The Human Liver-uPA-SCID Mouse

A Small Animal Model for the Study of Viral Hepatitis

Philip Meuleman

Thesis submitted to fulfill the requirements for the degree of
DOCTOR IN MEDICAL SCIENCES
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by Philip Meuleman

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# Table of contents.

Abbreviations .......................... 1

<table>
<thead>
<tr>
<th>Summary</th>
<th>Samenvatting</th>
<th>Résumé</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>7</td>
<td>9</td>
</tr>
</tbody>
</table>

**Chapter 1:** Introduction. .......................... 11
- Hepatitis B
- Hepatitis C
- Animal models for the study of HBV and HCV
- Research objectives

**Chapter 2:** A simple and rapid method to determine the zygosity of uPA-transgenic SCID mice. .......................... 71
P. Meuleman, P. Vanlandschoot and G. Leroux-Roels
*Biochemical and Biophysical Research Communications 308 (2003) 375–378*

**Chapter 3:** Morphological and Biochemical Characterization of a Human Liver in a uPA-SCID Mouse Chimera. .......................... 77
Philip Meuleman, Louis Libbrecht, Rita De Vos, Bernard de Hemptinne, Kris Gevaert, Jöel Vandekerckhove, Tania Roskams and Geert Leroux-Roels
*Hepatology 2005;41:847-856*

**Chapter 4:** Immune suppression uncovers endogenous cytopathic effects of the hepatitis B virus. .......................... 91
Philip Meuleman, Louis Libbrecht, Stefan Wieland, Rita De Vos, Nagy Habib, Anna Kramvis, Tania Roskams, Geert Leroux-Roels
*Journal of Virology, in press*

**Chapter 5:** Human hepatocytes secrete soluble CD14, a process not directly influenced by HBV and HCV infection. .......................... 105
Philip Meuleman, Sophia Steyaert, Louis Libbrecht, Sibyl Couvent, Freya Van Houtte, Filip Clinckspoor, Bernard de Hemptinne, Tania Roskams, Peter Vanlandschoot, Geert Leroux-Roels
*Clinica Chimica Acta, in press*

**Chapter 6:** General discussion and future perspectives. .......................... 115
### Abbreviations.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA:</td>
<td>amino acid</td>
</tr>
<tr>
<td>AIDS:</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ALT:</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>BVDB:</td>
<td>bovine viral diarrhea virus</td>
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<tr>
<td>cccDNA:</td>
<td>covalently closed circular DNA</td>
</tr>
<tr>
<td>CEA:</td>
<td>carcinoembryonic antigen</td>
</tr>
<tr>
<td>CK:</td>
<td>cytokeratin</td>
</tr>
<tr>
<td>COFRADIC:</td>
<td>combined fractional diagonal chromatography</td>
</tr>
<tr>
<td>CTL:</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DC-SIGN:</td>
<td>dendritic cell-specific ICAM3 grabbing nonintegrin</td>
</tr>
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<td>DHBV:</td>
<td>duck hepatitis B virus</td>
</tr>
<tr>
<td>DNA:</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>ELISA:</td>
<td>enzyme linked immunosorbent assay</td>
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<tr>
<td>ER:</td>
<td>endoplasmatic reticulum</td>
</tr>
<tr>
<td>FCH:</td>
<td>fibrosing cholestatic hepatitis</td>
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<tr>
<td>FDA:</td>
<td>food and drug administration</td>
</tr>
<tr>
<td>H&amp;E:</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HBeAg:</td>
<td>hepatitis B e antigen</td>
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<tr>
<td>HBsAg:</td>
<td>hepatitis B surface antigen</td>
</tr>
<tr>
<td>HBV:</td>
<td>hepatitis B virus</td>
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<tr>
<td>HCC:</td>
<td>hepatocellular carcinoma</td>
</tr>
<tr>
<td>HCV:</td>
<td>hepatitis C virus</td>
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<tr>
<td>HDL:</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HIV:</td>
<td>human immunodeficiency virus</td>
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<tr>
<td>HLA:</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HPCs:</td>
<td>hepatic progenitor cells</td>
</tr>
<tr>
<td>HVR:</td>
<td>hypervariable region</td>
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<tr>
<td>IFN:</td>
<td>interferon</td>
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<tr>
<td>Ig:</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IRES:</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>IU:</td>
<td>international units</td>
</tr>
<tr>
<td>kb:</td>
<td>kilo base</td>
</tr>
<tr>
<td>kDa:</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LDL:</td>
<td>low-density lipoprotein</td>
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<tr>
<td>L-SIGN:</td>
<td>liver/lymph node specific ICAM3 grabbing nonintegrin</td>
</tr>
<tr>
<td>MHC:</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA:</td>
<td>messenger RNA</td>
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<tr>
<td>mm:</td>
<td>nanometer</td>
</tr>
<tr>
<td>NS:</td>
<td>non-structural</td>
</tr>
<tr>
<td>OAS:</td>
<td>oligoadenylate synthetase</td>
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<tr>
<td>ORF:</td>
<td>open reading frame</td>
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<tr>
<td>ORO:</td>
<td>oil red O</td>
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<tr>
<td>PAS:</td>
<td>periodic-acid-Schiff</td>
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<td>PCR:</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PEG:</td>
<td>polyethylene glycol</td>
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<tr>
<td>pgRNA:</td>
<td>pregenomic RNA</td>
</tr>
<tr>
<td>RdRp:</td>
<td>RNA dependent RNA polymerase</td>
</tr>
<tr>
<td>RNA:</td>
<td>ribonucleic acid</td>
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<tr>
<td>sCD14:</td>
<td>soluble CD14</td>
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<tr>
<td>SCID:</td>
<td>severe combined immune deficient</td>
</tr>
<tr>
<td>SPF:</td>
<td>specific pathogen free</td>
</tr>
<tr>
<td>SR-B1:</td>
<td>scavenger receptor B1</td>
</tr>
<tr>
<td>uPA:</td>
<td>urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>VLDL:</td>
<td>very low-density lipoprotein</td>
</tr>
<tr>
<td>WHV:</td>
<td>woodchuck hepatitis virus</td>
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Summary.

A small, non-primate animal model for the study of HBV and HCV infections would be of great benefit to the scientific community. These viral pathogens can only be studied in chimpanzees, which is a highly expensive experimental approach that raises a lot of ethical concerns.

By backcrossing uPA-transgenic mice with SCID mice, we have generated uPA-transgenic SCID mice. These mice suffer from a severe liver disease caused by the overexpression of uPA and a severe immune deficiency, derived from the SCID background. Hepatocytes of human origin are therefore not rejected by these mice. The severe and chronic liver disease allows the human hepatocytes to repopulate the mouse liver in their attempt to restore normal liver function. Because only mice homozygous for the uPA transgene turn out to be ideal acceptors for xenogeneic hepatocyte transplantation and because the uPA+/+-SCID mice must be transplanted within two weeks after birth to achieve optimal liver regeneration, we have designed a one day screening method that allows for the unambiguous discrimination between negative, heterozygous and homozygous uPA-SCID mice. This assay consists of a multiplex PCR that is based on the presence of the uPA transgene and the absence of a small part of the mouse genome that was deleted at the site where the uPA transgene cassette had integrated.

We transplanted homozygous uPA-SCID mice with primary human hepatocytes and analyzed the mouse plasma and liver architecture at selected time points. Immunohistochemical analysis of the liver demonstrated that the repopulation process occurred in a progressive and organized fashion, until up to 87% of liver parenchyma was repopulated by human hepatocytes. The human hepatocytes formed functional bile canaliculi that connected to the mouse biliary system. The functionality of the human hepatocytes was corroborated by a proteomic analysis of the mouse plasma that showed the presence of at least 21 proteins of human origin in addition to human albumin. The concentration of the latter protein in the mouse plasma was measured using an in-house ELISA and used as an in vivo marker of liver cell expansion and function.

A remarkable observation was that, besides mature human hepatocytes, human hepatic progenitor cells also contributed to the liver regeneration process.

HBV infections could be easily achieved in successfully transplanted animals. Interestingly, infection with HBV resulted in severe hepatocellular damage.
that ultimately led to the premature death of the infected animals. HBV is generally considered to be a non-cytopathic virus, but in our immune deficient animal model it replicated in an unrestrained fashion and became cytopathic for the infected cell. The pathology observed in HBV infected animals closely resembled that of chronic HBV patients that suffer from fibrosing cholestatic hepatitis. This frequently fatal syndrome occasionally occurs in HBV patients undergoing severe immune suppression, for example in the context of organ transplantation.

uPA mice harboring a human hepatocyte graft could also be infected with HCV. Although we have never been able to directly visualize the virus in the infected liver cells, infection and active replication of HCV in chimeric mice is beyond any doubt based on the convincing increase of viral load in the plasma of infected mice and the fact that minute amounts of infected mouse plasma sufficed to transmit infection to an uninfected chimeric recipient.

Chronic HBV and HCV patients have increased levels of soluble CD14 (sCD14) in their plasma. We wanted to investigate what the source of this sCD14 could be and if the increased levels are a direct result from the viral infection. Using our chimeric animal model we demonstrated that hepatocytes are a major source of sCD14. The secretion of this protein was not elevated in HBV or HCV infected mice, suggesting that these infections do not directly stimulate the sCD14 production by hepatocytes. We assume that in patients with chronic HBV or HCV infections other, indirect factors determine the plasma sCD14 levels.
Samenvatting.

De studie van het hepatitis B virus (HBV) en het hepatitis C virus (HCV) wordt momenteel bemoeilijk door het ontbreken van een klein en doeltreffend proefdiermodel. De chimpansee was tot op heden de enige diersoort die succesvol geïnfecteerd kon worden met HBV en HCV. Het gebruik van chimpansees is om ethische redenen echter zeer omstreden en bovendien zeer duur.

Daarom hebben we de uPA-SCID muis ontwikkeld. Deze muis is een kruising van een uPA (urokinase type plasminogeen activator) transgene muis met de SCID (severe combined immune deficient) muis. De uPA-transgene muis lijdt aan een chronische leverziekte als gevolg van lever-specifieke overexpressie van het uPA gen. Daardoor ontstaat er een chronische leverinsufficiëntie die kan gecorrigeerd worden door transplantatie met gezonde levercellen. De SCID mutatie zorgt ervoor dat de uPA-SCID muis bovendien een niet-functioneel adaptief immuun systeem heeft, waardoor levercellen van humane oorsprong getransplanteerd kunnen worden zonder risico op afstoting.

Enkel uPA-SCID muizen die homozygoot zijn voor het uPA-transgen kunnen met succes getransplanteerd worden met humane hepatocyten. Bovendien moeten deze dieren zo vlug mogelijk, liefst binnen twee weken na de geboorte, getransplanteerd worden. Het is daarom van cruciaal belang dat we kunnen beschikken over een vlugge en betrouwbare methode om het onderscheid te maken tussen uPA-negatieve, heterozygote en homozygote muisjes. We hebben hiervoor een multiplex PCR methode ontwikkeld die ons op één dag het antwoord brengt. Deze test is gebaseerd op de aanwezigheid van het uPA-gen en de afwezigheid van een fragment muis DNA dat gedeleteerd werd tijdens de integratie van het transgen-construct in het muisgenoom.

Op verschillende tijdstippen na de transplantatie met humane hepatocyten hebben we de lever en het plasma van homozygote uPA-SCID muizen onderzocht. Immunohistochemische analyse toonde aan dat de humane hepatocyten de zieke muislever op een georganiseerde en progressieve manier innemen. Tot 87% van het muislever-parenchym werd vervangen door gezonde hepatocyten van humane oorsprong. De humane levercellen vormden functionele galcanuliculi die verbindingen aangingen met galcanaliculi gevormd door muishepatocyten. Bovendien werden ook chimere canaliculi aangetroffen.

Analyse van het muisplasma toonde duidelijk aan dat de getransplanteerde humane hepatocyten goed functioneerden. Een proteoom-analyse leerde ons dat er, naast humaan albumine, nog 21 andere
humane eiwitten in het muizenbloed voorkwamen. De concentratie van humaan albumine in het muisplasma is bovendien een merker voor de hoeveelheid humane hepatocyten aanwezig in de muislever. Interessant was de vaststelling dat er, naast mature hepatocyten, ook humane leverstamcellen deelnamen aan het regeneratieproces.

Succesvol getransplanteerde uPA-SCID muizen konden we infecteren met HBV. Deze infectie veroorzaakte echter ernstige schade aan de menselijke levercellen die uiteindelijk leidde tot de vroegtijdige dood van de geïnfecteerde muizen. Dit is opmerkelijk aangezien HBV doorgaans wordt beschouwd als een niet-pathogene virus. Een belangrijk aspect is dat de geïnfecteerde muizen immuundeficiënt zijn waardoor de replicatie van het virus niet onderdrukt wordt. De pathologie die we observeerden in de HBV-geïnfecteerde muizen kan worden vergeleken met ‘fibroserende cholestatische hepatitis’. Dit syndroom kan optreden bij chronische HBV patiënten die een immuunonderdrukkende therapie krijgen, meestal in het kader van orgaantransplantaties. ‘Fibroserende cholestatische hepatitis’ staat bekend als een ernstige, dikwijls dodelijke aandoening.

Succesvol getransplanteerde muizen konden eveneens geïnfecteerd worden met HCV. Na injectie van het virus vonden we wekenlang hoge aantallen virusdeeltjes in het muisplasma terug. Immunohistochemische kleuring voor het virale E2 eiwit toonde duidelijk de geïnfecteerde humane hepatocyten aan. Met de elektronenmicroscoop konden we echter geen virale partikels detecteren. Injectie van een kleine hoeveelheid plasma van een geïnfecteerde muis in een nieuwe, chimere muis resulteerde opnieuw in een ‘chronische’ HCV infectie. De stijgende virale titers in de geïnfecteerde muizen en de overdraagbaarheid van de infectie naar nieuwe muizen zijn echter voldoende bewijzen voor een actieve virale infectie.

In het plasma van chronische HBV en HCV patiënten worden verhoogde concentraties sCD14 gedetecteerd. Wij hebben in onze uPA-SCID muis onderzocht wat de oorzaak kan zijn van deze verhoging en of deze een direct gevolg is van de virale infectie. Ons onderzoek toonde aan dat bij de mens de lever, meer bepaald de hepatocyten zelf, een belangrijke bron is van sCD14. HBV of HCV infectie had echter geen invloed op de hoeveelheid sCD14 die geproduceerd werd. Aangezien humane hepatocyten de enige bron van sCD14 zijn in ons muismodel moet de verhoging van sCD14 bij patiënten verklaard worden door een ander mechanisme. De activatie van monocyten/macrofagen is één mogelijkheid.
Résumé.

Un petit modèle animal, qui ne soit pas un primate, et qui permettrait l’étude des infections dues au virus de l’hépatite B (VHB) et C (VHC) serait d’un grand intérêt pour la communauté scientifique. Seul le chimpanzé permet l’étude de ces agents pathogènes, mais ce modèle expérimental est très coûteux et pose des problèmes d’éthique.

En croisant des souris transgéniques pour la molécule uPA avec des souris SCID, nous avons généré des souris SCID-uPA transgéniques. Ces souris souffrent d’une atteinte hépatique sévère due à la surexpression de l’uPA ainsi que d’une déficience immunitaire sévère due au fond SCID. Dans ces souris, des hépatocytes humains greffés ne sont donc pas rejetés. L’atteinte hépatique chronique sévère permet aux hépatocytes humains, dans leur effort de restaurer des fonctions hépatiques normales, de coloniser le foie de la souris. Seules les souris homozygotes pour le transgène uPA se sont avérées des réceptrices idéales de transplantation hépatique xénogénique. Par ailleurs, les souris uPA+/−-SCID doivent être transplantées dans un délai de 2 semaines après la naissance pour permettre une transplantation hépatique optimale. Ainsi, nous avons développé une méthode de criblage qui permette en 24 heures de discriminer sans ambiguïté entre des souris uPA-SCID négatives, hétérozygote et homozygotes. Cette méthode repose sur un test de PCR multiplex basé sur la présence du transgène uPA et l’absence d’une petite portion du génome murin qui a été délaité du site ou la cassette du transgène uPA a été intégré.

Nous avons transplanté des souris homozygotes uPA-SCID avec des hépatocytes humains primaires et analysé le plasma des souris et l’architecture du foie en des temps différents. Des analyses immuno-histochimiques du foie de telles souris démontrent que la repopulation a lieu de façon progressive et organisée, jusqu’à que 87% du parenchyme soit colonisé par des hépatocytes humains. Les hépatocytes humains forment des canicules biliaires fonctionnelles qui se connectent au système biliaire de la souris. La fonctionnalité des hépatocytes humains est corroborée avec une analyse protéomique du plasma murin qui a montré la présence d’au moins 21 protéines d’origine humaine en plus de l’albumine humaine. La concentration de cette dernière protéine dans le plasma murin a été mesurée avec un ELISA maison et utilisée comme marqueur in vivo de l’expansion et la fonction de cellules hépatiques.

Une observation remarquable a été que, en plus d’hépatocytes humains matures, des cellules progénitrices d’origine
hépatique d'origine humaine ont aussi contribué au processus de régénération du foie.

Des infections par le VHB ont pu être facilement obtenues dans les animaux transplantés. De façon intéressante, l'infection par le VHB a résulté en une atteinte hépatique sévère conduisant en final à la mort prématurée des animaux infectés. Le VHB est typiquement considéré comme un virus non cytopathique, mais dans notre modèle murin présentant une déficience immunitaire, il se réplique de façon non contrôlée et devient cytopathique pour les cellules infectées. La pathologie observée dans les animaux infectés par le VHB ressemble fortement à celle observée chez des patients chroniquement infectés qui souffrent de fibrose hépatique cholestatique. Ce syndrome fréquemment fatal est souvent trouvé chez des sujets porteurs du VHB soumis à une déficience immunitaire sévère, par exemple dans le contexte d’une transplantation d’organe.

Les souris uPA portant une greffe d'hépatocytes humains ont également pu être infectées par le VHC. Bien que nous n'ayons jamais réussi à visualiser le virus dans les cellules infectées, l'infection et la réplication active du virus dans les souris chimériques ne fait aucun doute notamment de part l'augmentation évidente du titre viral dans le plasma des souris infectées et le fait que des quantités infimes de plasma de souris infectées permettent le transfert de l'infection à de nouvelles souris.

Les patients chroniquement infectés par le VHB et le VHC ont des taux élevés de CD14 solubles dans leur plasma. Nous avons voulu rechercher quelle était la source de ce CD14 soluble et si l'augmentation des titres est une signature de la réplication vitale. En utilisant notre modèle chimérique nous avons montré que les hépatocytes sont une source majeure de CD14 soluble. La sécrétion de cette protéine n’est élevée ni dans les souris infectées par le VHB ni dans celles infectées par le VHC suggérant que ces infections ne stimulent pas directement la sécrétion du CD14 soluble par les hépatocytes. Ainsi, notre hypothèse est que dans les infections naturelles dues au VHB ou au VHC, d’autres facteurs indirects sont responsables des taux plasmatiques du CD14 soluble.
Chapter 1: Introduction.

The Hepatitis B Virus.

The Hepatitis B Virus (HBV) is the causative agent of a major global health problem. Worldwide, more than two billion people show evidence of past or current infection. An estimated 360 million people suffer from a chronic infection, of which about 25% will die due to liver cirrhosis and/or hepatocellular carcinoma. Each year, the hepatitis B virus is responsible for more than 1 million deaths (1).

Despite these high numbers, the risk of getting chronically infected with HBV is quite low. Only 5 to 10% of adults become persistent carriers of the virus after infection. However, the rate of chronicity after infection is significantly higher in infants and neonates, 30 - 50% and 90% respectively.

Classification.

The Hepatitis B Virus (HBV) is a small partially double-stranded DNA virus belonging to the family of the Hepadnaviridae. This family contains two genera: the Orthohepadnaviridae and the Avihepadnaviridae. The members of the first genus are viruses that can infect mammals like humans (HBV), woodchucks (Woodchuck Hepatitis Virus, WHV), squirrels and primates. The latter genus includes viruses that can infect birds such as ducks (Duck Hepatitis B virus, DHBV).

Genomic organization and viral proteins.

The infectious HBV particle has a 3.2 kb long circular DNA genome that is only partially double stranded. While the minus (-)-strand is complete, the plus (+)-strand is always incomplete and variable in length. Despite this small genome, the virus is able to produce a remarkable variety of proteins. This is achieved by combining overlapping open reading frames (ORFs) with the use of different translation start sites within one ORF, thus producing several related gene products from one specific ORF. The HBV genome contains four ORFs: the S-, the C, the P- and the X-ORF (Fig. 1).

The S region encodes the three viral envelope proteins by differential initiation of translation at three in-frame initiation codons with one stop codon. Of these three proteins the S protein is the most abundant. This 24 kDa protein is also known as the hepatitis B s antigen (HBsAg) and shares its sequence (226 AA) with the two other proteins derived from the S gene. The middle (M) protein is identical to the S protein but contains an extra 55 amino acids, known as the PreS2 sequence, at the amino-terminal side. S and M are translated from two distinct 2.1 kb mRNAs (preS2/S mRNA) produced by a common promoter with heterogeneous start sites around the
initiation codon at the 5’ end of preS2. The L protein, which besides the preS2 sequence also contains the 128 AA preS1 sequence, is translated from a 2.4 kb mRNA (preS1 mRNA) starting upstream of preS1 of the envelope ORF. This protein is considered to interact with the receptor on the host cell (2, 3) and plays an important role in the assembly of the virion and its release from the cell (4).

The C region encodes for two proteins: the core and precore protein. The latter being the precursor of the soluble hepatitis B e antigen (HBeAg) while the core protein constitutes the nucleocapsid. The function of the HBeAg is somewhat unclear, however experiments in mice suggest that this protein may induce tolerance to the virus (5, 6). The precore, core and polymerase (P ORF) proteins are produced from the largest mRNA, the pregenome RNA (3.5 kb). The precore and core proteins are translated from distinct mRNA’s with heterogeneous 5’ ends (7), while the polymerase translation is initiated from a more downstream, less efficient, internal promoter in a different reading frame. The polymerase protein contains two main domains linked by a spacer region. The amino terminal domain plays a vital role in the priming of transcription and packaging while the carboxy terminal domain contains the reversed transcriptase and RNase H activity.

