Envelope coreceptor utilization was assessed with pre-trained classifier algorithms using consensus and clonal envelope sequences. For viruses from individuals 1756, HV52, Cin01, Cin02, Cin03, Cin04, and Cin05, the consensus envelope sequences from plasma and liver were predicted to utilize the CCR5 coreceptor. However, for subject 1580, the envelope sequences from plasma and liver were predicted to utilize the CXCR4 coreceptor. When clonal sequences were analyzed, the majority (64–100%) of viruses from the plasma and liver were predicted to utilize CCR5 for subjects 1756, Cin01, Cin03, Cin04, and Cin05. In contrast, two algorithms (C4.5 and C4.5–only p8 and p12) predicted CCR5 in all clones for subject 1580 regardless of the cell/tissue type, while three algorithms (PART, SVM, and the Charge Rule) predicted that only 0–22% of plasma-derived clones and 11–44% of liver-derived clones utilized CCR5. These findings are further supported by in vitro studies suggesting that liver cell infection may utilize CCR5 or CXCR4.\(^{16,17,19}\)

Analysis of compartmentalization

Phylogenetic trees were also reconstructed for each of the individuals with both plasma and liver biopsy variants (Fig. 2). In the analysis of intrapatient gag variability, three

![Phylogenetic trees](image)

FIG. 2. Patient-specific phylogenetic trees with gag viral variants from plasma (open circles) and liver biopsy tissue (closed circles). Shown in the upper right corner is a bar depicting the percent genetic distance for each tree. Only relevant bootstraps greater than 700 out of 1000 are shown. HXB2 (accession number K03455) and NL4-3 (M19921) are included as references.
distinct patterns were observed. Pattern 1 included one individual (HV52) with plasma- and liver-derived variants that were intermingled with no statistically significant clustering of these variants by sample source. Pattern 2 included four individuals (Cin01, Cin03, Cin04, 1756) with plasma- and liver-derived variants that were intermingled but with at least one statistically significant clustering of variants by sample source. Pattern 3 included two individuals (Cin05 and HV106) with complete, or near complete, separation of plasma- and liver-derived variants into distinct groupings that were supported by high bootstrap values.

In the analysis of intrapatient env variability, only patterns 2 and 3 were observed (Fig. 3). For example, in individuals Cin03 and 1580, some intermingling of plasma- and liver-derived variants was observed, although there were also smaller groupings of variants that clustered by sample source. In contrast, for individuals Cin01, Cin04, Cin05, and 1756, viral variants from the liver clearly clustered separately from viral variants from the plasma and were supported by high bootstrap values. For 1756, env sequences from peripheral blood mononuclear cells (PBMCs) were also available for analysis and demonstrated clear clustering of PBMC- and plasma-derived variants that was distinct from the cluster of liver-derived variants. A replicate RT-PCR of env using a second RNA extraction from the same liver biopsy, plasma, and PBMCs samples of subject 1756 also demonstrated evidence of significant compartmentalization between the liver and plasma/PBMCs (data not shown).

**FIG. 3.** Patient-specific phylogenetic trees with env viral variants from plasma (open circles) and liver biopsy tissue (closed circles). For subject 1756, viral variants from peripheral blood mononuclear cells (PBMCs) (closed triangles) were also included. Shown in the upper left corner is a bar depicting the percent genetic distance for each tree. Only relevant bootstrap values greater than 700 out of 1000 are shown. HXB2 (accession number K03455) and NL4-3 (M19921) are included as references.
HIV VARIABILITY IN THE LIVER

We further explored the potential compartmentalization of HIV in the liver using Mantel’s test as shown in Table 2. When comparing matched plasma- and liver biopsy-derived viral variants in the gag region of HIV, the results of Mantel’s test were consistent with significant quasispecies compartmentalization for Cin05 (p = 0.0009) and HV106 (p = 0.0027), while Cin04 (p = 0.066) and HV52 (p = 0.091) showed a trend toward compartmentalization. No significant compartmentalization in gag was observed for 1756, Cin01, or Cin03. S-M test results demonstrated statistically significant evidence of gag compartmentalization for Cin04, Cin05, HV52, and HV106.