The last ORF, X, encodes for the Orthohepadnaviridae unique X protein. The function of this protein is still unclear. It is however necessary for in vivo infection (8, 9) and can activate the transcription of host (10-12) as well as viral genes (13, 14).

The virion.

The infectious HBV particle, also known as the Dane particle, has a diameter of 42 nm (15) (Fig. 2). It consists of an outer phospholipid membrane composed of primarily HBsAg. The L and M envelope proteins are present in roughly the same
concentrations, while the S protein is three times as abundant. Although antibodies directed against the S protein are protective against infection, the preS1 domain is thought to be the ligand for the, still unidentified, human receptor (3). Inside the envelope, the icosahedral nucleocapsid encloses the viral DNA and polymerase.

![Image of viral and subviral particles found in plasma](image)

*Figure 2: Viral and subviral particles found in plasma.* Complete HBV virions with a size of 42 nm (white arrows) and 20 nm spherical particles (arrowheads) as well as filamentous structures of up to 200 nm long (black arrow), both composed of HBsAg, can be detected in the plasma of chronic HBV patients. (Adapted from Ganem D. and Prince A., NEJM 2004; 35:1118-29)

The capsid is composed of 120 core dimers and has a diameter of 37 nm (16). As mentioned above, the viral DNA is only partially double stranded and maintains a relaxed circular DNA structure. While the minus strand is complete, the plus strand is always incomplete and variable in length.

The viral DNA polymerase is covalently linked to the 5’ end of the minus strand and the 5’ end of the plus strand is preceded by a 17 bp RNA sequence.

Besides the Dane particle, other subviral HBV particles are present in the circulation of patients with acute or chronic HBV infection. These can be formed by the S protein alone, or in combination with the other HBs proteins. Two different forms of these non-infectious particles can be identified: a 22 nm large spherical particle (Fig. 2) and filamentous structures of 20 nm in diameter and up to 200 nm long (Fig. 2). These particles are more than 100 times more abundant in the plasma of infected individuals than the virions, and do not contain any DNA.

**Replication cycle.**

The virion attaches to the hepatocyte membrane through interaction of the preS1 sequence with the yet unidentified receptor. Recently it was shown that also a domain within the S protein may facilitate this interaction (17). After membrane fusion, the core particle is translocated to the nucleus where the shorter positive DNA strand is completed and the nick in the minus DNA strand is repaired after removal of the covalently attached viral polymerase (Fig. 3). This generates a fully double-stranded covalently closed circular genome (cccDNA) (18) that forms a supercoil and serves as the template for the transcription of viral mRNAs by the host RNA polymerase II (19). All RNA’s have a common 3’-termination
site and use the same polyadenylation site. These mRNA's are transcribed from four different promoters (core/precore, preS1, preS2/S and X) and are regulated by two enhancers upstream the core/precore and X promoters. These enhancers and promoters are subsequently regulated by general and liver-specific transcription factors. This explains the liver tropism of HBV.

The largest mRNA, the pregenomic RNA, which besides containing the code for the core/precore and polymerase also serves as template for the synthesis of the minus DNA strand (20).

The first step in the replication cycle involves the binding of a newly synthesized viral polymerase to a secondary RNA structure at the 5’ end of the pgRNA, called

![Figure 3: The replication cycle of HBV.](image)

After attachment to the, still unidentified, receptor the HBV virions are internalized. The viral core particles migrate to the nucleus of the hepatocytes where the viral genome is repaired to form a covalently closed circular DNA (cccDNA) that serves as the template for mRNA transcription. The viral mRNA is translated in the cytoplasm to produce the viral proteins. The progeny viral capsids assemble and incorporate the viral pregenomic RNA (pgRNA). This pgRNA is then reverse transcribed into viral DNA. The core particles can then either bud into the endoplasmatic reticulum to be enveloped and secreted into the plasma or they can recycle to the nucleus to be converted to cccDNA. (Adapted from Ganem D. and Prince A., NEJM 2004; 35:1118-29)
epsilon (ε). This triggers encapsidation of the complex by the core protein. The bulge of the ε structure acts as a template for a 3-4 nucleotide long DNA primer that is covalently attached to the polymerase. The polymerase–primer complex then translocates to the 3' end of the pgRNA where the 4 bases can anneal with a complementary sequence. During elongation of the minus DNA strand towards the 5' end of the pregenome, all but the final 17-18 ribonucleotides are degraded by the RNase H activity. The remaining fragment translocates to the 5' end of the newly synthesized (-)-strand where it anneals to the homologous DR2 region and then serves as primer for the (+)-strand synthesis.

In about 10% of all replication cycli, the translocation of the RNA primer does not occur and the (+)-strand synthesis occurs through an in situ priming reaction (21). This results in an infectious particle with a double stranded linear genome that can form cccDNA through illegitimate recombination (22).

All the replication events occur in a closed capsid that allows the entry of nucleotides through pores. However, once the capsid buds through the endoplasmatic reticulum (ER) it acquires an envelope that is impermeable. Once the nucleotide pool inside the capsid is depleted it cannot be replenished explaining the variable length and incomplete nature of the (+)-strand. Besides budding through the ER and subsequent export from the cell, intact capsids can also recycle to the nucleus where their genome can be converted to cccDNA to maintain a stable intranuclear pool of transcriptional templates (18).

**Serotypes and genotypes.**

Mutations in the HBV genome can arise during two steps in the HBV replication cycle (23). First, during the RNA pregenome synthesis by the host-derived RNA polymerase II and second, during the reverse transcription of the viral polymerase which lacks a proof-reading function. Genome analysis shows that all HBV isolates have a sequence homology of about 85%.

This variability is reflected in the presence of distinct HBV serotypes. Serologic analysis with subtype-specific antibodies against the HBsAg showed that all serotypes contain the 'a' determinant in combination with two pairs of mutually exclusive subdeterminants (d or y and w or r). The resulting nine major HBV serotypes...
(ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adrq+ and adrq-) can also be deduced from the amino acid composition at positions 122 (K for d and R for y), 160 (K for w and R for r) and 127 (P for w1/2, T for w3 and L for w4) (24, 25).

Based on the complete genetic diversity, different HBV strains can be classified into 8 genotypes, A to H, with an inter-genotype divergence of at least 8%. These genotypes have a more or less well defined geographic distribution (Fig. 4). Genotype A (serotypes adw2 and ayw1) is most prevalent in the United States (26, 27), north-west Europe and South Africa (28). Genotypes B and C (serotypes adw2, adr and ayr) predominate in Asia (29), while genotype D (serotypes ayw2 and ayw3) is found in the Mediterranean region as well as in the Middle East. Genotype E (serotype ayw4) is confined to western Africa (30-34). Genotype F (serotype adw4) is mainly present in South and Central America (25, 35-37). Genotype G (serotype adw2) has been reported recently in France (38) and the USA (39). Genotype H (serotype adw4) is restricted to Central and South America (40, 41). The geographic distribution should, however, not strictly be interpreted and can change due to the migration of chronically infected individuals (42).

The knowledge about the clinical significance of these genotypes has been rather limited. Several studies, conducted primarily in Asia, showed that genotype B, in contrast to genotype C, is associated with spontaneous HBeAg seroconversion, less pronounced liver disease, a slower progression to liver cirrhosis and a less frequent progression to hepatocellular carcinoma (HCC) (43-48). Interferon-α therapy is more successful in patients chronically infected with HBV of genotype A and B compared to genotype C and D (49-51). This was recently confirmed for treatment with pegylated interferon (52). Several studies were undertaken to find a correlation between genotype and response to lamivudine and adefovir treatment. Although no correlation could be demonstrated (53, 54), lamivudine resistant mutations arose more rapidly in patients infected with genotype A compared to genotype D (55).

**Transmission routes.**

HBV can be transmitted by mucous and percutaneous membrane exposures to infectious blood, serum, semen and saliva. The presence of HBeAg in serum correlates with higher titers of HBV DNA and greater infectivity. Mucous membrane exposures to infectious blood or body fluids through sexual and perinatal contact are extremely efficient modes of HBV transmission. No infections have been reported through oral exposure to HBsAg-positive saliva. Sexual contact is the predominant transmission route in adults.

Percutaneous exposures leading to transmission include transfusion of blood or blood products from infected donors, therapeutic injections with contaminated
equipment, intravenous drug use, cosmetic related procedures like tattooing and traditional shaving, and injuries with contaminated sharp instruments. The impact of contaminated blood products are nowadays limited in the industrialized world due to routine screening with sensitive methods. In addition, the hepatitis B virus is an extremely stable virus (more than 7 days at room temperature and resistant to drying, simple detergents and alcohol), thus increasing the risk of infection from contaminated environmental surfaces.

**Natural history.**

Once a person is infected with HBV, the infection can be characterized by three successive phases. The first, immunotolerant or viral replicative phase is characterized by the presence of HBsAg, HBeAg and high levels of HBV DNA in the plasma. In adults, this phase generally lasts about 2-4 weeks, while in perinatal acquired infections it often last for decades. At this point an immune response is generally absent or weak, resulting in the absence of symptoms, normal or slightly elevated transaminases and only minimal hepatocellular damage and infiltration. Immunostaining shows a predominant nuclear localization of HBcAg (56), while HBsAg is found in the cytoplasm of some hepatocytes and near the membrane of numerous cells forming a honeycomb-like pattern (57). A strong cytoplasmic HBeAg expression can be observed and is a marker for a high viral replication (56, 58).

The second, immunoreactive or viral clearance phase is associated with a decrease of HBV DNA, an increase of serum transaminases and ultimately seroconversion of HBeAg to anti-HBe (59). Liver histopathology consists of more severe lesions, like confluent necrosis of variable extent (60). Immunostaining reveals nuclear, cytoplasmic and membranous HBcAg, while HBsAg shows a membranous staining pattern and sometimes a weak cytoplasmic positivity (61). In an acute HBV infection, clinical symptoms and jaundice now appear and usually last for 3-4 weeks. At this point, more than 95% of acute infected adults will mount an HBV specific immune response of sufficient magnitude and complexity, and will recover spontaneously from the viral infection (acute self-limited hepatitis B). In less than 5% of adults, the anti-viral immune response is not adequate enough to suppress the viral replication and to eliminate HBV from most infected hepatocytes. Here the immunoreactive phase may last for decades, resulting in chronic hepatitis B.

The majority of chronically infected HBV patients who seroconvert to anti-HBe remain HBeAg negative and anti-HBe positive with normal transaminase levels and minimal to no necroinflammation on liver biopsy. The seroconversion is usually preceded with a decrease of HBV DNA
levels to below $10^5$ copies/ml. This phase has been referred to as the low or non-replicative phase. At this point integration of the virus in the host genome is not uncommon (viral integration phase) and HBsAg remains detectable in the serum. HBsAg accumulates in clusters of hepatocytes, while HBcAg is usually undetectable. Mild inflammation might persist for some time without substantial necroinflammation. This inactive carrier state may last for lifetime and if the damage during the viral clearance phase was not too severe, the liver may recover with only minimal abnormalities. Up to 20% of inactive carriers, however, experience exacerbations of hepatitis, as determined by ALT elevations exceeding 5 to 10 times the upper limit of the normal value. This HBV reactivation can occur with or without seroconversion of anti-HBe to HBeAg. Repeated flare-ups generally result in progressive fibrosis and eventually cirrhosis.

Some patients suffer from ongoing liver disease, even after HBeAg seroconversion. This particular disease course is frequently associated with the occurrence of a precore HBV mutant that is unable to produce HBeAg (62-66).

**HBV in immune suppressed patients.**

In immune competent subjects, a chronic HBV infection may induce a varying degree of liver damage depending on the vigour of the host’s immune response towards the virus. This immune response also suppresses the viral replication and maintains the viral load in the circulation between undetectable levels with unamplified assays (<$10^5$ copies/ml) and 1 or 2 log higher ($10^6$-$10^7$ copies/ml). However, when a state of severe immune deficiency occurs, e.g. by medical immune suppression or by progressive immune failure in the context of acquired immune deficiencies (e.g. HIV/AIDS), the viral load of HBV carriers increases dramatically (> $10^8$ copies/ml) and even reactivation of seemingly recovered or silent HBV infections can occur (67-69). In a minority of HBV carriers who undergo an immunosuppressive therapy for an autoimmune disorder (70) or after liver (71, 72), renal (73, 74) or bone marrow (75-77) transplantation or who are treated with chemotherapy for non-Hodgkin lymphoma (78, 79), the clinical course may severely worsen and eventually the clinico-pathophysiological picture of fibrosing cholestatic hepatitis (FCH) may arise. FCH is an aggressive and mostly fatal form of viral hepatitis associated with increased viral replication (80) and expression of intrahepatic viral proteins (81). Diffuse hepatocyte ballooning, the presence of ground-glass hepatocytes, cholestasis and periportal fibrosis are prominent histological features of FCH (74, 80-83).

**Prevention of HBV infection.**

The most important tool for the prevention of HBV infection is vaccination. The first vaccine was available in 1981, and
was derived from plasma from chronically infected HBV patients. Because of practical, financial and safety concerns, this plasma-derived vaccine was gradually replaced from the mid 80’s by a safe and efficacious yeast-derived recombinant vaccine. This vaccine is solely composed of HBsAg (20 µg) in combination with an aluminum adjuvant. Three doses of this vaccine, given at months 0, 1 and 6, suffice to adequately protect the majority of vaccinated adults from HBV infection. However, 1 to 3% of neonates and up to 10% of healthy adults is unable to produce protective levels of anti-HBs antibodies. These non-responders do not suffer from a general immune deficiency, but their unresponsiveness is related to their HLA haplotypes (84). Although HBsAg non-responsiveness affects only a minority of the population, the search for more immunogenic vaccines is still going on.

It is clear that due to massive vaccination programs, the amount of newly infected people will dramatically decrease, but the effects of vaccination on the incidence of HBV related HCC will only be visible within decades due to the long incubation period.

**Therapy.**

Although the hepatitis B virus has been discovered in the mid sixties (85), no successful therapy is currently available. At present, four therapeutic agents have been approved by the Food and Drug Administration (FDA) for the treatment of chronic hepatitis. The aims of treatment are the sustained suppression of HBV replication and remission of liver disease. To assess the response to therapy, the following criteria are evaluated at the end points of therapy: normalization in serum ALT levels, undetectable serum HBV DNA by an unamplified assay (i.e. <10^5 viral copies/ml), loss of HBeAg with or without the appearance of anti-HBe antibodies and the improvement of liver histology.

**a. Interferon.**

Interferons (IFN) have a dual mode of action. Besides a direct anti-viral effect they also have an immunomodulatory effect (86, 87). IFN-alpha has now already been used for more than 20 years for the treatment of chronic HBV patients. Nevertheless, the efficacy of this treatment is only limited to a small percentage of selected patients. In HBeAg-positive chronic HBV patients with persistent or intermittently elevated ALT levels, 37% of individuals responded to therapy (criteria described above) compared to 17% of controls (spontaneous improvement) (88). Therapy-induced HBeAg clearance appears to be durable in 80 to 90% of responders for at least 4 to 8 years (89, 90). Using sensitive PCR methods, HBV DNA remains detectable in the serum of these patients. High pre-treatment ALT and low serum HBV DNA level are the most important predictors of response to IFN-alpha therapy (91-93). In addition, IFN-α treatment reduces the risk of hepatocellular carcinoma in cirrhotic patients (94).
In HBeAg negative chronic HBV patients, the HBeAg seroconversion can not be used as an end-point to evaluate therapy. Therefore HBV DNA levels below $10^5$ copies/ml and the normalization of ALT levels are use as indicators of response. Results of four randomized trials showed that the end-of-treatment response ranged from 38% to 90% in treated patients compared with only 0% to 37% in controls. The 12 month sustained response rates varied from 10% to 47% (95-98). A major problem with the IFN-α treatment of HBeAg-negative chronic HBV patients is a high relapse after discontinuation (approximately 50% of responders). Although relapses can occur up to 5 years after therapy, 15% to 25% show a sustained response, while 15% to 30% of sustained responders cleared HBsAg (99).

A major drawback to IFN therapy is the occurrence of adverse effects like flu-like symptoms, fatigue, myalgia, thrombocytopenia and depression that may result in the discontinuation of therapy. Many patients undergoing therapy experience an acute flare of liver injury, generally preceding the clearance of HBeAg. This probably reflects the immunomodulatory effect of IFN which up-regulates MHC class I antigen presentation. Therefore, IFN-α therapy is generally contraindicated for patients with advanced cirrhosis and decompensated liver disease, since these flares of liver injury can result in total liver failure.

**b. Pegylated Interferon.**

Pegylated interferon α-2a is a modified form of conventional interferon. A large 40 kDa branched polyethylene glycol (PEG) molecule is attached to the base of the IFN molecule. This procedure results in a compound with a prolonged half-life, thus requiring only a weekly administration of the drug without high fluctuations in serum concentration (100). Two studies showed a superior efficacy to PEG-IFN therapy compared to conventional IFN α-a2 (36% vs. 25%) (52, 101). The frequency and severity of adverse effects are, however, comparable to conventional IFN therapy (101).

**c. Lamivudine.**

Lamivudine (Epivir-HBV, 3TC) is a cytidine analogue ((-)-enantiomer of 2’-3’ dideoxy-3’-thiacytidine) that upon incorporation into the viral DNA strand leads to premature chain termination, thus inhibiting DNA synthesis. The anti-HBV potency of lamivudine was discovered when HIV patients, coinfected with HBV displayed a reduction in serum HBV DNA during treatment (102). This reduction in HBV viremia was also observed in chronic HBV patients without HIV infection (103, 104).

Virologic response after a one year lamivudine therapy ranges from 16 to 18% in HBeAg positive patients with elevated ALT levels compared to 4% to 6% in controls (104-106). In chronic HBV patients...
with relatively normal or slightly elevated ALT levels, virologic response was lower than 10% (107). In HBeAg negative patients, a virologic response was achieved in 63% of patients treated for 24 weeks (placebo 6%) (108). The same rate of response was also observed after 12 months of therapy (109, 110), but 90% of individuals relapsed after cessation of therapy.

Combination therapy of Lamivudine with PEG-IFN is not superior to PEG-IFN monotherapy (52).

A major advantage of lamivudine is that it is usually well tolerated. It has no immunomodulatory effect and therefore can be used to treat HBV patients with decompensated cirrhosis (111).

The main drawback of lamivudine is the appearance of resistant mutants during treatment. The most frequent mutation affects the YMDD motif of the viral polymerase (112, 113). Lamivudine resistant viruses appear in 15 to 20%, 40% and even in 67% of patients after one, two and four years of treatment respectively (104-106, 114, 115), resulting in the reappearance of HBV DNA in the serum using unamplified assays. Most patients who continue treatment still have lower HBV DNA and ALT levels than before treatment due to the impaired replication of the mutant virus variants (116, 117).

d. Adefovir dipivoxil.

Adefovir is an adenosine monophosphate analogue that acts as an inhibitor of both the viral reversed transcriptase and DNA polymerase activity. A 48 week treatment of HBeAg positive chronic HBV patients with adefovir showed normalization of ALT levels in 48% of treated patients versus 16% in the placebo group (118). Twelve and 21% of treated patients experienced HBeAg seroconversion and a decrease of serum HBV DNA to below 400 copies/ml respectively (placebo 6 and 0%) (118). HBeAg negative chronic HBV patients showed a normalization of serum ALT levels in 72% of cases (placebo 29% and a reduction of viral DNA in the serum to below 400 copies/ml in 51% of treated patients (placebo 0%) (119). The safety profile in all adefovir treated chronic HBV patients was similar to the placebo group, while a 3 to 4 log median reduction of viremia and a histologic improvement of liver abnormalities was evident (118, 119).

A major advantage of adefovir dipivoxil is that it can be successfully applied to treat patients with lamivudine-resistant HBV mutants (120, 121). To date, the emergence of adefovir-resistant HBV mutants has not been reported after the first year of treatment (118, 119, 122, 123). However, a drug-resistant mutation (N236T) downstream the YMDD motif of the polymerase has been reported during the second year of therapy in 2 of 79 (2.5%) HBeAg negative chronic HBV patients (124). Patients harboring this mutant virus can be successfully treated with lamivudine (124).
**e. Entecavir.**

Entecavir (Baraclude™) is a guanosine nucleoside analogue with activity against the HBV polymerase. After initial testing in two animal models for HBV (125-127), entecavir was successfully used for the treatment of chronic HBV in nucleoside-naïve patients infected with wild-type HBV (128). Treatment for 22 weeks with entecavir reduced HBV DNA levels 4.7 log₁₀-fold and was shown to be superior to lamivudine treatment (128). A dose-ranging efficacy and safety study in chronic HBV patients who were considered to be lamivudine refractory showed an up to 5.06 log₁₀-fold reduction in viremia after 48 weeks of treatment with 1.0 mg entecavir (129). In addition, 68% of patients achieved normalization of alanine aminotransferase levels.

**f. Other antiviral compounds.**

It is clear that there still is need for improvements in the current therapies. Therefore, the search for more affordable and effective antiviral compounds that induce fewer side-effects is essential. Several candidates, like tenofovir (130-132), emtricitabine (133), clevudine (134) and several other compounds and compound combinations are currently in (pre)clinical phase for evaluation, but although the initial results from *in vitro* and *in vivo* experiments are promising, the promotion to approved standard therapy is still far away.
The Hepatitis C Virus.

In the mid-1970s, several patients were diagnosed with a transfusion-associated form of hepatitis that could not be attributed to an infection with hepatitis A virus, hepatitis B virus, Epstein-Barr virus, cytomegalovirus and other viruses known to cause liver inflammation (135, 136). Therefore these cases were termed non-A, non-B hepatitis (NANBH). It was only in 1989 that Choo et al. (137) were able to identify the causative agent of NANBH by molecular techniques. They isolated a cDNA clone from the plasma of a chimpanzee experimentally infected with NANBH, that encoded for an antigen specifically associated with NANBH infections. The etiologic agent of NANBH was designated hepatitis C virus (HCV). Screening of plasma of patients diagnosed with NANBH showed that the majority of these patients were infected with HCV (138, 139).

According to the World Health Organization, approximately 170 million people are chronically infected with HCV worldwide. Each year an additional 3 to 4 million people become newly infected.

Classification.

The hepatitis C virus belongs to the genus Hepacivirus (140), a member of the family of the Flaviviridae. This family also includes two other genera: the Pestivirus and the Flavivirus.

The Pestivirus contains viruses like the bovine viral diarrhea virus (BVDV) and the classical swine fever virus, while yellow fever virus, Dengue virus, Tick-borne encephalitis virus and Japanese encephalitis virus are members of the Flavivirus genus.

Although the sequence homology between HCV and the other members of the Flaviviridae is rather low, their genomic organization is very similar. Therefore these viruses and in particular BVDV, are often used as a model system to elucidate the replication.

Genomic organization and viral proteins.

a. The HCV Genome.

All members of the Flaviviridae are small-sized, enveloped viruses containing a (+)-sense single stranded RNA encoding a viral polyprotein. The HCV genome consists of about 9600 nucleotides and is composed of a 5' noncoding region (5’NCR), a long open reading frame (ORF) that encodes for a polyprotein of 3010 to 3033 amino acids long, depending on the strain, and a 3' noncoding region (3’NCR) (Fig. 5).

The highly conserved 5’NCR is 341 nucleotides long and contains an internal ribosomal entry site (IRES) that is essential for cap-independent translation of the viral RNA (141, 142). The 3’NCR is approximately
230 nucleotides long and is composed of a short variable region, a poly(U) tract of heterogeneous length and a highly conserved 98 nucleotide sequence essential for replication (143-145).

b. Viral proteins.

Translation of the HCV ORF yields a polyprotein that is co- and post-translationally processed by cellular and viral proteinases into ten distinct mature structural and non-structural proteins. The first three proteins from the polyprotein are the nucleocapsid C protein (21 kDa) and the two viral envelope glycoproteins E1 (37 kDa) and E2 (61 kDa). The C protein is highly basic and binds the viral RNA. Overexpression of the core protein has an impact on several host cell functions, like gene transcription, cell proliferation, cell death and lipid metabolism (146-149). The two envelope proteins, E1 and E2, form noncovalent heterodimers (150), and are essential for the interaction between the virus and the host cell (151, 152). Within the E2 sequence, two hypervariable regions have been identified: HVR1 and HVR2. This hypervariability is driven by continuous antibody selection and immune-escape. HVR1 shows a sequence variability of up to 80% between the HCV genotypes and contains the only in vivo HCV neutralization epitope thus far discovered (153).

The remaining seven proteins are non-structural proteins: p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. The p7 protein is a small protein that can form an ion channel composed of p7-hexamers (154, 155). These properties suggest that p7 belongs to the viroporin family and therefore might be important for viral particle assembly and release.

![Figure 5: HCV genes and gene products.](image)

The NS2 is a metalloproteinase that, in collaboration with the N-terminal part of NS3, participates in the proteolytic cleavage of the polyprotein at the NS2-NS3 junction (156, 157). The NS3 protein comprises two domains: the N-terminal (189 AA-long) serine proteinase and the C-terminal (442 AA-long) helicase-NTPase. The NS3 protease domain associates with its co-factor NS4A (54 AA-long) (158, 159) and this complex is responsible for the cleavage
of NS4A, 4B, 5A and 5B from the polyprotein (160). The NS3 helicase-NTase domain is responsible for RNA unwinding, a process driven by NTP hydrolysis (161-163). It was recently shown that NS4B can induce the formation of an ER-derived membranous web that is composed of all HCV structural and nonstructural proteins (164), and replicating RNA (165). It seems that the membranous web acts as a scaffold for the formation of the viral replication complex. The function of the NS5A protein remains inconclusive. It has been reported that a certain region within NS5A, termed the interferon sensitivity determining region, might play a role in the response to interferon alpha therapy (166, 167). In addition, the NS5A protein has been reported to interfere with the activity of the double-stranded-RNA-activated protein kinase (168). The NS5B protein plays a central role in the HCV replication cycle. Using the viral genome as a template, this RNA dependent RNA polymerase (RdRp) synthesizes a new viral (+)-strand RNA genome via an intermediate minus-strand RNA (169, 170). The RdRp can produce new RNA strands without the need of a primer, but it lacks a proofreading function. Recently, a new and alternate reading frame within the HCV core region was proposed (171). This reading frame is the result from a frame shift of the ribosome and generates a new protein called F (frame shift) or ARFP (alternate reading frame protein) (172, 173). Antibodies (174) and memory T-cell-mediated immune (175) responses against this protein can be detected in the sera of chronically infected HCV patients, which indicates the expression of this protein during a natural infection. The function of this protein remains to be determined.

The HCV virion.

HCV particles are thought to have a diameter of about 56 to 60 nm (176-178). The virions have an outer double-layered, host-derived, lipid envelope in which E1-E2 heterodimers are anchored. These viral envelope proteins are thought to form spikes on the virion surface (Fig 6). Inside the viral membrane an icosahedral nucleocapsid composed multimers of the viral core protein, encapsidates the viral RNA. Complete virions can only be rarely observed inside hepatocytes of HCV infected chimpanzees and humans (179).

Viral infection and replication cycle.

Although HCV RNA has been detected in several extrahepatic locations, like circulating lymphocytes, antigen presenting cells, bone marrow and even brain tissue (180-187), the hepatocyte is the primary target of the virus. Due to the lack of an efficient cell culture system the knowledge on viral attachment and host-cell entry was progressing slowly. However, since the development of infectious HCV pseudoparticles, new information on this subject is continuously being generated (151, 152). During the last decade, several candidate receptors were proposed. The
Figure 6: HCV virions inside infected hepatocytes. (a) A single virus-like particle composed of a relatively dense core and surrounded by a spiked envelope (arrow) can be seen inside a hepatocyte from a chronically infected chimpanzee. The peripheral cell membrane is indicated by a thick arrow. (b) A small group of HCV virus-like particles (arrows) can be noticed in a hepatocyte from a cirrhotic, HCV infected human liver. (Magnification x72,500; adapted from De Vos R. et al., J. Hepatology; 37:370-379)

Current consensus is that not one, but a combination of receptors, forming a receptor complex, are required to achieve infection.

One of the first receptor candidates put forward was CD81. This was based on the observation that recombinant HCV E2 could bind to the major extracellular loop of the CD81 molecule (188). A second receptor candidate was the low-density lipoprotein (LDL) receptor (189, 190). In serum obtained from chronically infected patients, HCV particles can be found associated with both LDL and VLDL (191-193). The LDL receptor was shown to facilitate the internalization of LDL-associated virions. Negative arguments for CD81 and the LDL-R as a receptor for HCV are the broad tissue distribution of these proteins.

Another putative receptor is the scavenger receptor class B type I (SR-BI), which is also able to bind the HCV E2 protein via interaction of the HVR1 (194). SR-B1 is highly expressed on hepatocytes and is also known as the receptor that is responsible for the internalization of HDL (195). In addition, HDL has been shown to enhance the infectivity of HCV pseudoparticles \textit{in vitro}, and to protect the virus from neutralizing antibodies (196).
The E2 HVR1 is essential in these processes. This is in accordance with the observed binding of recombinant HCV envelope proteins to HDL (197). While human and tupaia SR-B1 are able to bind HCV E2, this interaction is not observed with mouse SR-B1 (198). This might explain why mouse hepatocytes are resistant to HCV infection in contrast to human and tupaia hepatocytes.

HCV glycoproteins also interact with DC-SIGN and L-SIGN, two membrane-associated C-type lectins that are expressed on dendritic cells and hepatic sinusoidal endothelial cells respectively (199, 200). These interaction might also contribute to the capture and delivery of the virus to the hepatocytes (201).

After attachment of the hepatitis C virus to its receptor(s), the virus still has to enter the target cell (Fig. 7). Studies with the HCV pseudoparticle system showed that this entry is pH-dependent, strongly supporting a receptor-mediated endocytic route of entry (151, 202). After endocytosis, the viral glycoproteins probably undergo a low-pH induced conformational change that promotes fusion with the endosomal membrane. Once the viral RNA has entered the cytoplasm it is immediately translated near the rough endoplasmatic reticulum (ER). The ribosomal complex is directed by an IRES, and the produced polyproteins are co- and posttranslationally processed by host cell and viral proteinases (see above). The viral proteins, and probably also cellular proteins, form a replication complex which associates with the intracellular membranes. This replication complex is known as the membranous web (164, 165). The (+)-strand viral RNA is also used as template for the viral polymerase to produce new (+)-strand RNAs via intermediate (-)-strand RNAs. These progeny (+)-strand RNAs can be used either as new templates for translation, replication or for the assembly into new virions. The factors that regulate these processes are unknown.

![Figure 7: HCV life cycle](image)
How a new virion is assembled is currently unknown. Probably, virion assembly is started by an interaction of the viral core protein with a (+)-strand RNA strand. The core proteins interact with a region in the 5’ UTR of the HCV genome (203), a process that also inhibits translation from the IRES (204). The HCV envelope proteins E1 and E2 are retained in the ER due to retention signals in their transmembrane domains (205, 206). This might indicate that the viral nucleocapsids acquire their envelope through budding into the ER. Thus, virus secretion can occur using the constitutive secretory pathway.

Mathematical analysis of the viral decline in 23 chronically HCV infected patients during interferon-α-2b therapy showed that the virion half-life in serum was approximately 2.7 hours (207). Before treatment, $10^{12}$ new virions were produced on a daily basis, which means that an infected hepatocyte produces 10 to 100 virions daily. This calculation depends on the assumption that 5% to 50% of the $2\times10^{11}$ hepatocytes composing the human liver are infected. However, the actual amount of hepatocytes that are infected in a chronic HCV patient is difficult to determine since viral proteins are difficult to visualize on human liver biopsies.

**In vitro infection and replication of HCV.**

Until recently, in vitro studies of HCV were limited to the subgenomic replicon system (208). However, these HCV replicons do not replicate efficiently without cell culture-adaptive mutations (209, 210). Full-length replicons with adaptive mutations replicate efficiently, but are unable to produce infectious particles (211, 212) and are severely attenuated in vivo (213). This limits the use of the replicon system to the study of the replication and the evaluation of drugs with possible antiviral activity.

Recently, a major breakthrough allowed the in vitro study of the complete viral life cycle. Three groups almost simultaneously described the production of infectious HCV in cell culture (214–216). The system was based on a subgenomic replicon derived from a Japanese patient suffering from a fulminant hepatitis (JFH1), which replicated efficiently without cell culture-adaptive mutations (217). Transfection of full-length JFH1 or chimeric J6/JFH1 into Huh7 or Huh-derived hepatoma cells resulted in high replication and the production and secretion of complete virions (HCVcc) into the culture medium. The culture supernatant could be used to infect naïve hepatoma cells (214–216), and also induced a mild and transient HCV infection in a chimpanzee (216).

We recently demonstrated that cell culture grown chimeric FL-J6/JFH could establish a long-term HCV infection in chimpanzees and chimeric human liver-uPA/SCID mice (218). The animal-derived virus showed increased infectivity compared to the HCVcc, which correlated to a lower buoyant density of the virions. After re-
culture in Huh7.5 cells the infectivity decreased again to levels of the initial cell culture virus.

It is clear that the HCVcc system is a major step forward that will enable the scientific community to study all steps of the viral life-cycle in a very efficient way. The main drawbacks are that the HCVcc system is, thus far, limited to the JFH1 strain or chimeric viruses that contain the JFH1 non-structural machinery. The fact that HCVcc are considerably less infectious than in vivo generated HCV indicates that cell culture conditions are not completely comparable to what happens in life situation.

**HCV Genotypes, subtypes and quasispecies.**

Based on sequence analysis, the different HCV strains can be classified within 6 major genotypes (1 to 6) comprising different subtypes (a, b and c). These genotypes have now been designated ‘clades’. The new genotypes 7a, 8a, 9a and 11a have been assigned to clade 6, while genotype 10a has been classified under clade 3 (140, 219).

The divergence over the complete genome between different genotypes ranges between 30 and 35%. Higher variability can be noticed in the region comprising the envelope glycoproteins (>50%), whereas the nucleotide sequences of most of the nonstructural proteins are more conserved. The 5’UTR and the core region are highly conserved (220). The sequence difference between subtypes of the same genotype ranges from 20 to 25%.

The most common genotypes in Western Europe and the US are genotype 1a and 1b (together these represent 70% with equal distribution), followed by genotype 2 and 3. Genotype 1a and 3 are associated with intravenous drug abuse. In Europe, HCV prevalence ranges from 0.5% in northern countries to 2% in the Mediterranean and eastern regions. Genotype 4 is primarily found in Egypt, where about 22% of the population is infected with HCV, most likely as a consequence of unsafe parenteral antischistosomal therapy (221). Infections in western Africa are caused primarily by HCV genotype 2, whereas infections in central Africa are caused by genotypes 1 and 4. Genotype 5 is most prevalent in South Africa, while genotype 6 is ubiquitous in Southeast Asia but also in Australia (Figure 8).

The determination of HCV genotype is definitely of clinical significance. Response to therapy varies among different genotypes. IFN monotherapy and IFN/ribavirin combination therapy are much more efficacious against genotype 2 and 3 compared to genotype 1 (222). Infections with genotype 3 have a higher incidence of steatosis (223), probably due to virus-induced block in lipoprotein secretion (224).
Within a chronically infected patient, a pool of genetically distinct but closely related variants coexist as quasispecies (genomic diversity <10%). This phenomenon has several causes. First of all, HCV is an RNA virus and its replication is mediated by a polymerase without proofreading. Considering that RNA polymerases have a typical error rate between $10^{-4}$ and $10^{-5}$ substitutions per nucleotide copied (225), and that every day up to $10^{12}$ (207) new virions are produced in the liver, this degree of diversity is not surprising. The existence of the quasispecies might also be a strategy of the virus to evade the immune system and thus persist in the infected host. One target of the humoral immune system is the HVR1 of the E2 protein. The existence of neutralizing antibodies to the envelope proteins and especially to this specific region (153, 226) might be the driving force to the variability of the HVR1. Studies in patients with agammaglobulinemia support this theory (227). In these patients, less mutants were observed compared to immune competent patients; probably because their occurrence does not represent any advantage in the absence of immunoglobulins. Additionally, the sequence diversity in the HCV population within the patient might also be a way to alter epitopes that are strongly recognized by the cellular immune system, or to avoid presentation by the MHC complex.

**Transmission routes.**

The main routes of HCV transmission are intravenous drug abuse and transfusions of blood or plasma-derived product. This latter mode of infection is nowadays rather limited in developed countries through the screening of blood products. People undergoing repeated medical interventions, like renal dialysis, also are at substantial risk of acquiring an HCV infection if the primary prevention strategies are not rigorously applied.

Vertical transmission is limited, but rates of transmission of 5% have been reported in babies of otherwise healthy mothers and up to 30% in babies from HIV coinfected mothers. Transmission through sexual contact is still controversial but may occur at very low frequencies.

The stability of HCV on environmental surfaces is relatively low compared to HBV. Nevertheless, in an experimental setting, viral transmission to chimpanzees still can occur 16 hours after
infectious plasma was dried in a vacuum chamber, but not after 4 days (228).

**Natural history.**

The majority of people acutely infected with HCV experience no or only mild symptoms, like jaundice, nausea, abdominal pain, fatigue and depression. Chronic HCV infections are also characterized by a prolonged period without specific symptoms. Therefore most HCV infections are not recognized before the onset of advanced or end stage liver disease, several decades later.

Between 70% and 85% of the people infected with HCV evolve towards chronicity (229). Acute self-limited hepatitis is rare, but spontaneous elimination of the virus occurs in about 10 to 20%. About half of the chronically infected people have persistently normal ALT levels. Liver biopsy analysis reveals only minimal chronic hepatitis combined with mild fibrosis; cirrhosis is rare. The long-term outcome is generally favorable in this group. The other half of the chronically infected patients have intermittently or continuously elevated transaminase levels. Histological evaluation of liver biopsies shows marked necroinflammatory lesions and extensive fibrosis. In this group, cirrhosis will be diagnosed in more than 35% after 25 years of chronic infection. Overall, about 20% of chronically infected people will develop cirrhosis after 20 years. Once cirrhosis is established, the risk of developing hepatocellular carcinoma is approximately 3% per year.

Besides liver disease, HCV is also responsible for several extrahepatic diseases. About half of HCV infected people suffer from essential mixed cryoglobulinemia (type II). Cryoglobulins usually contain large amounts of HCV virions captured in antigen-antibody complexes (230). Type II cryoglobulinemia can induce purpura, arthritis, hypertension, vasculitis, peripheral neuropathy and in the most severe cases in membranous and membranoproliferative glomerulonephritis (in 5% of infected people). In about 5% of all cases, porphyria cutanea tarda can develop (231). Treatment consists of antiviral therapy or phlebotomy to deplete the excess of iron that can exacerbate the porphyria.

Other extrahepatic manifestations possibly linked with HCV infections are lymphoproliferative disorders (5%-35%), autoimmune thrombocytopenia (10%-15%) and arthralgias and myalgias (20-30%).

**Immune evasion of HCV.**

The hepatitis C virus generally induces a persistent infection, presumably because the virus has developed different strategies to evade the host’s immune system.

*a. Intracellular host defense.*

The hepatocyte, the prime target for HCV, has several defense mechanisms to protect itself against viral infections. Viral infections are usually detected through pathogen-associated molecular patterns (PAMP), which include viral proteins,
Figure 9: Overview of the intracellular events in the host response to HCV infection and possible points in this signaling cascade where the virus may interfere. (Adapted from Gale M.Jr. and Foy E.M., Nature 2005; 436:939-945)

Single-stranded (ss) and double-stranded (ds) RNA and polyuridine structures (232-234). Upon binding to PAMP-receptors, like Toll-like receptors (TLR) and nucleic acid binding proteins, a series of intracellular proteins become activated that lead to the induction and expression of several antiviral proteins.

Two independent pathways become triggered once dsRNA is recognized: a TLR3 pathway and a retinoid-acid-inducible gene I (RIG-I) pathway (Fig. 9). Both TLR3 and RIG-I can activate interferon regulatory factor (IRF)-3 which then migrates to the cell nucleus and activates the production of IFN-β (235-237). Activation of IRF-7 by TLR3 results in the production of IFN-α (238, 239). Both IFN-α and β are secreted by the activated hepatocyte, resulting in an autocrine and paracrine activation of the Jak-STAT pathway, which results in the transcription of IFN-stimulated genes (ISG) like IRF-7, OAS (2',5'-oligo-adenylate synthetase), PKR (double stranded RNA-activated protein kinase R), etc. (240). This increases the antiviral capacity of the infected hepatocyte, but also triggers nearby uninfected hepatocytes to prevent further spread of the virus. Besides inducing ISG expression, IFN-α also plays a role in the modulation of the adaptive immune response against the invading pathogen. In addition, RIG-I and TLR3 can activate NF-κB that activates the transcription of several chemokines and cytokines (232, 241).
b. Evasion mechanisms.

HCV has devised several ways to interfere in the intracellular signaling, thus preventing, or at least reducing, the antiviral potential of the hepatocyte.

The NS3/4A protease can block IFN-β production by inhibiting the activation of IRF-3 by RIG-I and by cleavage of TRIF (Toll-interleukin-1 receptor-domain-containing adaptor inducing IFN-β), a TLR-3 adaptor protein (242-244). Cleavage of TRIF also abolishes the activation of NF-κB (243).

The HCV core protein has been shown to increase the expression of SOCS-3 (suppressor of cytokine signaling-3) (245). SOCS proteins are inhibitors of the Jak-STAT signaling pathway (246).

Both the envelope protein E2 and NS5A are inhibitors of PKR (168, 247, 248), but NS5A has also been implicated in the inhibition of 2′,5′ OAS (249). HCV of genotype 1 contains less OAS/RNaseL cleavage sites in its genome, which might be another way to be more resistant to this ribonuclease (250).

Antiviral therapy.

a. Evolution of therapy.

At the time when HCV was still termed non-A, non-B hepatitis, interferon alpha therapy was initiated with limited success (251). Large randomized trials were started that showed the beneficial effect of IFN on serum alanine aminotransferase levels and liver histology, but most of the treated patients relapsed after cessation of therapy (252, 253). Depending on therapy duration, only 5 to 16% showed a sustained response defined as a normalization of serum aminotransferase levels.

A major improvement in HCV therapy consisted of the combination of IFN with ribavirin. Combination therapy significantly increased the amount of patients with a sustained virologic response (HCV RNA negative) up to 40% after 48 weeks of treatment (254, 255). Therapy was most successful in people infected with HCV genotype 2 and 3 (sustained response of 65%).

Recently, the treatment of HCV was largely improved by the replacement of ‘traditional’ IFN by pegylated IFN. A 48 week therapy with pegylated IFN alone, compared to ‘traditional’ IFN therapy, increased the sustained virologic response from 19% to 39% in non-cirrhotic patients (256) and from 8% to 30% in people with advanced liver disease (257).

The current standard therapy consists of a 48 week combination therapy of pegylated IFN with ribavirin. The overall sustained virologic response rate is 55% (258, 259). People infected with HCV genotype 1 have a 40% to 50% sustained response rate, depending on the dose of ribavirin administered, while non-genotype 1 infections can be cured in 75% of all cases (260). Treatment of chronic hepatitis of genotype 1 with PEG-IFN plus ribavirin will be discontinued after 12 weeks of therapy if a drop in HCV RNA titer of at least 2 log(10)
fold is not achieved (261). About 20% of treated patients do not meet this criterion and only have 1.6% chance of achieving a sustained virologic response. Therefore, the use of this early stopping rule prevents that 20% of treated patients undergo the side effects associated with treatment without a significant chance of success. In addition the use of this early stopping rule reduces treatment costs by at least 19%.

Desombere et al. recently showed that the decision to proceed or discontinue treatment could already be made after one week of therapy (personal communication). Chronic HCV patients who do not have antibodies against NS4A before start of therapy, and who still have a HCV RNA level exceeding $10^5$ IU/ml have 0% chance of achieving a sustained virologic response.

b. Active compounds.

**Interferon.** The antiviral action of IFN is caused by two complementary mechanisms: a direct nonspecific antiviral effect and an immunomodulatory effect. In the first case, IFN induces the expression of 2'-5' oligoadenylate synthetase (2'-5' OAS), Mx proteins and double-strand RNA-dependent protein kinase (PKR). 2'-5' OAS, which is also activated by double-stranded RNAs, in turn activates RNase L, a cellular endoribonuclease (262). RNase L degrades cellular and viral single-strand RNA. The Mx proteins directly inhibit viral polymerases, but are less important in HCV infections (263). PKR phosphorylates eIF-2 which then inhibits nonspecifically all protein synthesis (264). The immunomodulatory effects of IFN-alpha comprise increased MHC class I expression, activation of macrophages, NK cells and cytotoxic T-cells and promotion of the type 1 T-helper (Th1) pathway.