Evidence of significant compartmentalization in env was observed for 1580 (p = 0.0043), 1756 (p = 0.0001), Cin01 (p = 0.0001), Cin04 (p = 0.0005), and Cin05 (p = 0.0002) using Mantel’s test, while Cin03 (p = 0.060) also showed a trend toward compartmentalization in env. S-M test results similarly demonstrated statistically significant evidence of env compartmentalization for 1580, 1756, Cin01, Cin02, Cin03, Cin04, and Cin05. Thus, all nine individuals with HIV detectable in the liver demonstrated evidence of gag and/or env compartmentalization between the plasma and liver biopsy with at least one statistical test of compartmentalization.

**Signature sequence analysis of liver-specific amino acids**

To identify specific amino acids associated with HIV in the liver, signature sequence analysis was performed using gag and/or env sequences for matched plasma-biopsy samples. A significant difference in amino acid frequency was identified in a single individual when analyzing gag variants in the liver biopsy compared to those in the corresponding plasma samples. For HV52, a 12 amino acid insertion was present in the liver biopsy that was present only at very low frequency among variants from the corresponding plasma. For the other five individuals with matched sequence data, no other liver-specific signature amino acids were identified in the region of gag analyzed.

In contrast, five of six individuals (Cin01, Cin04, Cin05, 1580, and 1756) had evidence of distinct amino acid frequencies when analyzing env data. For these five individuals, a total of 52 liver-specific signature amino acids (mean 10.4; range: 1–18) was identified (Fig. 4). Of these signature amino acids 14 (26.9%) were located within the V3 loop. Four amino acid signatures were shared by at least two individuals.

**Discussion**

To date, only one published study has examined HIV variability in the liver. Van’t Wout et al. assessed HIV proviral DNA variability from a single individual who died of AIDS-related complications. They concluded that the presence of HIV in nonlymphoid tissues was likely the result of the late disease stage of the samples examined, although samples collected from earlier disease stages were not included. Similarly, Donaldson et al. reported that infection of nonlymphoid organs, such as the liver, occurred only in individuals with AIDS-defining illnesses and not among asymptomatic individuals, however, HIV variability was not explored. In contrast, in the current study, HIV RNA was detected in nine individuals with a wide range of CD4 cell counts (0 to 601 cells/mm³) suggesting that HIV infection of the liver may occur at varying levels of immunosuppression.

Importantly, several lines of evidence suggest that the liver may represent a potential site of HIV compartmentalization. First, the presence of distinct consensus sequences in the liver compared to the corresponding plasma/PBMCs argues against simple contamination of biopsy tissues with peripheral lymphocytes and/or cell-free virions from the peripheral blood supply. Second, patient-specific clonal analysis frequently demonstrated distinct HIV variants in the liver compared to the corresponding plasma. These findings were further supported statistically by the results of both Mantel’s and Slatkin–Maddison tests. Third, signature sequence analysis identified 52 amino acid residues in the region of env sequenced that were associated with HIV detection in the liver. These amino acids imply adaptation of HIV for infection of the liver and may impact HIV replication and/or cell tropism.

Despite frequent observation of liver enzyme abnormalities in those with HIV infection, HIV treatment providers rarely include liver biopsy in the evaluation process of these patients. Liver biopsy is more commonly employed in patients with HBV and/or HCV infection; therefore, few investigators have had access to samples that would permit assessment of the direct effects of HIV on the liver in vivo. While our methodology does not provide any information regarding the specific cell type(s) that may be infected by HIV or the overall level of HIV replication in the liver, there is considerable evidence suggesting that several liver cell types can support HIV replication as reviewed elsewhere. Thus, it is reasonable to assume that there is at least one cell type in the liver that is capable of supporting HIV replication. Although we cannot

**Table 2. p-Values from Mantel’s Test and Slatkin–Maddison Test for HIV Compartmentalization in the Liver Compared to the Plasma**