Interferon therapy is associated with several side effects. Influenza-like symptoms, fatigue, fever, rigors, myalgia and thrombocytopenia are very common. Insomnia, lack of motivation, inability to concentrate, emotional instability and depression also occur frequently.

**Ribavirin.** Ribavirin is a guanosine analogue with possibly four different antiviral characteristics (265). Ribavirin can directly inhibit the viral polymerase and can act as a competitive inhibitor of inosine monophosphate dehydrogenase. The latter functions results in a depletion of the cellular GTP pool, thus inhibiting viral RNA synthesis. In addition, ribavirin causes a shift towards Th1-type immune responses (266) and is an RNA mutagen (267).

Ribavirin is much more tolerable than interferon, but nausea, anemia, pruritus and hemolysis are common adverse effects.

**Pegylated interferon.** Pegylated interferon is conventional interferon that is covalently attached to a polyethylene glycol molecule. This increases the half-life of the IFN molecule from 4 to 6 hours to 96 hours, permitting a once-a-week dose regimen. Two forms of PEG IFN exist: IFN-α2b
linked to a 12 kDa polyethylene molecule (PEG-INTRON, Schering-Plough) and IFN-α2a coupled to a 40 kDa branched polyethylene molecule (Pegasys, Roche). Pegasys has a longer half-life and is mainly cleared by the liver, while PEG-Intron is removed primarily by the kidney.

The frequency and severity of adverse effects of pegylated interferon are comparable to those associated with standard interferon therapy.

**New antiviral compounds.**

Since currently approved therapies are not adequate enough to cure the majority of chronically infected patients, new antiviral compounds are designed that specifically inhibit the viral replication. Candidate targets are of course all viral proteins that are indispensable for viral replication, like the protease, helicase and polymerase.

Since protease inhibitors have proven their effectiveness in HIV therapy, this strategy also became the most promising in anti-HCV drug design. One of the first promising protease inhibitors tested in humans was BILN 2061 (Ciluprevir; Boehringer Ingelheim Pharmaceuticals Inc.) (268). This small organic molecule, originally based on a hexapeptide, was administered to ten patients chronically infected with HCV genotype 1. Ten patients were treated with 200 mg BILN 2061, administered twice daily for two days. A dramatic 2 to 3 log10-fold or greater reduction in viral load was observed in all treated patients 24 to 28 hours after oral administration of the compound. This impressive viral decline was followed by a viral rebound in all treated persons with HCV RNA levels returning to pre-treatment levels within 6 to 13 days after initiation of the treatment. Three other clinical trials, comprising in total 51 patients, confirmed the efficiency of the BILN 2061 compound (269). Unfortunately, this NS3-serine protease inhibitor has only minimal effect in patients chronically infected with HCV of genotypes 2 and 3 (270). Nevertheless, additional and larger clinical studies are currently on hold due to cardiac toxicity of the compound in animals.

Another viral protease inhibitor, VX-950 (Vertex Pharmaceuticals Inc.), already proved its effectiveness in a Phase Ib study. Patients treated with this compound achieved a median 4.4 log10-fold reduction of HCV RNA at the end of 14 days of treatment (271). These results definitely encourage the start of a Phase II trial.

Another potential target for antiviral therapy is the viral polymerase NS5B. Two compounds are currently in Phase II clinical trials: NM283 (Valopicitabine; Idenex Pharmaceuticals/Novartis) and JTK-003 (Japan Tabacco). The former compound is a chain-terminating cytidine analogue while the latter is a non-nucleoside allosteric inhibitor. Another non-nucleoside allosteric inhibitor HCV-796 (ViroPharma/Weyth) is currently in a Phase Ia trial.
Animal models for the study of HBV and HCV.

It is clear that current therapies against HBV and HCV need to be improved. Still, a majority of people chronically infected with one of these hepatotropic viruses cannot be cured. New antiviral compounds are continuously being designed and even existing compounds that are effective against other viruses, like HIV, are evaluated for the treatment of HBV and HCV. Therefore suitable in vitro and in vivo models should be available. This has been a major problem for a long time due to the lack of a simple and robust cell culture system (208). Chimpanzees were the first, and probably the best model animals for the study of HBV and HCV. However, ethical, practical, and financial constraints have hampered the use of these primates. Alternative animal models were sought and used, some with more success than others. Here we give an overview of the different animals and in vivo strategies applied for the study of HBV and HCV infections.

The chimpanzee.

The chimpanzee genome (Pan Troglodytes) has a 98.77% sequence homology with the human genome (272). Therefore the chimpanzee has been routinely used for the study of human diseases. Despite this high genomic homology, some important immunological differences are present. Humans and chimpanzees do not share any HLA class I alleles (273); HLA-A2 alleles are absent in chimps (274) but they have more sequence diversity in the MHC region (274, 275). In addition, there are also considerable differences in the MHC class II region (276-278). These and other differences make that the disease pattern and outcome in chimpanzees are not necessarily the same as in humans.

a. Hepatitis B infection in the chimpanzee.

The first indications that the chimpanzee could be a useful animal for the study of hepatitis B virus infections came when HBsAg appeared to be present in the serum of a considerable amount of animals caught in the wild (279). In 1972, Robert Purcell’s group was the first to demonstrate successful de novo infection of chimpanzees with HBV (280). They inoculated two chimpanzees with plasma from HBV infected humans and these resulted in a transient appearance of HBsAg in the blood which was followed by anti-HBs seroconversion. In 1977, the chimpanzee played a major role in the identification of the infectious virion (281).

Like humans, chimpanzees can experience the two known types of HBV infection: acute self-limited hepatitis and chronic hepatitis (282). In the acute phase, chimpanzees mount a cellular immune
response similar to what is observed in acutely infected humans (283), which indicates that the chimpanzee is a valuable and adequate animal to study immune responses towards HBV and to evaluate the efficacy of vaccines. However, the absence of any overt chronic liver disease prevents the study of liver cirrhosis and hepatocellular carcinoma. It is however useful to study the early events after viral infection. In humans, such studies are nearly impossible since the acute phase in HBV infection is generally asymptomatic (284).

During the eighties, large numbers of chimpanzees were not only used to assess the immunogenicity and protective effectiveness of HBV vaccines, but also to evaluate the safety of plasma derived vaccines. All new batches from different manufacturers needed to be injected in more than 100 chimps without causing infection, before these could be approved for human use (283). The safety of blood derived products, such as coagulation factors, and new sterilization protocols for the inactivation of virions, without loss of the biological activity of these derivatives, were also evaluated in these highly evolved primates (285).

Since the availability of a safe and efficacious recombinant yeast-derived vaccine and the accessibility of very sensitive methods to detect minute amounts of viral particles in blood products, the use of chimpanzees for the study of HBV has been drastically reduced.

b. Hepatitis C infection in the chimpanzee.

The chimpanzee also played a crucial role in the discovery of HCV. In 1978, chimps were found to suffer from non-A, non-B hepatitis (286-288). This was only three years after this disease was first observed (136). Non-A, non-B hepatitis was called HCV after the causative agent was partially cloned in 1989 (137).

The cloning of the virus was a major breakthrough in the molecular characterization of HCV. This consequently led to the development of molecular clones that have been used as well defined infectious agents. Direct intrahepatic injection of in vitro synthesized RNA transcripts (289-292) results in acute hepatitis. In contrast to virus isolated from acute and chronic plasma samples, molecular clones have the advantage that the resulting infection is caused by a single, well characterized HCV sequence instead of different quasispecies. Molecular clones with artificially introduced mutations or deletions are excellent tools for the functional analysis of the HCV genome (293, 294), and to define the impact of naturally occurring mutations.

As with HBV infections, the initial phase after HCV infection is very difficult to study since the infection will only be recognized at the onset of acute hepatitis, 6 to 8 weeks after infection. Because acute HCV infection in humans is generally asymptomatic, chimpanzees are the only tool to study the events early after infection. In chimpanzees it is easier to repeatedly
take blood and liver biopsies, thus providing a detailed overview of the events during HCV infection. This way it was discovered that only three days after inoculation of the virus, viremia became detectable in the serum. Three days later, viral proteins could be discerned in the cytoplasm of the infected hepatocytes. ALT values were elevated from two weeks after exposure, while antibodies to structural and non-structural proteins appear after at least 10 weeks (295-299). The amount of viral particles present in the serum ranges between $10^5$ and $10^7$ genome equivalents per milliliter. The study of cellular responses showed that an early, strong and multispecific intrahepatic T cell response is indicative for acute resolving hepatitis (300-302).

The disease progression of HCV in chimpanzees closely resembles that observed in humans. The chronicity rate of HCV infections in humans is estimated between 70 and 85% (229). In chimpanzees this is only 39% according to some (303), and 60% according to others (296, 299, 304). The liver disease in chimpanzees is significantly milder than in humans. Fibrosis and cirrhosis have never been observed, and the development of hepatocellular carcinoma has only been detected in one animal (305).

The chimpanzee was and is still being used to evaluate several HCV inactivation protocols (306-309). The animal is still instrumental for the development of prophylactic and therapeutic vaccines against HCV (310-314).

Besides humans and chimpanzees, several other primates like cynomolgus monkeys, green monkeys, rhesus monkeys, Japanese monkeys and the doguera baboon have been inoculated with HCV. However, none of these species were susceptible to HCV infection (315).

Although the chimpanzee is still the best available animal model for the study of viral hepatitis, the use of these animals has been hindered by several aspects. Due to large-scale hunting and the intensification of agriculture in their main habitats, the wild chimpanzee has been listed as an endangered species since 1988. The use of chimpanzees as laboratory animals is also very expensive as the result of strict regulations during (caging requirements, maintenance ...) and after (retirement in semi-wild sanctuaries) experimentation. In addition, their high similarity to humans undoubtedly raises serious ethical concerns.

To circumvent these obstacles, alternative animal models were sought that were smaller, cheaper, easier to handle, raised less ethical questions and, of course, that allowed infections with HBV and HCV.

**Tree shrews.**

Tree shrews are small non-rodent mammals, originally considered to be lower non-human primates, but later classified into a separate order Scandentia (316). The
family Tupaiidae is divided in two subfamilies: the diurnal Tupaiinae that contains the genera *Anathana, Dendrogale, Lyonogale, Tupaia* and *Urogale*, and the nocturnal Ptilocercinae that contains *Ptilocercus* as the sole genus. Tree shrews are geographically distributed in an area extending from India to the Philippines and from Southern China to Java, Borneo, Sumatra and Bali (316).

Yan et al. were the first to report *in vivo* infection of *Tupaia belangeri chinensis* with human HBV (317). Animals captured in the wild can be infected with HBV and approximately 55% of those inoculated with HBV show signs of infection for more than 41 weeks. Successive infections from one tree shrew to another were effective in 94% of cases, indicating adaptation of the virus to the new host. However, administration of a hepatitis B vaccine to the tree shrews prevented the successful establishment of an experimental HBV infection with a protection rate of 89%.

These researchers were also able to demonstrate a synergistic role of exposure to HBV and aflatoxin B1 (AFB1) in the development of hepatocellular carcinoma (HCC) (318). Tree shrews infected with HBV and exposed to AFB1 suffered from an increased incidence of HCC (52.94%) compared to animals solely infected with HBV (11.11%) or exposed to AFB1 (12.50%) (318), supporting an etiological relationship between HBV, AFB1 and HCC.

Almost simultaneously Walter *et al.* confirmed the results of Yan and colleagues. They showed that newborn tupaias could be easily and reproducibly infected with HBV, resulting in a serologic course typical of acute self-limited HBV infection. Two to 4 weeks after inoculation, HBsAg could be detected in the serum, followed by the appearance of antibodies against HBeAg, HBeAg and HBsAg (319). By contrast, infection of adult animals resulted in a very short, transient production of viral antigens and a rapid seroconversion to anti-HBc, anti-HBe and anti-HBs antibodies after 2 to 4 weeks. While immunosuppressive treatment prior to inoculation augmented the infection, still only 0.1 to 1% of hepatocytes in the liver showed a positive nuclear staining for HBcAg while HBsAg could never be detected immunohistochemically, demonstrating the weakness of the generated infection.

The tree shrew has recently been used to evaluate the protective capacity of a new DNA vaccine (320).

The logical next step after HBV infection was, of course, to examine HCV infections of tupaias. This was successfully accomplished in 1998 by Xie *et al.* (321). In 34.8% of *Tupaia belangeri chinensis* inoculated with genotype 1b, HCV RNA could be detected in serum. Viremia was transient (25%) or intermittent (75%) in a period of 2 to 47 weeks post inoculation. In only 37.5% of HCV RNA positive animals HCV-specific antibodies could be detected, while in 15% of all animals HCV-specific antibodies could be detected in the absence
of viral RNA. In a second study from the same group only 20% of tupaias, inoculated with a mixture of genotypes 1a, 1b and 3, became infected. Total body irradiation (750 cGy) augmented the infection rate to 50%, viremia was prolonged and the antibody response was augmented. Nonetheless, viral titers were on the low side, equal to or below \(10^4\) to \(3.5 \times 10^5\) viral copies per ml serum.

Tupaias may become a useful alternative for chimpanzees in the study of hepatitis B and C. These small animals, which have the size of a small rat, adapt easily to the environment in animal facilities and retain a high reproducibility in captivity. This results in lower costs and overcomes the problem that wild tupaias are frequently already infected with viruses, such as the tree shrew herpes virus which may cause spontaneous hepatitis (322).

**Surrogate models for HBV.**

**a. The woodchuck.**

Infection of woodchucks (Marmota monax) with the woodchuck hepatitis virus (WHV) was first described by Summers et al. (323). In a colony of woodchucks high rates of chronic hepatitis and even hepatocellular carcinoma were observed. Viral particles with properties similar to those of HBV were observed in the serum of these animals.

The WHV is a member of the Orthohepadnavirus genus, and has a genetic organization and replicative cycle similar to HBV. The disease progression in woodchucks with a natural chronic WHV infection resembles that in humans infected with HBV. Chronic, active hepatitis with portal mononuclear cell infiltration, bile duct proliferation, hepatic neoplasms consisting of well-differentiated HCC’s, and even fibrosis are observed (324, 325).

Woodchucks with a natural chronic WHV infection were soon replaced by animals wherein the infection was introduced experimentally. That way the duration of infection was well known and any influence of unknown nutritional and environmental factors on the disease progression was avoided. In addition, hepatic lesions caused by other pathogens, like certain nematode species, were common in wild woodchucks (326), complicating the interpretation of experimental results.

Experimental WHV infection in adult animals routinely results in an acute self-limited infection (327-329). Unless the animals are undergoing an immunosuppressive regimen, the WHV infection rarely evolves towards a chronic infection (330, 331). A broad and robust humoral and cellular immune response is responsible for the clearance of the virus from the infected hepatocytes (332-334). This is really impressive since virtually all the hepatocytes become infected (335).

Because a chronic WHV infection was difficult to induce in adult animals, newborn woodchucks were inoculated with virus. The rate of chronicity in these
neonates was higher than 60% (336-338). Interestingly, all animals that suffered from a chronic WHV infection developed HCC, resulting in a reduced life expectancy (336, 339). While HCC was not observed in uninfected control animals, 17% of woodchucks with a resolved WHV infection still developed HCC (339). These data provided the first experimental evidence of the carcinogenesis of mammalian hepadnaviruses.

Since the pathology and severity of disease of a chronic WHV infection correlated very well with the disease pattern of HBV, the woodchuck became one of the most attractive animal models. It was extensively used to study the development of HCCs and to elucidate the impact of WHV gene expression, viral DNA integration into the host genome and certain environmental factors (340). In addition, it also proved to be a valuable tool in the preclinical evaluation of new antiviral drugs like lamivudine (341-343), famciclovir (343), emtricitabine (344), entecavir (126, 345), clevudine (346, 347), telbivudine (348, 349) and tenofovir (350)

b. The duck.

Another practical surrogate model for the study of HBV is the Peking duck, which can be infected with the duck hepatitis B virus (DHBV). Congenital infection or infection at early age usually results in a chronic infection, but is not associated with severe liver disease and HCC (351-354). Like the WHV, infection of adult ducks results in infection of almost all the hepatocytes but resolves rapidly thereafter without substantial hepatocellular damage (355).

The DHBV model has been very important in the study of hepadnaviral replication. The first functional description and further characterization of cccDNA in the viral life cycle was done in this model (18, 356-358). Of course, the duck also has been used to evaluate the efficacy of new antiviral compounds (127, 359-365). A serious disadvantage is that Peking ducks are less sensitive to toxic effects of chemical compounds, thus preventing the prediction of potential severe side effects in humans (366, 367).

Surrogate models for HCV.

a. The tamarin.

In the eighties, several reports described successful HCV infection in two tamarin species: Sanguinus mystax and S. labiatus (368-371). However, the infection rate in these small New World primates was disappointingly low and the experimental results were rather inconsistent. Later, another tamarin species, Sanguinus Oedipus Oedipus proved resistant to an experimental HCV infection (372).

Although tamarins cannot be infected with HCV, they can be infected with the hepatitis GB virus B (GBV-B) (373, 374). The GB virus was discovered after injection of sera from patients suffering from hepatitis into tamarins. One sample,
isolated from a surgeon with initials G.B., caused an acute hepatitis in these animals and could be passaged to new tamarins. Whether the GB virus was indeed present in the original serum sample from that patient or whether it was already present in the animals used remains unclear.

GBV-B belongs to the Flaviviridae family and is phylogenetically the closest related virus to HCV: it has a similar genomic organization and the viral polyprotein has a 27% sequence homology (375). GBV-B causes an acute infection in tamarins characterized by increased ALT levels and transient high viremia ($>10^9$ genome equivalent/mL). Ultimately, the viral infection is cleared between 10 to 26 weeks (373, 374). The duration of viremia can be prolonged to 46 weeks if the animals are undergoing an immunosuppressive therapy at the moment of GBV-B infection (376). Eighteen weeks after the end of immunosuppressive therapy the tamarins cleared the virus. During clearance there was no increase in ALT levels in the plasma, indicating a non-cytolytic mechanism.

Marmosets (Callithrix jacchus) infected with GBV-B experience high viremia ($10^8$ to $10^9$ genome copies/mL) for a period of 40 to 60 days. The infection is cleared 60 to 80 days postinfection. A 4 day therapy of GBV-B infected marmosets with a NS3 protease inhibitor resulted in a 3-log-fold decrease of viremia (377).

This shows that tamarins and marmosets can be successfully used for the evaluation of new antiviral compounds.

**Rodent models for HBV and HCV.**

*a. Rats.*

The last decades, rats have proven their usefulness in several research areas. Like mice, these rodents are easily maintained, bred and handled at a reasonable price in standard animals facilities and do not require the special care like chimpanzees do. In addition they overcome several limitations, like blood volume, associated with mouse studies.

Several attempts were done to induce active HBV infection in rats, mostly with limited success. Takahashi et al. first demonstrated HBV infection in rats by direct injection in the liver of a replication competent cloned HBV construct (378). Three days after transfection viral DNA and RNA could be demonstrated inside the liver and HBeAg and HBV virions were present in the sera of the animals. From then on, the amount of virions rapidly declined while HBeAg could be detected up to day 7, followed by the appearance of HBeAg specific antibodies. Transfection with HBV genomes of athymic nude rats prolonged the presence of HBV virions to up to 21 days (378).

One year later, an alternative rat model was presented. Here, high titered serum from a chronic HBV patient was injected in the portal and caudal veins (379). One to 2 months later, the presence of HBV DNA and HBSAg could be demonstrated inside the liver but neither viremia nor antigenemia were detected, limiting the usefulness of the model.
Wu and colleagues created a novel rat model by tolerizing immunocompetent rats to human cells by injecting primary human hepatocytes through the uterine wall into the peritoneal cavity of fetal rats on day 17 of gestation (380). One day after birth, these rats were again transplanted with human hepatocytes and one week later HBV was administered to the chimeric animals. From day three after infection, increasing amounts of HBsAg could be detected in serum, reaching maximal levels of 0.75 pg/ml and remaining constant throughout the 60 day observation period. About 30% of human hepatocytes residing in the liver, of which the total amount was not mentioned, stained positive for HBsAg. Active infection was confirmed by the presence of HBV DNA in serum (ranging from 5,000 to 50,000 copies per ml) from 1 to 15 weeks post infection, and by cccDNA in liver tissue as well as in serum. Elevations of serum alanine aminotransferase levels were indicative for a host immune response towards the infected hepatocytes (381). Since there is certainly no match between the human MHC on hepatocytes and the T-cell receptor on the rat leukocytes, the hepatocyte damage is probably the consequence of non-specific immune mediated toxicity.

Recently, this model was adapted for the study of HCV (382). Immunocompetent rats were tolerized and subsequently transplanted with Huh-7 hepatoma cells. One week later, the transplanted rats were inoculated with HCV. Viral infection persisted for several weeks but serum levels of HCV RNA reached maximum levels of only $2 \times 10^4$ copies/ml. Human serum albumin levels, indicative for the amount of human cells present in this rat chimera, ranged from 1 to 10 µg/ml, which is very low compared to other chimeric animal models (see below).

It is clear that viral replication can be achieved in this rat model, but the low levels of hepatocyte engraftment, the use of hepatoma cells and the low viremia are weaknesses that limit the suitability of this model for the study of hepatotropic viral infections. The mismatch between human and rat MHC molecules definitely prevents the study of adaptive immune responses towards the infected hepatocytes. In addition, the presence of a functional rat immune system excludes the transplantation of selected human lymphoid cells, like HCV-specific T-cell clones.

b. Transgenic mice.

Transgenic mice are produced by embryo microinjection of gene constructs. In principle, any gene can be used under the control of any specific promoter. This way, targeted expression of HBV or HCV genes to the liver can be achieved.

HBV transgenic mice. The first HBV transgenic mice were created in 1985. Two independent groups simultaneously published the development of HBsAg transgenic mice (383, 384). These mice produced subviral HBsAg particles and
secreted them in high concentrations into the circulation. Remarkably, the transgenic mice were tolerant to HBsAg and no liver disease was observed. Overexpression of large envelope protein resulted in the accumulation of the viral envelope proteins in the cell (385). Later it became clear that this retention had a disastrous effect on the physical condition of the hepatocytes (386). Long filamentous HBs particles accumulated in the dilated endoplasmatic reticulum. The hepatocytes became enlarged and acquired the characteristics of “ground glass” hepatocytes. Subsequently, these cells became necrotic and multifocal nodular hepatocellular hyperplasia became apparent. Ultimately, this led to the development of hepatocellular carcinomas (387). This was the first evidence that overexpression of the large envelope protein can be directly cytopathic, and could lead to the development of HCCs. Ground glass hepatocytes can also be found in human livers chronically infected with HBV, albeit at lower frequencies (388). Transgenic expression of the HBV X protein also can lead to the development of HCCs, suggesting an oncogenic effect of this viral protein (389). This probably occurs by inactivation of the tumor suppressor p53 after binding with the X protein (390). These results were contradicted in another HBV-X transgenic mouse model (391). HBV precore and core transgenic mice have also been generated (5, 392).

Although HBV transgenic mice have been very useful to study the biochemical properties of the viral proteins and their interactions with other cellular proteins, they cannot be used to study the complete replication cycle of the virus. Viral replication has been demonstrated in several different HBV transgenic mice (393-396), but the episomal HBV cccDNA has never been detected. Viral particles derived from transgenic mice were infectious in chimpanzees (284). The animals can however be used to study the immune responses towards the viral proteins. Although the transgenic mice themselves are immunotolerant to the transgenes expressed, selected lymphocytes from non-tolerant mice can be adoptively transferred to the transgenic mice. After transplantation with HBsAg-specific cytotoxic T lymphocytes (CTL), the HBsAg transgenic mice suffered from CTL-induced necroinflammatory liver disease (397). While a direct effect of the CTLs can be observed, the majority of liver disease is caused by an indirect, antigen-nonspecific cytotoxic effect of other inflammatory cells that are recruited and activated by the CTLs (398). Although the liver disease induced is only transient and generally mild, retention of HBsAg can cause a fulminant hepatitis resulting in the death of the animals. Antibodies against IFN-γ or inactivation of the resident macrophages completely abolishes this fulminant hepatitis proving their essential role in this process (398). Immunodeficient HBV transgenic mice also support HBV gene expression and replication (399). Plasma from these mice
contained between $10^5$ and $10^7$ genome equivalents/ml but adoptive transfer of unprimed, syngenic splenocytes resulted in a chronic hepatitis that persisted for over 9 months.

Using HBeAg transgenic mice, the tolerogenic capacity of HBeAg was demonstrated (5). T-cells from the non-transgenic progeny of these transgenic mice were tolerant to HBeAg and HBcAg, probably through thymic deletion of nucleocapsid-specific T-cells during development.

**HCV transgenic mice.** Mice containing the genomic code for one or more HCV proteins have also been produced. Koike et al. produced a transgenic mouse containing both viral envelope proteins E1 and E2 (400). Long-term follow-up of these mice did not show any evidence of hepatocellular damage. Several other HCV transgenic mice, containing one or both of the envelope proteins in combination with the core gene under the control of a liver specific promoter, also did not suffer from any liver disease (401, 402). In contrast, two other groups described progressive hepatic steatosis in HCV transgenic mice containing the genetic code of either the viral core protein, the nonstructural proteins or even the entire HCV ORF under the control of liver specific promoters (403, 404). At later age, these animals developed hepatic tumors (404, 405). The reasons for these conflicting data are unknown, but the level of gene expression and the different mouse strains used in this research may have been contributing factors.

Using a NS5A transgenic mouse, it was shown that this viral protein can inhibit TNF-mediated hepatic apoptosis by interference in the TNF-signal transduction pathway (406).

A general major drawback on studies conducted with HBV and HCV transgenic mice is that the viral proteins a usually overexpressed in an uncontrolled fashion. The concentration of viral proteins during a natural infection may be a lot lower and well controlled. Therefore the results must be interpreted with caution. Certain qualities attributed to these viral proteins might be related to this artificial overexpression.

Another limitation of transgenic mice is that viral infection, entry into the hepatocyte and viral spread to other neighboring cells cannot be studied.

**Trimera mice.** The first human-mouse chimera suitable for viral hepatitis studies was the Trimera mouse. This mouse was originally developed for the engraftment of human hematopoietic cells and tissues (407, 408). The name ‘Trimera’ refers to the three different genetic backgrounds of the tissues used to create this mouse model. After lethal total body irradiation, normal or BNX (beige/nude/X-linked immunodeficient) mice are rescued
with SCID bone marrow and subsequently small human liver fragments are transplanted under the kidney capsule.

When HBV infected or *ex vivo* HBV infected human hepatocytes are transplanted in these animals, viremia can be detected from day 8 on and lasts for about one month after transplantation (409). The mean viral load ranges from 7x10^4 copies/ml to 2.5x10^5 copies/ml, depending on the time point. This model was used to evaluate the antiviral activity of two reverse transcriptase inhibitors (Lamivudine and β-L-5-fluoro-2,3-dideoxycytidine) and the therapeutic effects of human polyclonal anti-HBs antibodies (Hepatect) (409) and two monoclonal anti-HBs antibodies (410).

A few years later, Ilan and coworkers demonstrated that the Trimera mouse could also be used for the evaluation of compounds with possible anti-HCV activity (411). Therefore, human liver fragments, infected *ex vivo* with HCV, were transplanted under the kidney capsule. Viremia also lasted for about one month and viral titers ranged between 10^4 and 6x10^4 HCV RNA copies/ml. In this HCV-Trimera model, an HCV IRES inhibitor and a monoclonal antibody against the HCV E2 envelope protein were shown to selectively reduce the viral load during therapy.

Both the HBV- and HCV-Trimera mice are useful instruments to analyze the possible antiviral activities of certain compounds. However, the low viral titers and major histological changes within the transplanted tissue, like ischemia, fibrosis, loss of lobular architecture and necrosis are main drawbacks (409, 411, 412). The occurrence of these histologic abnormalities is not surprising since the liver fragments are transferred to an extrahepatic location. Therefore, long-term studies to assess viral hepatotoxicity are excluded.

In addition, it is impossible to use this model to study viral entry and neutralization since *in vivo* infection of Trimera mice transplanted with healthy human hepatocytes has been unsuccessful until now.

**uPA transgenic mice.** Perhaps one of the most promising small animal models for the study of viral hepatitis is based on the Alb-uPA mouse. The Alb-uPA mouse is a transgenic mouse carrying the murine urokinase-type plasminogen activator (uPA) gene linked to the albumin enhancer/promoter. This mouse was originally developed in 1990 in Ralph Brinster’s lab, for the study of plasminogen hyperactivation and the treatment of bleeding disorders (413). The overexpression of the uPA gene in the liver resulted in high plasma uPA levels and hypofibrinogenemia, ultimately leading to severe and sometimes fatal intestinal and abdominal bleeding, early after birth. Another consequence of the hepatocyte specific expression of the uPA-transgene is extensive liver toxicity ultimately leading to hepatic insufficiency (414). This functional
liver deficit provides a chronic, supportive niche for liver regeneration by transplanted hepatocytes of murine origin (415).

To allow liver repopulation by xenogeneic cells, the Alb-uPA transgene had to be backcrossed onto a genetically immunodeficient mouse strain. This approach allowed that rat hepatocytes could almost completely repopulate the diseased mouse liver in Swiss athymic (nu/nu) Alb-uPA mice (416), while woodchuck hepatocytes repopulated up to 90% of the liver of uPA-RAG2 mice (417, 418). The first report of successful transplantation of human hepatocytes was announced in 2001 by Dandri et al. (419). The amount of human hepatocytes present in the mouse liver (up to 15%) was rather low compared to the transplantation efficiencies previously reported with rat and woodchuck hepatocytes. Apparently the quality and the period of ischemia of the human liver fragments from which the primary hepatocytes were isolated played a major role in that. Later it would become clear that also the zygosity of the animals was an important contributor to these low engraftment levels. Dandri et al. only used animals heterozygous for the uPA-transgene. Early regeneration by mouse hepatocytes that have selectively deleted the transgene (414), prevented a more pronounced and stable engraftment by the human hepatocytes. However, the amount of liver tissue of human origin was sufficient to establish an HBV infection by injecting serum from a chronic HBV carrier. HBV infection was demonstrated by the presence of viral DNA in the serum of the infected mice (4.5 to $10^8$ genome equivalents/ml), and was confirmed by immunohistochemical detection of HBcAg in liver sections.

A few months later, a Canadian group utilized homozygous uPA+/−-SCID/Beige mice for the transplantation of primary human hepatocytes (420). The transplantation of homozygous uPA animals is technically more difficult due to the narrow transplantation window early after birth and the susceptibility to bleeding, but the engraftment rate is much higher. Histologic analysis showed that more than 50% of mouse liver parenchyma was replaced by hepatocytes of human origin, resulting in high serum levels of human albumin, sometimes exceeding 1 mg/ml. Intraperitoneal injection of HCV containing human serum into successfully transplanted animals resulted in long-term HCV infection. The mouse serum contained viral RNA levels ranging from $10^3$ to $10^6$ RNA copies/ml; negative strand HCV RNA could be detected in the chimeric mouse livers and the viral infection could, in some cases, be maintained for up to 21 weeks.

A nice application of the human liver-uPA-model was the evaluation of an apoptosis-inducing molecule specific for HCV infected hepatocytes (421). An apoptosis inducing molecule (BID) was modified to contain a cleavage site specifically recognized by the HCV NS3/NS4A protease. Cleavage of the BID
precursor molecule by the viral protease activated the cell-death pathway, resulting in apoptosis of the target cell. The efficacy of this approach was evaluated by injecting three doses of an adenovirus, expressing this modified BID, into six HCV infected human liver-uPA mice. This resulted in a two to three fold log decrease of viral RNA in the mouse serum during treatment, and even cleared the infection in animals with low viremia at the onset.

It is clear that the uPA-model is a very useful instrument for the study of viral hepatitis, but it may have numerous applications in other fields as well. Tateno et al. recently reported that this model could also be used for the preclinical evaluation of pharmacological responses of human hepatocytes to new, candidate drugs (422). This is a major step forward compared to the current in vitro cultures of human hepatocytes. In these cultures, the phenotype of the hepatocytes changes rapidly due to the artificial culture matrices. This most likely has a significant influence on the function and behavior of the hepatocytes.
Research objectives.

The *in vivo* study of HBV and HCV has been severely hampered by the lack of an adequate small animal model. Only humans and chimpanzees can acquire a ‘natural’ HBV or HCV infection. It is clear that ethical, practical and financial constraints limit the use of humans and chimpanzees for such studies. We therefore wanted to develop a mouse model that could be used for the study of viral hepatitis, without the limitations mentioned before.

Our immediate goals are:

1. To back-cross the uPA transgene from the B6SJL-TgN(Alb1Plau)144Bri mouse onto the SCID background. This way we will generate a mouse that suffers from a severe liver disease and that has, in addition, a non-functional adaptive immune system. Both these qualities are necessary to allow successful repopulation of the diseased mouse liver by xenogeneic (human) hepatocytes.

2. To devise a method to easily and quickly discriminate between negative, heterozygous and homozygous uPA-SCID mice. Only the livers of homozygous animals can be successfully repopulated by human hepatocytes, provided that transplantation occurs within two weeks after birth.

3. To transplant uPA+/+~SCID mice with freshly prepared primary human hepatocytes and analyze in detail the function, organization and morphology of these human hepatocytes in the chimeric mouse liver.

4. To establish a chronic HBV and HCV infection in the human liver uPA-SCID mice, and to evaluate any possible effect of HBV and HCV infection on the function, organization and morphology of the human hepatocytes in the chimeric mouse liver.
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Chapter 2: A simple and rapid method to determine the zygosity of uPA-transgenic SCID mice.

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A simple and rapid method to determine the zygosity of uPA-transgenic SCID mice

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Abstract

Successful transplantation of xenogeneic hepatocytes into uPA-transgenic SCID mice depends on the zygosity of the recipient mice. Normally, the difference between homozygous and heterozygous animals is determined via a quantitative Southern blot. We sequenced a part of the mouse genome that is eliminated upon integration of the transgene in the genome. Based on that sequence we developed a multiplex PCR that allows the unambiguous discrimination of negative, heterozygous, and homozygous uPA-transgenic SCID mice in a single day procedure. The speed of the procedure is an essential quality because transplantation of xenogeneic hepatocytes into uPA-SCID mice should be done as soon as possible after birth.

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Keywords: uPA; SCID; Transplantation; Hepatocytes; Genotyping; PCR; Animal model; HBV; HCV

Infections with the hepatitis B virus (HBV) and the hepatitis C virus (HCV) are major health problems. Worldwide chronic infections with HBV and HCV affect an estimated 350 and 170 million people, respectively, and are causing over a million deaths annually [1]. In the past two decades major progress has been made in the prevention and treatment of these infections but the search for more efficacious, tolerable, and affordable treatments for both ailments as well as the quest for a vaccine for HCV go on. This research is hampered by the lack of simple in vitro culture systems and reliable small animal models. Both HBV and HCV have a very narrow tropism and only man and chimpanzee are natural hosts of these viruses. Ethical and financial constraints limit the use of chimpanzees for biomedical research in general and hepatitis-related research in particular. Therefore a small animal model to study HBV and HCV is badly needed. Mice or rats are the prime candidates to create such a model but the stringent tropism of HBV and HCV imposes that hepatocytes from man or chimpanzee are transferred in these rodents. To allow the survival and expansion of xenogeneic donor hepatocytes in mouse or rat, the recipient animal must provide an environment that is permissive for the engraftment and the expansion of the liver cells. Both criteria are met in an immune deficient animal that suffers from a severe liver disease. An animal with both ailments is not available but can be created by crossing the Alb-uPA transgenic mouse with a severely immune deficient mouse strain such as the nude (nu/nu), the SCID/beige, or the RAG-/- mouse [2-4].

The Alb-uPA transgenic mouse, developed in 1990 by Heckel et al. [5] to study plasminogen hyperactivation and therapeutic protocols to prevent bleeding, contains a tandem repeat of four murine uPA genes under the control of an albumin promoter. The transgene overexpression results in profound hypofibrinogenemia and accelerated hepatocyte death. Homozygous animals can be rescued by transplantation of normal hepatocytes from mouse or rat into the Alb-uPA transgenic mouse [6,7]. The Alb-uPA transgenic mouse, developed in 1990 by Heckel et al. [5] to study plasminogen hyperactivation and therapeutic protocols to prevent bleeding, contains a tandem repeat of four murine uPA genes under the control of an albumin promoter. The transgene overexpression results in profound hypofibrinogenemia and accelerated hepatocyte death. Homozygous animals can be rescued by transplantation of normal hepatocytes from mouse [6,7], rat [2], woodchuck [3] or man [4,8] which undergo rapid proliferation to replace the dying hepatocytes. Transplants of hepatocytes from species susceptible to hepatitis viruses can be infected with the homologous hepatitis virus. This was first accomplished with woodchuck hepatocytes infected with the woodchuck hepatitis virus [3] and more recently with human hepatocytes which could be infected with HVB [8] and
HCV [4]. Animals heterozygous for the uPA transgene spontaneously lose the transgene at low frequency and the ensuing ‘rescued’ mouse hepatocytes then repopulate the liver [9]. For this reason heterozygous animals are inferior recipients of xenogeneic hepatocyte grafts compared to uPA homozygous mice. A rapid and reliable screening method to discriminate homozygous from heterozygous transgenic mice is therefore important for successful hepatocyte transplantation.

Initially this analysis of zygosity was performed by quantitative comparison of the Southern blot signal produced by probes for an endogenous gene and the transgenic uPA gene [2]. This method is time consuming and may generate ambiguous results. Rhim et al. also noted that the insertion of the uPA transgene into the mouse genome resulted in the deletion of endogenous DNA at the integration site. These investigators picked-up part of this sequence in plasmid p3371C and used this as a probe to analyze the genotype of the animals. Using a multiplex PCR based on this deleted endogenous sequence we designed a fast and reliable assay that allows for the unambiguous determination of the zygosity of the transgenic animals.

Materials and methods

**Breeding of the uPA-SCID mice.** B6SJL-TgN(Alb1Plau)144Bri mice were back-crossed on CBySmn.CB17-Prkdcscid mice. Both strains were purchased from the Jackson Laboratories (Bar Harbor, Maine, USA). Screening for the SCID background was done with an in-house mouse sandwich Elisa. Screening for the uPA transgene was done by PCR, initially using the genotyping protocol described by the Jackson Laboratories [10] and subsequently by the in-house method described herein. All mice were bred and handled under specific pathogen free (SPF) conditions in the SCID mouse facility of the Department of Clinical Chemistry, Microbiology and Immunology, Ghent University.

**Amplication, isolation, and sequencing of plasmid p3371C.** *Top 10* *Escherichia coli* cells (Invitrogen, Merelbeke, Belgium) were transformed with plasmid p3371C (kindly provided by Dr. R. Palmiter) by heat shock and grown overnight on LB-plates containing Ampicillin. Resulting colonies were resuspended in LB-broth containing ampicillin and incubated overnight at 37°C on a shaking platform. Cells were centrifuged and plasmid was isolated with a HiSpeed Plasmid purification kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol.

Plasmid p3371C was then sequenced with an ABI 310 sequencer (Applied Biosystems, Lennik, Belgium). Briefly, 500 ng plasmid, 3.2 pmol primer, and 8 μl of the Terminator Ready Reaction Mix (Applied Biosystems, Lennik, Belgium) were denatured at 90°C for 30 s. Annealing (15 s at 50°C) and polymerization (4 min at 60°C) were repeated for 25 cycles. The resulting product was then purified using ethanol/sodium acetate precipitation and analyzed on the sequencer. For the unambiguous determination of the zygosity of the transgenic animals.

**Isolation of mouse genomic DNA.** Genomic DNA was extracted from the mouse tail using the Nucleospin Tissue Kit (BD Biosciences Clontech, Erembodegem, Belgium) according to the manufacturer’s protocol. Briefly, tissue from the mouse tail was digested with Proteinase K at 56°C for 3 h or until the sample was completely lysed. After addition of a chaotropic agent the samples were incubated for 10 min at 70°C. Then ethanol was added and the sample was loaded on the Nucleospin columns and centrifuged for 1 min at 11,000g. The column containing the trapped DNA was washed twice to remove any contaminating proteins and other cellular debris. After contaminants were spun through the filter, nucleic acids were eluted in 100 μl of a Tris-buffered solution. All enzymes and buffers were provided by the manufacturer.

**PCR.** The PCR mix consists of 49 μl of thermophilic DNA polymerase buffer supplemented with 200 μM dNTPs (Roche Diagnostics, Vilvoorde, Belgium), 2.5 mM MgCl₂, 1 μM of each primer, and 2.5 U Taq DNA polymerase (Promega, Leiden, The Netherlands). To this mixture 1 μl of mouse genomic DNA was added. The PCR cycling profile was as follows: an initial denaturation step of 5 min at 94°C was followed by 30 cycles of 30 s denaturation at 94°C, 30s primer annealing at 62°C, and 30s primer extension at 72°C. To promote completion of partial extension, the samples were held at 72°C for 5 min. The samples were then stored on ice until analysis on a 2.5% agarose gel.

Results

**Sequencing of plasmid p3371C.**

Rhim et al. [2] noted that the insertion of the uPA transgene into the mouse genome resulted in the deletion of endogenous DNA at the integration site. These investigators picked up part of this sequence in plasmid p3371C (Fig. 1) and used the BamHI-fragment of this region as a probe for detection via Southern blotting. The sequence of the deleted region was unknown but part of the plasmid has been sequenced. Starting from this known sequence we performed serial overlapping sequence reactions until we completely elucidated the code of the deleted region. All the known restriction sites inside p3371C were used as positive markers during sequencing. The *BamHI* fragment (underlined in Fig. 1), previously reported to be approximately 550 bp long, consisted of the following 664 bp long sequence:

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**Fig. 1. Map of plasmid p3371C containing a 2 kb part of the mouse genome which has been deleted at the uPA transgene integration site.** The grey bar represents a known sequence which was used as the starting point for the sequencing reactions. All unknown parts are represented by a black bar. Enzymatic digestion of plasmid p3371C with *BamHI* produces a 664 bp fragment which was previously used as a probe for Southern blotting.
GGATCCAGCTCAAGGGAAGGCTCCAAGGCCT
GACACTTACTGATGCTATGGTGAATATTCTCTC
ATTATAAGGGTGACAGAAATAAGAGAAGAAT
TTACATAAAAAGTAAAAGGAACAATGAGTTT
AAAGCAGGGGAGTGTATCCACAGTTGAAATA
ACTACACATTTGCTTTAAAACGTGGAATTAATC
TTCTCTTCTCTTGCCCTCTCACATCATTGAATT
TTGCATATTAAGATATTTGCATGTCCAAGAAG
CAGCTGTTTTTGGAAACTGTTATACTTTCTACT
TTTCTCCATTGCTACTCCAATTGCAAAGACAT
CATCACACAATGTGTGAATGATCCAGAGAGTC
TGATAGCATATCTCCCAAAGGCAGTCATTTCA
AAACTCTAGACAATCACCATCTGCATTGCATG
GGCCAGATTTTCAAACAGGAGGCTCAAAAGTAG
ACTCTGGAAATTTGCTGTGTATTGCACAGA
CAGTAAGTTATGAATTTACCAAGACTTAGAAT
TCCTGCAGCCGGGATCC.

**Multiplex PCR**

Based on the sequence of the deleted region and the nature of the transgene cassette we developed a multiplex PCR based on three primer pairs.

Primer pair one, ApoA#1: 5'-CATCTCGCACCTTTAGCCAT-3' and ApoA#2: 5'-TCTCTGTGCCCAGGAAGGTA-3', is a control primer pair amplifying a 226bp region of the mouse Apolipoprotein A1-gene. This is a control primer pair that should always result in a positive signal.

Primer pair two, uPA#1: 5'-CATCCCTGTGACCCCTCC-3' and uPA#2: 5'-CTCCAAAACCACCCCCTC-3', amplifies a 151bp region within the 3'-untranslated region of the human growth hormone which is present in the transgene cassette. This PCR product allows us to discriminate between uPA negative and positive animals.