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Gag Mantel’s test</th>
<th>Gag Slatkin–Maddison test</th>
<th>Env Mantel’s test</th>
<th>Env Slatkin–Maddison test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cin01</td>
<td>NS*</td>
<td>NS</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cin03</td>
<td>NS</td>
<td>NS</td>
<td>0.060</td>
<td>0.049</td>
</tr>
<tr>
<td>Cin04</td>
<td>0.066</td>
<td>0.0109</td>
<td>0.0005</td>
<td>0.0001</td>
</tr>
<tr>
<td>Cin05</td>
<td>0.0009</td>
<td>0.0103</td>
<td>0.0002</td>
<td>0.0001</td>
</tr>
<tr>
<td>1580</td>
<td>Not done</td>
<td>Not done</td>
<td>0.0043</td>
<td>0.0104</td>
</tr>
<tr>
<td>1756</td>
<td>NS</td>
<td>NS</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HV52</td>
<td>0.091</td>
<td>0.0023</td>
<td>Not done</td>
<td>Not done</td>
</tr>
<tr>
<td>HV106</td>
<td>0.0027</td>
<td>0.0003</td>
<td>Not done</td>
<td>Not done</td>
</tr>
</tbody>
</table>

*NS, nonsignificant (p > 0.10).
definitely rule out contamination by PBMC-derived HIV, this is unlikely for two reasons. First, several studies have shown little or no compartmentalization, or similar mutational patterns, in PBMCs compared to plasma/serum. Secondly, when PBMCs were included from subject 1756, PBMC- and plasma-derived viruses grouped together but were separate from liver-derived sequences. Several limitations of the current study warrant further discussion. While the population size is modest, these data represent the largest study of HIV variability in the liver ever performed. Additionally, while several distinct methods to detect viral compartmentalization are available, there is no one gold standard or preferred approach. For example, Zarate et al. compared multiple methods for detecting HIV compartmentalization and found that discordant predictions by distinct methods may occur; therefore, utilizing several complementary methods, as performed here, provides the most reliable assessment of viral compartmentalization. Furthermore, the cross-sectional nature of this analysis does not permit a detailed examination of liver-specific HIV variants over time or provide important data on the possible trafficking of liver-specific HIVs into the peripheral circulation. Similarly, it is possible that our cloning strategy may not have amplified all minor variants present in a given tissue/cell type.

FIG. 4. Signature sequence analysis showing amino acid positions at which the distributions of liver- and plasma-specific envelope variants are significantly different. Numbers in parentheses indicate the number of amino acid signatures present in the liver. Shown are the consensus amino acids at each position. In several instances, a given amino acid may appear to be identical between the two compartments; however, the frequency distribution of the viral variants that make up that consensus is different between the two compartments. Asterisks denote amino acid signatures that were shared by at least two individuals. All sequences are shown relative to their position within X82 with the single underlined amino acid residues denoting the V3 loop (amino acids 296–330) and the V3 tip (amino acids 312–315) double-underlined.

Our study is the first to explore HIV RNA variability in the liver and to demonstrate distinct HIV variants in liver biopsy tissues. However, it is important to note that HIV was not amplifiable in all samples examined, although we did not directly quantify intrahepatic levels of HIV in the current study. Thus, identification of the virologic, immunologic, and genetic factors that impact HIV detection in the liver will require additional study. Moreover, exploring the variability of additional HIV genomic regions amplified from the liver, as well as detecting low-frequency viral variants by single-genome amplification, is warranted and may enhance our understanding of HIV pathogenesis. Finally, additional studies are currently underway to determine the relative contributions of distinct liver cell types to HIV pathogenesis and viral diversity, as well as the impact of liver-derived HIV on liver damage and fibrosis progression.
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Author Disclosure Statement

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References


Supplementary Data

SUPPLEMENTARY FIG. S1. Intrapatient genetic distance (A, B), entropy (C, D), and dN-dS (E, F) values for gag (left panels) and env (right panels) nucleotide sequences in liver biopsy tissue (closed circles) and plasma (open circles). NS, not significant ($p > 0.10$).