The amplification product of primer pair three, uPA#3: 5'-TTCTCTTCTCTTGCCCTCTACA-3' and uPA#4: 5'-TTGAGACCCCTCAAGACAGCCA-3' (both underlined in the above shown sequence), is 301 bp large and is the product of a region within the mouse genome which was deleted when the transgene had entered.

The amplification product of primer pair three, uPA#3: 5'-TTCTCTTCTCTTGCCCTCTACA-3' and uPA#4: 5'-TTGAGACCCCTCAAGACAGCCA-3' (both underlined in the above shown sequence), is 301 bp large and is the product of a region within the mouse genome which was deleted when the transgene had entered.

The combination of the three above-mentioned primer pairs allowed us to determine unambiguously the zygosity of the transgenic animals. Fig. 2 clearly shows that in case of a uPA negative animal (lane 2) two bands appear (226 and 301 bp). Because the transgene is absent the 151 bp band is not present. Lane 4 shows the PCR products of a homozygous uPA animal. Here also only two bands are visible, the 226 control band and the 151 bp transgene band. There is no amplification product from primer set uPA#3 and #4 because the transgene has been inserted in both copies of the chromosome. In case of a uPA heterozygous animal (lane 3) all three bands appear. Heterozygous animals still have one chromosome left where the endogenous DNA has not been deleted, explaining the presence of a 301 bp product. This clearly demonstrates the simplicity and the clarity of this method.

**Discussion**

The need for an affordable, reliable, and ethically acceptable in vivo model to study HBV and HCV infections is high. The uPA-mouse model first described in 1990 represents one of the most promising tools to create this research tool. uPA-transgenic mice bred on an immune incompetent background like SCID, SCID/beige or RAG/C0 allow a repopulation of the sick liver by allogeneic and xenogeneic hepatocytes. When transplanted with human hepatocytes these animals become susceptible to infections with HBV and HCV [4,8]. However, the outcome of the xenogeneic liver cell graft in these animals is largely dependent on the zygosity of the uPA transgene in these animals. Whereas the survival and expansion of the liver cell graft is poor in heterozygous mice, an excellent and sustained engraftment is observed in homozygous animals. Since the success of the transplantation increases when the donor hepatocytes are introduced early after birth, preferably at the age of one to two weeks, a fast and unambiguous method to determine the zygosity of the animals is very important. We designed a one day method based on the loss of a small portion of the mouse genome as a consequence of the transgene integration. We have sequenced this region and used selected fragments from
it as templates for a PCR. Together with two control PCRs, one to detect the mouse Apo A1 region and one to detect the uPA transgene itself, this single tube PCR method meets the requirements mentioned above.

A search in the mouse genome database revealed that a region homologous \( p = 8, \ 9E-58 \) to the deleted sequence was located on chromosome 10, indicating that this is the integration site. Finally it must be stressed that with this method it is impossible to misclassify a negative or a heterozygous uPA mouse as a homozygous animal because the screening method is based on the absence of a signal. Any possible contamination of \( uPA^{+/+} \) chromosomal DNA with, e.g., wild type DNA will result in a heterozygous result. This method thus prevents the occurrence of such errors and as a consequence thereof the waste of very scarce and valuable hepatocytes in erroneous transplantations.

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References

Chapter 3: Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera.

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Morphological and Biochemical Characterization of a Human Liver in a uPA-SCID Mouse Chimera

Philip Meuleman,1 Louis Libbrecht,2 Rita De Vos,2 Bernard de Hemptinne,3 Kris Gevaert,4 Jo¨el Vandekerckhove,4 Tania Roskams,2 and Geert Leroux-Roels1

A small animal model harboring a functional human liver cell xenograft would be a useful tool to study human liver cell biology, drug metabolism, and infections with hepatotropic viruses. Here we describe the repopulation, organization, and function of human hepatocytes in a mouse recipient and the infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) of the transplanted cells. Homozygous urokinase plasminogen activator (uPA)-SCID mice underwent transplantation with primary human hepatocytes, and at different times animals were bled and sacrificed to analyze plasma and liver tissue, respectively. The plasma of mice that were successfully transplanted contained albumin and an additional 21 human proteins. Liver histology showed progressive and massive replacement of diseased mouse tissue by human hepatocytes. These cells were accumulating glycogen but appeared otherwise normal and showed no signs of damage or death. They formed functional bile canaliculi that connected to mouse canaliculi. Besides mature hepatocytes, human hepatic progenitor cells that were differentiating into mature hepatocytes could be identified within liver parenchyma. Infection of chimeric mice with HBV or HCV resulted in an active infection that did not alter the liver function and architecture. Electron microscopy showed the presence of viral and subviral structures in HBV infected hepatocytes. In conclusion, human hepatocytes repopulate the uPA+/−,SCID mouse liver in a very organized fashion with preservation of normal cell function. The presence of human hepatic progenitor cells in these chimeric animals necessitates a critical review of the observations and conclusions made in experiments with isolated “mature” hepatocytes. 

Supplementary material for this article can be found on the HEPATOLOGY website (http://www.interscience.wiley.com/jpages/0270-9139/suppmat/index.html). 

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See Editorial on Page 703

Despite decades of research, the processes that govern liver development and regeneration are only partially understood. A good understanding of the mechanisms that play a role in these processes is important because it may lead to new treatments of human liver diseases. Several in vivo experimental conditions have been used to study liver regeneration and repair and the interaction of different cell types in these processes. These include partial hepatectomy, administration of toxic compounds, or a combination of both, and transgenic expression of certain proteins.1,2 In contrast to in vitro experiments, these approaches raise fewer questions concerning the influence of artificial matrices on the function and behavior of hepatocytes and other liver cell types. Although these procedures have generated useful information on rodent liver regeneration, stem cell activation, and other processes, extrapolating these findings to the
human situation would be imprudent. Therefore, a suitable small animal model to study human liver regeneration is needed.

Successful transplantation of human hepatocytes into mice or rats requires that the recipient animals do not reject the graft and provide a "supportive niche" that promotes engraftment and expansion of the liver cells. The former condition can be attained by using recipient animals that have an inborn (genetic) or acquired (drug-induced) immune deficiency. An environment that favors hepatocyte engraftment is encountered in animals with severe, chronic liver disease caused by the overexpression of a "noxious" protein, as in the urokinase plasminogen activator (uPA)-transgenic mouse.

uPA transgenic mice backcrossed with "severe immune deficient" mice, such as Swiss athymic nude (nu/nu) mice, recombination activation gene 2 (RAG-2) knockout mice, or SCID/beige mice, allow the engraftment and repopulation by xenogeneic hepatocytes. In 2 of these studies, hepatocytes of human origin were used that could be infected with hepatitis B virus (HBV) or hepatitis C virus (HCV) after colonization. These 2 studies provided ample molecular and serological proof of successful repopulation and infection of human hepatocytes. However, they paid less attention to the architecture and organization of the human hepatocytes in this xenogeneic environment.

We backcrossed the same Alb-uPA mouse on the immunodeficient SCID mouse (CBBySmn.CB17-Prkdcscid) and examined with great care the repopulation and organization of the human hepatocytes in this xenogeneic environment.

Materials and Methods

Breeding of the uPA-SCID Mice

B6SJ-L-TgN(Alb1Plau)144Bri mice were, at least 7 times, back-crossed on CBBySmn.CB17-Prkdcscid mice. Both strains were purchased from The Jackson Laboratories (Bar Harbor, ME). Screening for the SCID background was done with an in-house mouse immunoglobulin M (IgM) sandwich ELISA. Genotyping of the uPA-SCID mice was performed via a multiplex polymerase chain reaction as described before. Human hepatocytes were transferred only in animals homozygous for the uPA-transgene. The study protocol was approved by the Animal Ethics Committee of the Faculty of Medicine of the Ghent University.

Isolation of Human Hepatocytes and Transfer into uPA-SCID Mice

Human liver specimens were collected from patients undergoing a partial hepatectomy for the resection of metastatic disease. All patients gave written, informed consent, and all experiments were approved by the Ethical Committee of the Ghent University Hospital. Hepatocytes were isolated from tumor-free sections via a collagenase digestion. Briefly, tissue was first perfused with Liver Perfusion Medium (Invitrogen, Merelbeke, Belgium) and then with Liver Digest Medium (Invitrogen). Subsequently, the digested liver was placed in Hepatocyte Wash Medium (Invitrogen). The cell suspension released from the liver was filtered through a 70-μm cell strainer (BD Falcon, Erembodegem, Belgium) and hepatocytes were separated from non-parenchymal cells via three low-speed centrifugation steps (5 minutes, 50g). Cell viability, measured using the Trypan Blue exclusion test, generally exceeded 90%. One million hepatocytes were injected into the spleen of 6- to 14-day-old uPA-/-+ -SCID mice. At selected moments, mice were bled, and plasma was stored at −70°C until further analysis.

Quantification of Human Albumin

Human albumin in mouse plasma was measured using an in-house sandwich ELISA. Maxisorp Immunoplates (Nunc, Roskilde, Denmark) were coated with goat anti-human albumin antibodies (Bethyl Laboratories, Montgomery, TX). After blocking and washing, diluted samples or calibrators were added, and human albumin bound to the plate was detected with a HRP-conjugated goat-anti-human albumin antibody (Bethyl Laboratories).

HBV and HCV Infection. Five weeks after transplantation, chimeric mice were infected with either HBV or HCV. As a source of infectious HBV, serum from a patient suffering from a chronic HBV infection (hepatitis B surface antigen [HBsAg+]), hepatitis B e antigen [HBeAg+], and anti-HBs) with a high HBV DNA content (107 cop/mL) was used. One hundred microliters of this serum was injected intraperitoneally. To induce an HCV infection, serum from an acutely infected chimpanzee was used (gift from Dr. J. Bukh). Twenty microliters of this serum (strain J4, 3.16 × 104 cop/mL) was injected intraperitoneally.

Detection and Quantification of Viral DNA/RNA and Proteins

HBV DNA and HCV RNA levels were quantified using the Cobas Amplicor HBV Monitor test and the Cobas Amplicor HCV Monitor test v2.0 (Roche Diagnostics, Mannheim, Germany), respectively. HBsAg and HBeAg
were determined with the Axsym HBsAg V2 and the Axsym HBe 2.0 system (Abbott, Chicago, IL), respectively. The antigen levels were expressed as Signal/Noise values.

Detection of Human Proteins in Chimeric Plasma

Fifty microliters unfractionated mouse EDTA plasma was used for a non-gel proteome analysis. The proteins were digested with trypsin, and combined fractional diagonal chromatography (COFRADIC™) was used to isolate the amino terminal peptides out of this complex peptide mixture. The isolated peptides were further analyzed by LC-MS/MS using a Q-TOF1 mass spectrometer (Micromass UK Limited, Cheshire, UK) and the obtained MS/MS spectra were linked to peptide sequences stored in a tailor-made database (http://www.expasy.ch) containing human and murine full and sequentially ragged protein sequences, using the MASCOT database search engine (http://www.matrixscience.com). Peptides identified by a MASCOT score that exceeded the identity threshold score of MASCOT at the 95% confidence level were considered as positively identified.

Table 1. Proteome Analysis of Chimeric Mouse Plasma

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession Number</th>
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<tbody>
<tr>
<td>1 Afamin precursor</td>
<td>P43652</td>
</tr>
<tr>
<td>2 Alpha-1 antitrypsin</td>
<td>P01009</td>
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<tr>
<td>3 Alpha-1-antichymotrypsin precursor</td>
<td>P01011</td>
</tr>
<tr>
<td>4 Alpha-2-HS-glycoprotein precursor</td>
<td>P02765</td>
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<tr>
<td>5 Apolipoprotein A-II precursor</td>
<td>P02652</td>
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<tr>
<td>6 Apolipoprotein E precursor</td>
<td>P02649</td>
</tr>
<tr>
<td>7 Complement C2 precursor</td>
<td>P06681</td>
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<tr>
<td>8 Complement C4b-binding protein alpha chain precursor</td>
<td>P04003</td>
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<tr>
<td>9 Complement C3 precursor</td>
<td>P01024</td>
</tr>
<tr>
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<td>P00751</td>
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<tr>
<td>11 Chloride channel protein 7</td>
<td>P51798</td>
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<tr>
<td>12 Eukaryotic translation initiation factor 4 gamma</td>
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<td>13 Fibrinogen alpha/alpha-E chain precursor</td>
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<td>P02675</td>
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<td>16 Kininogen precursor</td>
<td>P01042</td>
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<tr>
<td>17 Prostaglandin F2 receptor negative regulator precursor</td>
<td>Q0P282</td>
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<td>18 Protein-tyrosine phosphatase delta precursor</td>
<td>P23468</td>
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<td>19 RNA-binding protein 5</td>
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<tr>
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<td>22 Zinc finger protein 70</td>
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NOTE. Overview of the 22 human proteins detected in the plasma of transplanted uPA-SCID mice using a mass spectrometric technique. Most of these proteins are produced exclusively or nonexclusively by hepatocytes. However, in this context, hepatocytes can be the only source. Some proteins are generally regarded to have a membrane or nuclear localization and are probably remnants of dying hepatocytes. Additional information on the above mentioned proteins can be retrieved via the corresponding SwissProt (http://www.expasy.ch) accession numbers.

Results

Plasma Analysis of Chimeric Mice

The success of human liver cell engraftment and expansion in the transplanted uPA-SCID transgenic SCID mice was evaluated by quantifying human albumin in mouse plasma at regular intervals. Figure 1 shows that human albumin concentrations reached a median level of 3 mg/mL by week 4 after transplantation and increased to 7 mg/mL by week 7. Thereafter the albumin levels remained quite constant until at least 14 weeks after transplantation.

To evaluate whether the transplanted human hepatocytes were functional and behaved like mature, normal hepatocytes, we analyzed the human proteome of the mouse plasma. Seven weeks after transplantation, plasma from a successfully transplanted mouse was analyzed by COFRADIC™. We decided to use this particular proteomics method because an a priori in silico study indicated that alike plasma proteins of different origin (mouse and human) mainly had sequence differences at their N-
terminal extremity. We unambiguously identified 22 different human proteins in this mouse plasma (Table 1). Besides these 22 proteins of human origin, several other plasma proteins were detected that were either specifically from murine origin or of which the identified peptides did not allow discrimination between a human or murine origin (data not shown).

Histological Studies of Uninfected Chimeric Livers

Chimeric uPA-SCID Mice 36 and 78 Days After Transplantation. Massive numbers of mature human hepatocytes were identified in the livers of transplanted mice. These could easily be discriminated from their murine counterparts by their larger size and the peculiar appearance of their cytoplasm (Fig. 2A). The human hepatocytes were somewhat swollen and had a rarefied cytoplasm with wisps and small clumps of eosinophilic material in an otherwise empty-appearing cytoplasm. The cell membranes appeared mildly thickened. The empty-appearing areas of the cytoplasm on hematoxylin and eosin staining corresponded to areas of glycogen storage, as shown by periodic-acid-Schiff (PAS) staining (Fig. 2B). The stainings for albumin, mitochondria, pan-cytokeratin, and CK18 confirmed the human origin of these hepatoocytes.
tocytes (Fig. 2C-F), and the cytoplasmic staining pattern was also in wisps and small clumps, indicating that the organelles were displaced and clumped together by the excessive glycogen accumulation. The morphological features of the hepatocytes are identical to the morphology of hepatocytes in all glycogen storage diseases, except type IV.11 This was confirmed by comparing our present findings with 4 archival clinical liver biopsy specimens from patients with glycogenosis and with descriptions of glycogenosis in the literature.11

Apart from this massive glycogen storage, the human hepatocytes appeared quite “healthy” and showed no signs of damage or degeneration. This is in sharp contrast with the extensive damage of mouse liver parenchyma, where ceroid macrophages formed large clusters (Fig. 3A) and focal confluent necrosis of mouse hepatocytes was visible, both indicating moderate to severe hepatocyte damage and loss. Occasionally, human hepatocytes contained a mitotic figure, and steatosis was minimal.

In the liver sections of the 44-day-old mouse, also two red foci were seen. Hepatocytes in red foci were larger than the surrounding mouse hepatocytes. The red foci contained no ceroid macrophages and were devoid of oval cells. The foci were rather sharply demarcated and slightly compressed surrounding mouse parenchyma. These findings indicate that the loss of transgene expression in the cells of these foci leads to disappearance of cell damage and to a growth advantage compared with transgene-expressing parenchyma. Interestingly, most hepatocytes within the red foci had a cytoplasm that was partly positive on PAS staining, whereas surrounding, transgene-expressing hepatocytes were generally PAS negative.

Fig. 3. Five weeks after transplantation, approximately one fourth of liver parenchyma is repopulated by pale human hepatocytes that are organized in nodules. (A) Ceroid macrophages are restricted to the areas occupied by diseased mouse hepatocytes, as shown by PASα staining. (B) Staining with a polyclonal anti-CEA antibody clearly shows connections between mouse canaliculi (arrows) and canaliculi that are somewhat bigger and that are formed by the surrounding human hepatocytes (middle). (C) Staining of the liver with an antibody specific for human Ki-67 showed that proliferating human hepatocytes were preferentially localized at the edge of the nodules. The scarce presence of sinusoids lined by spindle-shaped human endothelial cells was visualized by staining for human CD34 (D). (Original magnifications: A, ×100; B, ×400; C and D, ×200)

Fig. 4. Human hepatic progenitor cells (HPCs) were identified in the livers of chimeric mice at the interface between human (H) and mouse (M) hepatocytes. (A) HPCs are small cells with scanty cytoplasm and a relatively large oval nucleus and strongly immunoreactive for pan-cytokeratin (black arrows). Intermediate hepatocyte-like cells (white arrows) could be recognized as polygonal cells with a size and immunophenotype intermediate between that of HPCs and hepatocytes. Some mature hepatocytes display a decreased and granular staining pattern, possibly as a consequence of the glycogen accumulation. (B) Staining with an anti-CK7 antibody confirms the presence of both these cell types, which are surrounded by mature hepatocytes that do not stain for CK7. Staining of the liver of a non-transplanted uPA-SCID mouse with anti-pan-cytokeratin (C) and anti-CK7 (D) antibodies served as a negative control. (Original magnifications: A and B, ×400; C and D, ×100)
The transversal section through the liver of the 44-day-old mouse showed that the human hepatocytes were organized into approximately 80 nodules, which were well demarcated with sometimes a singular hepatocyte infiltrating into the surrounding mouse liver parenchyma. Between 10 and 90 human hepatocytes were present in the section through the nodules. Between the nodules, there were occasionally singular human hepatocytes or small groups of, maximally, 5 hepatocytes. Overall, 25% of the liver parenchyma consisted of human cells. In contrast to mouse liver parenchyma, these nodules rarely contained ceroid macrophages (Fig. 3A). Only 5 of approximately 80 nodules contained 1 or 2 ceroid macrophages that were not located in the center of the nodule, suggesting that these ceroid macrophages are rather transiting than resident. There was no preferential localization of the human hepatocyte nodules in the liver; they were observed as well periportal as around the centrolobular vein. Areas of confluent necrosis of mouse liver parenchyma always contained human hepatocyte nodules at their periphery.

As shown in Fig. 3B, human hepatocytes formed normal canalicular structures that connected to mouse canaliculi that were smaller. Ki-67 staining showed that 18% of human hepatocytes were proliferating, and these cells were preferentially localized at the edge of the nodules (Fig. 3C).

In the liver of the 90-day-old mouse, human hepatocytes no longer formed nodules but appeared as large areas separated from each other by small areas of mouse liver parenchyma. Overall, the human hepatocytes represented 66% of the mouse liver parenchyma. Again, human areas contained very few ceroid macrophages; 6% of the human hepatocytes were Ki-67 positive and showed no preferential distribution. In both mice, areas consisting of human hepatocytes contained scarce mouse oval cells, at numbers that appeared lower than in mouse liver parenchyma. Sinusoids lined by human endothelial cells were present but rare (Fig. 3D).

Fifteen nodules in the 44-day-old mouse contained, in addition to mature hepatocytes, 1 to 5 human hepatic progenitor cells (Figs. 2F and 4A-B) that were recognized on the basis of their morphology and immunohistochemical phenotype. Hepatic progenitor cells were present both in the center and at the edge of the nodules. Reactive bile ductules containing human cells were not observed. Occasionally, human intermediate hepatocyte-like cells were seen neighboring human hepatic progenitor cells located at the human–mouse parenchymal interface. Similarly, singular human hepatic progenitor cells and intermediate hepatocyte-like cells were focally present at the human–mouse parenchymal interface in the 99-day-old mouse liver. In this animal, one reactive bile ductule with a dilated lumen was completely lined by human cells.

We never observed dysplasia or malignant transformation of human hepatocytes, nor tumor cells of the type for which the patient underwent liver surgery.

**Ultrastructural Analysis.** Human hepatocytes had a normal canalicular and sinusoidal pole and showed no abnormalities, besides the glycogen accumulation. Hepatic progenitor cells of types I (most undifferentiated type) and III (having some hepatic features) were present in the chimeric liver. Some progenitor cells showed signs of damage, indicating a mouse origin, and were forming a chimeric canalicular structure with a human hepatocyte (Fig. 5A). Healthy type III hepatocyte-like cells formed part of a canaliculus with adjacent mature human hepatocytes. The absence of any damage strongly suggests that these cells were of human origin (Fig. 5B).

**In Vivo HBV Infection**

Five weeks after successful transplantation with cells from 3 different donors, 9 animals were infected with HBV. Twenty days later, mouse plasma was analyzed for viral proteins and HBV DNA. All animals showed signs of infection, but in 3 mice the serum markers were rather low, namely, an HBsAg level of 52.5 S/N, HBeAg below the detection limit, and HBV-DNA levels of approximately $2 \times 10^5$ cop/mL. These values changed very little until the animals died. In the remaining 6 mice, the HBV infection was highly replicative, with HBsAg and HBeAg levels of, on average, 1,130 S/N and 663 S/N, respectively, and HBV-DNA levels exceeding $2 \times 10^{10}$ cop/mL.

Twenty-five days after HBV inoculation, 1 animal was sacrificed for histological analysis of the liver. Human hepatocytes were loaded with glycogen and formed large areas occupying 87% of mouse liver parenchyma. No other signs of damage or cell loss were observed. No human hepatocytes had a “ground glass” appearance. Almost all human hepatocytes had a granular cytoplasmic staining for HBsAg, and many were also strongly positive near the cell membrane (Fig. 6A). Figure 6B shows that a large majority of the human hepatocytes displayed a strong nuclear and a moderate cytoplasmic staining for hepatitis B core antigen.

At the interface between red foci and human hepatocytes, there was no sharp border, no compression, and the two cell types appeared to infiltrate each other’s areas. These findings suggest that the human hepatocytes and the “transgene-free” mouse hepatocytes profit equally from the growth stimuli present.

Ultrastructural analysis of the liver of an HBV-infected mouse showed that the nuclei of numerous hepatocytes...
contained noncoated virus particles with a diameter of 21 to 24 nm corresponding to HBV core particles. In the cisternae of the endoplasmic reticulum, 1 or 2 longitudinally transsected tubules and cross-sectioned spheres of HBsAg were present. In some cisternae, core particles surrounded by a clear halo and a dark ring of approximately 40 nm in size, corresponding to Dane particles, were observed (Fig. 6C).

**In Vivo HCV Infection**

uPA+/+_.SCID mice, untransplanted or successfully transplanted with human hepatocytes, were infected with HCV of genotype 1b. In untransplanted but HCV-injected animals, HCV RNA was never detected. Two weeks after infection, the plasma of transplanted animals contained \( \geq 2.35 \times 10^6 \) IU/mL HCV RNA. The viral load increased over time and reached levels of up to \( 8.1 \times 10^7 \) IU/mL by week 10. Plasma from these animals was used to infect other transplanted mice. Three microliters infectious mouse plasma (containing approximately \( 10^5 \) IU HCV RNA) sufficed to establish a new infection. Animals infected in this way also displayed the same massive increase in viral load. Recalculating all viral titers with respect to the blood volume of the infected animals, and taking into account the serial infections, we achieved a \( 10^9 \)-fold amplification of viral RNA. The in vivo HCV infection could be maintained for at least 4 months.

Six weeks after infection, one HCV-infected animal was sacrificed for histological examination. Seventy-seven percent of liver parenchyma consisted of healthy human glycogen accumulating hepatocytes, which formed large areas separated from each other by small areas of diseased liver cells.
mouse hepatocytes. Almost all human hepatocytes showed a granular cytoplasmic staining with the anti-HCV E2 antibody (Fig. 6D). This granular staining pattern has always been observed in liver biopsy specimens from chronic HCV patients. As in the livers of non-infected and HBV infected mice, singular human hepatic progenitor cells and intermediate hepatocyte-like cells were focally present at the human–mouse parenchymal interface. Moreover, some of the ductular clusters consisted of ductules that were lined by a mixture of bile ductular cells of mouse and human origin. Despite careful ultrastructural analysis, no viral particles or viral tubular structures could be retrieved.

Discussion

Recently, two groups independently created chimeric mice harboring human hepatocyte grafts and successfully induced HBV and HCV infections in these animals. Both groups provided convincing evidence for solid hepatocyte engraftment and active viral replication but paid less attention to the functional integrity and structural organization of the transplanted hepatocytes in the xenogeneic environment. We wanted to fill this gap and present here a detailed description of the functional and morphologic characteristics of the chimeric liver before and during active HBV and HCV infections.

To evaluate the human hepatocyte graft-take and expansion in uPA+/−-SCID mice, the human albumin concentration in mouse plasma was monitored at regular intervals. In successfully transplanted mice, the albumin level reached a plateau value of approximately 7 mg/mL (median value) around week 7, and this level remained unchanged in the weeks that followed. Although the human albumin level is a reliable marker of the integrity and functional status of the human hepatocytes, we performed a proteomic analysis of the mouse plasma and demonstrated the presence of 21 additional and less abundant human proteins. Most of these proteins are known plasma proteins synthesized by the liver. Apart from these “classical” human plasma proteins, some human proteins with a membrane or nuclear localization could also be detected. Their presence in the plasma may be due to membrane turnover or release from dying hepatocytes. The presence of these atypical plasma proteins in our chimeric mouse plasma is not unexpected because such proteins are also found in the plasma of healthy blood donors.

The integrity and spatial organization of the human hepatocytes in the chimeric liver was also examined by histology. Early after engraftment, the human hepatocytes appeared as sharp nodules that expanded and became blurred as time went on and occupied up to 87% of liver parenchyma. Although the human hepatocytes resided in a xenogeneic environment, they showed no signs of damage or degeneration and appeared to have remodeled the diseased mouse liver in a progressive and organized fashion. We also demonstrated connections between human and mouse canaliculi. These chimeric ducts must be open and functional, because no signs of cholestasis were found.

The only, but striking, abnormality of the transplanted liver cells was the abundant accumulation of glycogen. The cause of this aberrant polysaccharide accumulation remains unclear, but this phenomenon is most likely attributable to inappropriate recognition of murine signals (e.g., hormones) by human hepatocytes. This feature is independent of HBV or HCV infection, and it is probably not just a compensation for the absence of glycogen in the diseased mouse hepatocytes because mouse hepatocytes in red foci have a normal appearance and normal intracellular glycogen content. The overall appearance of the chimeric mouse and the aspect of the liver cells in histology studies suggest that this metabolic disturbance has a negative effect on the animal as a whole and the grafted hepatocytes in particular. This is confirmed by the fact that uPA+/− mice transplanted with human hepatocytes never looked as healthy as their uPA+/− -SCID littermates that were “rescued” with a murine hepatocyte graft. Although the latter developed normally and after a few weeks could not be discriminated from uPA−/−-SCID mice (P. Meuleman, unpublished data, 2004), the former survived but suffered from severe growth retardation and failure to thrive. This can be a result of the observed glycogenosis, but it is plausible that also other biochemical pathways may not function optimally, possibly because of communication failure between mouse ligands or receptors and their human counterparts. The inconsistency between the albumin levels (approximately 20% of normal values) and tissue analysis (repopulation up to 87%), again indicates suboptimal communication between human cells and the mouse environment. These observations deserve further study considering the growing interest in transplantation of xenogeneic liver tissue or hepatocytes to treat hepatic failure in humans.

Once human hepatocytes had stably engrafted in the uPA+/− -SCID host, these animals could indeed easily be infected with hepatotropic viruses. After an infection with HBV, we could detect high levels of HBsAg, HBeAg, and HBV DNA in the mouse plasma. The concentrations of HBsAg and HBV DNA observed in our experiments largely exceeded those reported by Dandri et al., and these investigators were unable to find HBeAg. This may be linked to the inferior hepatocyte engraftment (5%-15%) obtained in heterozygous uPA animals. The immune-
nohistochemical staining pattern of the infected mouse liver corresponds very well to that observed in chronic hepatitis B patients during the viral tolerance/replication phase.19 However, the proportion of HBV-positive hepatocytes seen in our mouse model is considerably higher than routinely observed in biopsy specimens of such patients.19 This result correlates well with the extremely high amounts of HBV DNA values found in the infected mouse plasma.20,21 We were also able to visualize subviral HBV particles and infectious Dane particles inside the human hepatocytes of our chimeric mice.

Infections of chimeric mice with HCV were as successful as the HBV infections. Viral RNA could be detected in the plasma of all infected mice (n = 7), and HCV RNA levels were much higher than those routinely observed during chronic HCV infections in humans. High viremia of this magnitude can be observed, however, when HCV-infected patients are undergoing immune suppressive therapy after transplantation.22-24Histological analysis of the liver of an HCV-infected mouse showed that most human hepatocytes were infected. Despite careful electron microscopic analysis, no viral or subviral particles could be identified in human hepatocytes. This is not surprising, because these structures are also rarely seen in biopsy specimens of chronically infected patients.13 However, sequential infections proved the presence and de novo production of infectious HCV particles.

Although most human hepatocytes were infected with either HBV or HCV and although the viral replication was high, these viruses had no negative effects on the function, survival, or proliferation of the infected hepatocytes. This finding is in agreement with the current view that hepatocyte damage in patients with active chronic viral hepatitis is induced by the immune system rather than the virus itself.25 Whether long-term infections with HBV or HCV have any negative or cytopathic effect on the transplanted human hepatocytes is currently being explored.

A final, but very important, observation is the fact that the chimeric mouse liver contained human hepatic progenitor cells in addition to mature human hepatocytes. In close association with the human progenitor cells we noticed human intermediate hepatocyte-like cells, which suggests that the progenitor cells are differentiating toward mature hepatocytes through this intermediate stage. Occasionally, human hepatic progenitors were observed within murine ductular structures, illustrating the bipotential characteristics of these cells. The presence of progenitors in all the analyzed hosts indicates that these cells must have been present in the injected cell suspensions. This report suggests the presence of progenitor cells in a cell suspension obtained via a widely used technique for the isolation of hepatocytes. This observation is important because certain qualities attributed to mature hepatocytes, such as the capacity to trans-differentiate into biliary cells26,27 and the extensive potential to repopulate damaged liver,28,29 might be related to the presence of these progenitor cells. Data obtained in previous studies using so-called hepatocyte suspensions should at least be critically reviewed. The number of progenitor cells in the hepatocyte graft and their role in the repopulation of the diseased liver is being examined.

In conclusion, the uPA+/−.SCID mouse easily accepts human hepatocyte grafts, and the chimeric animals thus created represent a successful small animal model to study HBV and HCV infections and new therapeutic compounds to treat these infections. In addition, this model also may prove useful for the study of metabolic aspects of xenogeneic transplantation and the hepatic engraftment, maturation, and differentiation of stem cells of different origins.

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References


Additional Materials and Methods.


Preparation of tissues for histological study.
A part of each fresh mouse liver was snap-frozen in liquid nitrogen-cooled isopentane and stored at -70°C until further use. Another small part of the fresh liver was fixed overnight in 2.5% glutaraldehyde, 0.1 M phosphate buffer followed by a 1 hour postfixation in 1% osmiumtetroxide, 0.1 M phosphate buffer for standard ultrastructural evaluation. After fixation, the samples were dehydrated in graded series of alcohol and embedded in epoxy resins. Ultrathin sections were cut, stained with uranyl acetate and lead citrate and examined using a Zeiss EM 10 electron microscope. The remainder of the liver and the spleen was fixed in B5-fixative for approximately 3 hours. In some cases, a part of the liver was fixed in 6% formalin. After fixation, the tissue was embedded in paraffin.

Histology.
Four µm-thick sections from paraffin-embedded liver tissue were stained with hematoxylin and eosin (H&E) for overall histopathological evaluation, periodic-acid-Schiff (PAS) to assess cellular polysaccharide deposits, PAS after predigestion with amylase (PASα) to detect ceroid macrophages, Sirius red for fibrosis and Hall-Van Gieson to detect bilirubinostasis (1-3). Five µm-thick sections from frozen liver tissue were fixed in acetone for 10 min after overnight drying, followed by an oil red O (ORO) staining for the evaluation of steatosis (2). Frozen tissue was used for assessment of fat (3).

Immunohistochemistry.
Four µm-thick sections were deparaffinized and rehydrated. For most stainings, the sections were heated in a microwave oven in citrate buffer, pH 6.0 for 5 min at 500 Watt, followed by 20 min at ‘defrost’ setting and the slides were then cooled at room temperature for 15 min. This antigen retrieval method was used before the incubation with most of the primary antibodies, except for those against albumin, mitochondria, carcinoembryonic antigen (CEA) and hepatitis B surface and core antigen (HBsAg and HBCAg), since we always obtain adequate staining without antigen retrieval when performing immunohistochemistry with these antibodies on routine clinical liver biopsies processed in an identical fashion as the samples in the present study. Incubation with the primary antibodies was performed at room temperature for 30 min, followed by three washes with PBS. The different human cell types within the mouse livers were studied with a polyclonal rabbit anti-human antibody against albumin (dilution 1/200, Dako, Denmark) and monoclonal mouse anti-human antibodies against mitochondria (clone 113-1, dilution 1/100, Chemicon, USA), pan-cytokeratin (clone KL1, dilution 1/50, Immunotech, USA), cytokeratin 8 (CK8) (clone 35beta11, dilution 1/100, Dako), CK18 (clone CD10, dilution 1/10, Dako), CK7 (clone OV-TL12/30, dilution 1/50, Dako), CK19 (clone RCK108, dilution 1/20, Dako), vimentin (clone V9, dilution 1/30, Dako), and CD34 (clone My10, dilution 1/10, Becton Dickinson, USA) and Ki67 (clone Mib-1, dilution 1/100, Dako). Albumin is a well-established marker of hepatocytes while vimentin is a marker of all mesenchymal liver cells (i.e. (myo)fibroblasts, hepatic stellate cells and endothelial cells (4), while CD34 only stains endothelial cells (5). The different anti-keratin antibodies were used to detect all human epithelial cells. Their staining intensity and pattern and the morphology of the positive cells makes it possible to recognize mature hepatocytes, bile duct epithelial cells and cells of the progenitor cell compartment (i.e. hepatic progenitor cells, intermediate hepatocyte-like cell and reactive ductular cells) (6). The monoclonal mouse anti-human antibody Ki67 (clone Mib-1, dilution 1/100, Dako) was used to assess the proliferative status of human hepatocytes, since it stains all nuclei that are not in the G0-phase (7). Expression of viral proteins was assessed using a polyclonal rabbit antibody against HBCAg (dilution 1/200, Dako) and monoclonal mouse antibodies against HBsAg (clone H1, dilution 1/80, ForLab, Belgium) and HCV E2 (clone IG222, 1/50, Innogenetics, Belgium). Immunohistochemical analysis of liver tissue with the antibody IG222 is a specific and sensitive method to detect HCV infection (8). To evaluate both human and mouse cells, polyclonal rabbit anti-human antibodies against CEA (dilution 1/300, Dako) were used. The polyclonal antibody against carcinoembryonic antigen (pCEA) is a marker for canaliculi (9). For all mouse monoclonal antibodies, the secondary antibody was undiluted anti-mouse Envision (Dako). The secondary antibody was applied for 30 min,
followed by a wash in three changes of phosphate-buffered saline (PBS) for 5 min. A second step was not used for the anti-albumin antibody, since this antibody is peroxidase-labeled while the others are unlabeled. For the rabbit polyclonal antibodies against CEA and HBcAg, the second and third step consisted of swine anti-rabbit immunoglobulins and rabbit peroxidase-antiperoxidase (both from Dako) diluted in phosphate-buffered saline (PBS), pH 7.2, containing 10% normal human serum. The incubations were carried out for 30 min at room temperature and each incubation step was followed by a wash in three changes of PBS for 5 min. For all stainings, the brown reaction product was developed with the use of 3,3-diaminobenzidine tetrahydrochloride and H2O2. The sections were counterstained with hematoxylin.

Sections wherein the incubation with the first and/or secondary/tertiary antibodies were omitted and fully-processed sections of liver tissue from untransplanted animals served as controls. Positive controls consisted of selected liver biopsies from the clinical routine that were stained with these antibodies.

**Quantitative assessment of the different human liver cell types present in uPA-SCID liver.**

To measure the fraction of liver parenchyma represented by human hepatocytes, digital pictures (totally 7 to 8 pictures per liver) were taken with a 5x objective. Using Adobe Photoshop 7.0, the amount of pixels within the total liver parenchyma (excluding large portal veins, areas of calcifications, red foci and areas without tissue) was determined and compared to the amount of pixels within delineated human hepatocyte areas. To quantify proliferating hepatocytes the number of Ki67 nuclei positive hepatocytes was counted in random fields of at least 300 human hepatocytes.

**References.**

Chapter 4: Immune suppression uncovers endogenous cytopathic effects of the hepatitis B virus.

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Immune Suppression Uncovers Endogenous Cytopathic Effects of the Hepatitis B Virus

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It is generally accepted that the host’s immune response rather than the virus itself is causing the hepatocellular damage seen in acute and chronic hepatitis B virus (HBV) infections. However, in situations of severe immune suppression, chronic HBV patients may develop a considerable degree of liver disease. To examine whether HBV has direct cytopathic effects in severely immune compromised hosts, we have infected severe combined immune deficient mice (uPA-SCID), harboring human liver cells, with HBV. Serologic analysis of the plasma of HBV-infected animals revealed the presence of extremely high amounts of viral genomes and proteins. Histological analysis of the livers of uPA-SCID chimeras infected with HBV for more than 2 months showed that the majority of human hepatocytes had a ground-glass appearance, stained intensely for viral proteins, and showed signs of considerable damage and cell death. This histopathologic pattern closely resembles the picture observed in the livers of immunosuppressed HBV patients. These lesions were not observed in animals infected with HBV for less than 1 month. Ultrastructural analysis of long-term-infected hepatocytes showed a highly increased presence of cylindrical HBsAg structures, core particles, and Dane particles compared to short-term-infected hepatocytes. These long-term-infected hepatocytes also contained elevated amounts of HBV cccDNA. In conclusion, HBV causes dramatic intracellular changes and hepatocellular damage in the human hepatocytes that reside in a severely immune deficient mouse. These lesions show much resemblance to the ones encountered in immunosuppressed chronic HBV patients. Our observations indicate that HBV may be directly cytopathic in conditions of severe immune suppression.

Hepatitis B virus (HBV) is considered to be a noncytopathic virus, and the hepatocellular damage observed during acute and chronic HBV infections is thought to be mediated by the host’s immune response to the virus (6). More than 90% of immune competent adults who become infected with HBV display a vigorous, polyclonal, and multispecific antiviral immune response that results in a rapid reduction of the viral load and a long-lasting, protective immunity. About 5% of infected immune competent adults and a large proportion of neonates (up to 90%) and young children are unable to mount an immune response of sufficient magnitude and complexity to clear the virus and develop a chronic infection with various degrees of necro-inflammatory liver disease. Self-limiting and chronic HBV infections represent different stages of a dynamic equilibrium between the immune response of the host and the replicative capacity of the virus.

Suppression of the immune system by immunosuppressive agents or by progressive immune failure in the context of acquired immune deficiencies (e.g., human immunodeficiency virus/AIDS) may lead to reactivation of seemingly recovered or “silent” HBV infections (1). This generally presents as a reappearance of “classic” HBV markers such as HBsAg, HBeAg, and HBV DNA, in the absence of overt liver disease and/or elevated levels of transaminases. In addition, chronic HBV carriers who undergo an immunosuppressive therapy for an autoimmune disorder (62) or after liver (3, 11, 32), renal (5, 27), or bone marrow (43) transplantation or who are treated with chemotherapy for non-Hodgkin lymphoma (39) experience increased viremia accompanied by intensified viral protein expression in the infected hepatocytes. In a minority of these immunosuppressed patients, this liver disease may evolve toward fibrosing cholestatic hepatitis (FCH), an aggressive and mostly fatal form of viral hepatitis. FCH is associated with increased viral replication (42) and is characterized histologically by high intrahepatic expression of viral proteins, diffuse hepatocyte ballooning, the presence of ground-glass hepatocytes, prominent cholestasis, and periportal fibrosis (3, 5, 11, 27, 32, 42).

To examine whether this particular disease course is related to the severe immunosuppression encountered in these patients, we have studied the evolution of an experimental HBV infection in the human liver-uPA-SCID model (46). This model is based on the successful transplantation of uPA mice (24, 51, 52), backcrossed on the immune deficient SCID mouse, with primary human hepatocytes. We have recently shown that, after transplantation, up to 85% of the liver of these chimeric animals is repopulated by well-organized hu-
man hepatocytes that retain their normal cell functions and characteristics (46).

In addition, we compared the changes induced by HBV in this animal model with immunohistochemical and ultrastructural observations made in liver biopsies of an immunosuppressed patient with HBV-induced FCH and with literature data on this syndrome.

We show that HBV infection of human hepatocytes in immune deficient uPA mice induces a clinicopathological syndrome that closely resembles the picture in severely immunodepressed chronic HBV patients, which indicates that the hepatocellular damage in these HBV patients is caused by endogenous cytopathic effects of the virus itself.

MATERIALS AND METHODS

Patient with HBV-induced FCH. In the present study, we have examined histologically a needle liver biopsy and fragments from the explant liver of a patient with HBV-induced FCH. The patient was a 54-year-old female chronic hepatitis B carrier who developed hemolytic-uremic syndrome, possibly induced by a severe, acute gastroenteritis. She was treated with plasmaferesis, hemodialysis, and corticosteroids and intermittently with vincristine and cyclosporine. The immunosuppressive treatment elicited a FCH, which was confirmed by needle liver biopsy. Treatment with lamivudine could not prevent the development of subacute liver failure, and the patient underwent a liver transplantation. All patients gave written, informed consent, and the study protocol was approved by the Ethical Committee of the Ghent University Hospital. Within 2 weeks after birth, nine homozygous uPA-SCID mice (47) were transplanted with mouse hepatocytes that retain their normal cell functions and negative for anti-HBs and antibodies against HCV, HDV, and human immunodeficiency virus.

Production of human liver-uPA-SCID mice and infection with HBV. Human hepatocytes were isolated via a standard collagenase digestion (46) from tumor-free liver fragments, collected from three patients undergoing a partial hepatectomy. All patients gave written, informed consent, and the study protocol was approved by the Ethical Committee of the Ghent University Hospital. Within 2 weeks after birth, nine homozygous uPA-SCID mice (47) were transplanted with one million human hepatocytes (three mice with donor 1 hepatocytes, four mice with donor 2 hepatocytes, and two mice with donor 3 hepatocytes). At selected moments, plasma was taken and stored at −80°C until analysis. To evaluate graft take and survival, human albumin was determined by using an in-house enzyme-linked immunosorbent assay (46).

Five weeks after the transfer of hepatocytes and the demonstration of successful engraftment, these nine chimeric mice were infected with HBV via an intraperitoneal injection of 100 μl of serum from a patient that was suffering from a mild chronic hepatitis B infection for at least 17 years. This serum contained 10^8 copies of HBV DNA/ml and was positive for HBsAg and HBeAg and negative for anti-HBs and antibodies against HCV, HDV, and human immunodeficiency virus.

Detection and quantification of viral DNA, HBsAg, and HBeAg. HBV DNA levels in mouse EDTA plasma were quantified by using the Cobas Amplicor HBV Monitor test (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The most abundant DNA fragments were prepared for direct sequencing by using the BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif.) and sequenced on a Spectrophotometer (SpectraMaxx LLC, Pennsylvania). In addition to the primers used for amplification, HBV-specific primers (112(49)/H11001/H11002, 472(49)/H11001/H11002, 1009(49)/H11001/H11002, 1800(49)/H11001/H11002, 2396(49)/H11001/H11002) for the first round (I) and 1898(49)/H11001/H11002, 970(49)/H11001/H11002, 1373(49)/H11001/H11002, and 2498(49)/H11001/H11002) for the second round (II) (49). The amplicons were prepared for direct sequencing by using the BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif.) and sequenced on a Spectrophotometer (SpectraMaxx LLC, Pennsylvania). In addition to the primers used for amplification, HBV-specific primers (112(49), 970(49), 1373(49), 2807(49), 472(49), 1009(49), 1920(49) 5'-ATT TGG ACC TAC TGT GGA G3') and embedded in paraffin. Small fragments were snap-frozen or fixed in glutaraldehyde for electron microscopy. Five large biopsies were randomly taken from the explant liver and embedded in paraffin after fixation in 6% formalin. Small fragments were snap-frozen or fixed in glutaraldehyde for electron microscopy.

Histology. Four-micrometer-thick sections from paraffin-embedded liver tissue were stained with hematoxylin and eosin (H&E) for overall histopathological evaluation, periodic acid-Schiff (PAS) to assess cellular polysaccharide deposits, PAS after predigestion with amylase (PASOs) to detect ceroid macrophages, Sirius red for fibrosis, and Hall-Van Gieson to detect biliurinosis (2, 40, 41). Five-micrometer-thick sections from frozen liver tissue were fixed in acetone for 10 min after overnight drying, followed by an oil red O (ORO) staining to visualize steatosis (2).

Immunohistochemistry. Four-micrometer-thick sections from paraffin-embedded tissue were deparaffinized and rehydrated. Incubation with the primary antibodies used to detect and study the different cell types of human liver within the mouse livers were as follows: a polyclonal rabbit antibody to human albumin (dilution 1/200; Dako, Denmark) and monoclonal mouse antibodies to human hepatocytes (hepatocyte paraffin 1 or Heppar-1, dilution 1/50; Dako), mitochondria (clone 113-1, dilution 1/100; Chemicon), pan-cytokeratin (clone KL1, dilution 1/50; Immunotech), cytokeratin 8 (CK8; clone 35betah1, dilution 1/50; Dako), CK18 clone CD10, dilution 1/10; Dako), CK7 (clone OV-TL212/30, dilution 1/50; Dako), and CK19 (clone RCK108, dilution 1/20; Dako). The antibodies to albumin and Heppar-1 are well-established markers of hepatocytes (59). The different anti-keratin antibodies were used to detect all human epithelial cells. Their staining intensity and pattern and the morphology of the positive cells allowed each of the epithelial cells to be classified as hepatocytes, bile duct epithelial cells, or cells of the progenitor cell compartment (i.e., hepatic progenitor cells, intermediate hepatocyte-like cell and reactive ductular cells) (37). Expression of viral proteins was assessed by using a polyclonal rabbit antibody to HBsAg (dilution 1/200; Dako) and monoclonal mouse anti-human antibodies to HBsAg (clone H1, dilution 1/80; ForLab, Belgium). The monoclonal mouse anti-human antibody Ki67 (clone Mib-1, dilution 1/100; Dako) was used to assess the proliferative activity of human hepatocytes. To evaluate the presence of polyclonal rabbit antibodies against human cytokeratin (dilution 1/20; Dako) and CEA (dilution 1/300; Dako) were used (15, 36). The polyclonal antibody to CEA is a marker for canaliculi (33). For all mouse monoclonal antibodies and the rabbit polyclonal anti-cytokeratin antibody, the secondary antibodies were unlabeled anti-mouse and anti-rabbit Envision (Dako), respectively. Sections wherein the incubation with the first and/or secondary/tertiary antibodies were omitted, and fully processed sections of liver tissue from untransplanted animals served as controls. Positive controls consisted of selected liver biopsies from the clinical routine that were stained with these antibodies.

Quantitative assessment of the different human liver cell types present in uPA-SCID liver. To measure the fraction of liver parenchyma represented by human hepatocytes, digital pictures (total of seven to eight pictures per liver) were acquired with a ×0.5 objective lens. Using Adobe Photoshop 7.0, the number of pixels within the total liver parenchyma (excluding large portal veins, areas of calcifications, red foci, and areas without tissue) was determined and compared to the number of pixels within delineated human hepatocyte areas. To quantify proliferating hepatocytes, the number of Ki67 nucleus-positive hepatocytes was counted in random fields containing at least 300 human hepatocytes.

HBV genotyping and sequencing. Total DNA from the infecting inoculum and from mouse plasma was extracted by using the QIAamp DNA MiniKit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The HBV isolates were genotyped by using the method of Lindt et al. (38). Primers P7 and P8 were used to amplify nucleotides (nt) 256 to 796 of the S region. The amplicon was then cleaved by using restriction enzymes HinfI and Spel909l, in separate reactions to give the characteristic restriction fragment length polymorphism patterns for the different genotypes.

The full sequence of the HBV strains was obtained by amplifying overlapping regions of the genome as follows: (i) with a single-round PCR with the primer pairs P7 and P8 (nt 230 to 796) (38, 455(49)) and 1800(49) − 29, and 2408(49) and 1327(49) (49); (ii) with a nested PCR with primer pairs 1698(49) + 2498(49) − for the first round (I) and 1898(49) + 2396(49) − for the second round (II) (49, 1689(49) + 685(49) − (I) and 2267(49) + 2858(49) − (II), and 1730(49) + 2043(49) − (I) and 1763(49) + 1966(49) − (II) (49). The amplicons were prepared for direct sequencing by using the Big Dye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif.) and sequenced on a Spectrophotometer (SpectraMaxx LLC, Pennsylvania). In addition to the primers used for amplification, HBV-specific primers (112(49), 970(49), 1373(49), 2807(49), 472(49), 1009(49), 1920(49) 5'-ATT TGG ACC TAC TGT GGA G3').
and 2352(−) (5'-TCT AAC AGT ATT CTC-3') were used for sequencing. All sequences were analyzed in both the forward and reverse directions. Complete HBV genome sequences were compared to corresponding sequences of HBV from GenBank.

HBV RNA detection in the liver. Frozen liver tissues were dissolved in guanidine-isothiocyanate solution, and RNA was extracted by the acid-guanidinium phenol-chloroform method (8). Total RNA (10 μg) was analyzed by Northern blotting and probing for HBV and GAPDH exactly as described previously (21).

HBV DNA detection in the liver. Total liver DNA was extracted from frozen liver biopsy samples as described previously (22), and the levels of HBV DNA replicative intermediates were determined by quantitative real-time PCR using a Bio-Rad iCycler system exactly as described previously (56).

HBV cccDNA quantification in the liver. Covalently closed circular DNA (cccDNA) was isolated from frozen liver biopsies and analyzed by Southern blotting and by quantitative real-time PCR for quantification exactly as described previously (60). For quantification of mitochondrial DNA derived from the human hepatocytes, the following primers specific for human mitochondrial DNA were used: HsMito3362U, 5'-TCTAATGCTTACCGAACGA-3' and HsMito3442L, 5'-GGTCTACCGAAGGGTTGTAG-3'. The number of human hepatocytes represented in the total DNA samples was determined by quantitative real-time PCR of total liver DNA for the cellular gene IMAP1 (30) using primers specific for the human IMAP gene: hIMAP3652U, 5'-TTTTTCAGCT CCAAGTGGTCC-3'; and hIMAP5703L, 5'-GCCGAAGCAGGTAGCAGT-3', and human genome copy numbers were obtained by comparing the IMAP PCR results with a dilution series of purified genomic human DNA (Clontech, Mountain View, CA) under the assumption that ~6-pg genomic DNA represent one hepatocyte.

Statistics. Statistical analysis was performed by using GraphPad Prism v4. Before regression analysis, the normality of distribution of HBsAg, HBeAg, and HBV DNA was assessed by the Kolmogorov-Smirnov test. Survival curves were created by using Kaplan-Meier analysis.

RESULTS

Hepatocyte transplantation and engraftment. Nine uPA-SCID mice, successfully transplanted with primary human hepatocytes, were infected with 10^6 HBV genome copies. Seventeen days later, EDTA plasma was analyzed for the presence of HBsAg, HBeAg, and HBV DNA. Eight of the nine animals were positive for these viral markers. The one negative animal showed signs of infection 2 weeks later, when HBsAg, HBeAg, and HBV DNA appeared in the plasma.

Kinetic study of HBV markers. To study the progression of the HBV infections in uPA-SCID mice, we quantified the viral proteins and DNA in the mouse plasma. Figure 1A shows the evolution of the virologic parameters, together with the human albumin levels, obtained in six mice and shows that 2.5 weeks after injection of HBV the mean HBV DNA titer was 1.4 × 10^10 copies/ml, while HBeAg and HBsAg reached levels of 107 PEIU/ml and 788 IU/ml, respectively. Serologic HBV parameters peaked between 4 and 5 weeks after infection and reached a mean HBV DNA level of 5 × 10^10 copies/ml, a mean HBeAg level of 602 PEIU/ml, and a mean HBsAg level of 14,398 IU/ml. From then on three animals appeared clinically sick and died within 2 weeks. One month later, the surviving animals had either equal or lower HBeAg and HBsAg levels, and all had lower HBV DNA levels in their plasma. The clinical condition of two animals rapidly deteriorated, and they died 4 days later. Only one mouse survived beyond 12.5 weeks after infection. In all animals, clinical deterioration coincided with a dramatic drop of human albumin levels in plasma. The median life span of the HBV-infected animals was 50 days after infection or 85 days after transplantation. This is significantly shorter (P < 0.0001) than the survival of uninfected mice (Fig. 1B), which survived for at least 220 days after transplantation and maintained a human albumin concentration in their plasma ranging between 4 and 6 mg/ml.

Correlation between HBV DNA and viral proteins. When HBsAg levels were compared to the levels of HBeAg in the plasma of the infected animals, a strong and linear correlation was observed (r = 0.9808, P < 0.0001) (Fig. 2A). Figure 2B shows a linear correlation between HBV DNA and both HBsAg (r = 0.9808...
any viral marker and human albumin (HBV DNA and human albumin, 
(A) and between HBV DNA and HBsAg (P = 0.8710, P < 0.0001) and HBsAg (r = 0.9344, P < 0.0001) (B). (C) In contrast, no correlation between any viral marker and human albumin (HBV DNA and human albumin, r = 0.244, P = 0.4006) could be demonstrated.

Sequence analysis of HBV. The HBV strains amplified from the infecting inoculum and from the mouse plasma (88 days after inoculation) both belonged to genotype E, serological subtype ayw4. The complete genomes of the two HBV isolates were sequenced and compared by phylogenetic analysis to the 17 complete sequences of genotype E in the GenBank database (AB091255, AB091256, AB032431, X75657, X75664, AB106564, AY738144, AY739675, and DQ060822 to DQ060830 (A. Kramvis et al., submitted for publication, and data not shown). Both isolates had a genome length of 3,212 nt characteristic of genotype E, and their sequences were identical (GenBank accession no. AY935700).

The pre-S1 sequence was completely conserved, whereas a single missense mutation relative to other genotype E sequences was found in the pre-S2: T53C (Phe22Leu). The polymerase open reading frame (ORF) was well conserved relative to other genotype E sequences except for an amino acid substitution within the reverse transcriptase rtSer212Thr as a result of the T766A missense mutation. The following missense mutations were detected in the X ORF overlapping the basic core promoter: T1753C (Ile127Thr), A1762U (Lys130Met), and G1764A (Val131Ile). The core region contained four missense mutations: A2092T (Glu64Asp), C2093G (Leu65Val), A2099T (Thr67Ser), and A2159G (Ser87Gly).

Histology. The histological findings of the livers from uninfected, short-term HBV-infected and long-term HBV-infected animals are hereafter described in this order. However, we present the figures of short-term and long-term-infected animals side to side to illustrate the differences between both conditions in the best way. For this reason, the order of figures does not always follow the flow of the text.

Histologic analysis of the livers of transplanted, uninfected uPA-SCID mice. At 36 and 78 days after transplantation, the livers of two uninfected animals were examined. Mature human hepatocytes could easily be recognized because they are larger in size than their murine counterparts and, in addition, they stained positive for Heppar-1, human albumin, mitochondria, and CK8/18. All of these markers were negative on tissue derived from untransplanted control animals. We recently found (46) that the human hepatocytes tend to accumulate glycogen in the cytoplasm, which results in the typical “plant-like” morphology that is also seen in all glycogen storage diseases, except in type IV. Steatosis of human hepatocytes was minimal. At 25 days after transplantation, one-quarter of the liver parenchyma was occupied by nodules of human hepatocytes. Ki67 staining showed that 18% of human cells were proliferating. In contrast to the sick mouse liver parenchyma, the regions occupied by human hepatocytes contained almost no ceroid macrophages. At 73 days after transplantation, 66% of the liver parenchyma was of human origin. Only 6% of human cells were still proliferating, and ceroid macrophages were still rare within the human zones. Except for glycogen storage, human hepatocytes showed no abnormalities. Besides mature hepatocytes, the host liver also contained human hepatic progenitor cells, which were recognized on the basis of their characteristic morphology and immunohistochemical phenotype (37).

Histologic and ultrastructural analysis of the liver of short-term-infected animals. Two uPA-SCID mice, transplanted with human hepatocytes and subsequently infected with HBV, were sacrificed 21 and 25 days after infection. Figure 3A shows that also in this animal and in no way different from the livers of noninfected mice, human hepatocytes were excessively loaded with glycogen and formed large areas occupying 34 and 87% of mouse liver parenchyma, respectively. Apart from the glycogen storage, no other signs of damage or cell loss were seen. The human hepatocytes stained strongly for human albumin (Fig. 3B), and ceroid macrophages were confined to the mouse areas (Fig. 3C). Hepatocytes staining for Ki67 were scattered over the liver and accounted for 10% of all human hepatocytes. There were no human hepatocytes with a “ground-glass” aspect of the cytoplasm.

Figure 4A shows that 25 days after infection almost all human hepatocytes stained for HBcAg, displaying a strong signal in the nucleus and a moderate one in the cytoplasm. At 21 days after infection, 50% of the human hepatocytes stained positive (not shown). The cytoplasm of the human hepatocytes almost always had a granular staining for HBsAg, frequently with an accentuation near the cell membrane (Fig. 4B). Whereas almost all human hepatocytes stained positive for HBsAg 25 days after inoculation, only 10% stained positive 21 days after infection. The staining patterns and intensities are similar to those observed in liver biopsies of patients with chronic HBV in the viral replicative phase. The number of positive hepatocytes is usually lower in human liver biopsies (14).

In addition to human hepatocytes and diseased mouse hepatocytes, seven red foci (mouse regenerative nodules that have
deleted the uPA transgene) were also present in the liver of one short-term-infected animal. These consisted of 21 to 88 hepatocytes that were partly or completely surrounded by areas of human hepatocytes. The interface between red foci and human areas was neither sharply delineated nor compressed, and the two cell types seemed to infiltrate each other’s "territories." These findings suggest that the human hepatocytes and the transgene-free mouse hepatocytes are responding equally well to the available growth stimuli. As in noninfected mouse livers, human hepatic progenitor cells and intermediate hepatocyte-like cells were focally present at the human-mouse parenchymal interface.

Using electron microscopy, noncoated viral particles with a diameter of 21 to 24 nm, corresponding to HBV core particles, could be observed dispersed in the nuclei of several human hepatocytes. In the cisternae of the endoplasmic reticulum, one or two longitudinally transected tubules and cross-sectioned spheres of HBsAg were present. In some cisternae, core particles, surrounded by a clear halo and a dark ring approximately 40 nm in size, corresponding to Dane particles were observed. In addition to the presence of viral and subviral particles, no ultrastructural alterations could be observed in these hepatocytes compared to those of noninfected animals.

**Histologic and ultrastructural analysis of the livers of long-term-infected animals.** The clinical condition of the HBV-infected animals deteriorated over time, and some animals died spontaneously. Two mice that looked clinically “terminal” (infected for 73 and 88 days) were sacrificed to examine their livers. In these mice, the cytoplasm of most human hepatocytes had a “ground-glass” appearance (Fig. 3D) and no longer showed the excessive accumulation of glycogen, observed in uninfected or short-term-infected livers. The insert shows a magnification of human ground-glass hepatocytes. (E) Long-term HBV infection results in an overall deterioration of the condition of the cells as shown by the decreased albumin production. (F) Large amounts of ceroid macrophages are now also present in the regions occupied by human hepatocytes, indicating that long-term infection causes the death of human hepatocytes. Original magnifications: A, C, D, and F, ×200; insets, B, and E, ×400.
which is much less than in the uninfected and short-term HBV-infected livers containing a comparable amount of human cells. The liver sections also revealed seven red foci consisting of 16 to 43 hepatocytes. In contrast to the picture seen in short-term infection, these red foci were sharply delineated and mildly compressed the surrounding human parenchyma, which indicates that the hepatocytes in these red foci have a growth advantage over the long-term-infected human hepatocytes.

The immunohistochemical staining for HBcAg (Fig. 4C) showed a very intense staining of hepatocyte nuclei and a less intense, but still pronounced staining of cytoplasm and cell surface membranes. The staining for HBsAg (Fig. 4D) showed that the cytoplasm of most hepatocytes contained high amounts of this viral protein. In some hepatocytes with a less intense staining of the cytoplasm, membranous staining became apparent. Overall, the staining intensities were much higher than in the livers of the short-term-infected mice.

Several human hepatic progenitor cells and intermediate hepatocyte-like cells were present not only at the human-mouse parenchymal interface but also within the human parenchymal areas (Fig. 5). This leads to higher numbers of human progenitor and intermediate cells in long-term-infected animals than in short-term or noninfected animals.

Ultrastructural analysis showed that long-term HBV-in-

FIG. 4. Immunohistochemical staining for HBcAg (A, C, and E) and HBsAg (B, D, and F) on liver sections of short-term (upper panel) and long-term (middle panel) HBV-infected human liver-uPA-SCID mice and biopsies from a patient suffering from FCH (lower panel). We can clearly observe a highly increased presence and difference in the distribution of both viral proteins in long-term-infected tissue compared to short-term-infected tissue. The amount and pattern of HBcAg and HBsAg staining on the long-term-infected liver corresponds very well to the one observed in liver tissue derived from the FCH patient. Original magnifications, ×200.
Infected hepatocytes were swollen, ballooned, and considerably damaged. This was never observed in the livers of the short-term-infected mice. The nuclei of most human hepatocytes were completely filled with noncoated round HBV core particles with diameters of 21 to 24 nm. In the cytoplasm of these hepatocytes, nearly all of the cisternae of the endoplasmic reticulum displayed cystic dilations that contained numerous HBsAg tubules and spheres and complete Dane particles (Fig. 6A). Unlike the livers of the short-term-infected and noninfected mice, glycogen granules were scarce in these ballooned human hepatocytes loaded with virus. Human hepatocytes that contained fewer viral particles and showed less cell damage were scarce.

Dysplasia or malignant transformation of human hepatocytes, or tumor cells of the type for which the patient underwent liver surgery were never seen.

Histologic and ultrastructural analysis of liver tissue from a patient with HBV-induced FCH. The needle biopsy and the fragments of the explant liver showed periportal fibrosis with occasional formation of a septum, vague nodularity of the parenchyma, and perisinusoidal fibrosis. The periportal area contained an impressive ductular reaction that extended into

FIG. 5. Immunohistochemistry for human cytokeratin 7 on a liver section from a chimeric mouse long-term infected with HBV. Hepatic progenitor cells (arrows), as well as cells with a phenotype intermediate between progenitors and mature hepatocytes (arrowheads), are not only located at the interface between human (H) and mouse (M) hepatocytes but also within human parenchyma. Staining with anti-cytokeratin 19 confirms the presence of human hepatic progenitor cells (inset). Original magnifications: main image, ×200; inset, ×400.

FIG. 6. Electron micrograph of a human hepatocyte in the liver from a long-term-infected chimeric mouse (left) and in the liver of a patient with HBV-induced FCH (right). (A and B) Numerous amounts of HBV core particles (open arrows) are obvious inside the nucleus, whereas complete Dane particles (arrow) are present in the cisternae of the endoplasmic reticulum. (C and D) These cisternae are dilated and contain large amounts of HBV surface tubules. Original magnification: ×77,000.
the lobular parenchyma. Singular hepatic progenitor cells and intermediate hepatocyte-like cells were scattered throughout the parenchyma. The parenchyma showed several signs of considerable cell damage and loss: (i) some hepatocytes showed ballooning, (ii) there were numerous ceroid macrophages that sometimes formed clusters, and (iii) a few areas of confluent necrosis were present. The cytoplasm of a considerable proportion of hepatocytes had a “ground-glass” aspect. There was also prominent bilirubinostasis. Bilirubin droplets were present in hepatocytes, bile canaliculi, and frequently in bile ductules with dilated lumina and damaged epithelium. The parenchymal inflammatory infiltrate was only mildly increased.

The immunohistochemical staining for HBcAg (Fig. 4E) showed a very intense staining of nearly all hepatocyte nuclei and a less intense, but still marked, staining of cytoplasm and membrane. The cytoplasm of almost all hepatocytes stained intensely positive for HBsAg (Fig. 4F). Remarkably, some cells from reactive bile ducts were also positive for HBsAg and HbcAg, a feature that has been described previously in chronic HBV infection (13). Striking similarities between the livers of the long-term HBV-infected mice and the human liver with HBV-induced FCH were the presence of extensive hepatocyte damage and loss, the pattern and intensity of immunohistochemical HBV stainings, and the presence of only a mild inflammatory infiltrate.

Ultrastructural evaluation of the small biopsies fixed in glutaraldehyde showed findings almost identical to those seen in the long-term-infected animals (Fig. 6). Many hepatocytes contained nuclei filled with HBV core particles, while the cytoplasmic endoplasmic reticulum cisternae were dilated and packed with HBsAg tubules and spheres and complete Dane particles. Determination of HBV cccDNA, DNA, and RNA. The livers of one short-term-infected and one long-term-infected animal were also analyzed for the presence of HBV cccDNA, replicative intermediates, and RNA transcripts. Using Southern blotting, cccDNA could easily be identified in both chimeric livers (Fig. 7A). After EcoRI digestion, the cccDNA molecules were linearized and comigrated with the linear HBV DNA present in the total DNA isolated from the same tissue. cccDNA could also be detected in liver biopsies from a chronically infected chimpanzee and the FCH patient, but was absent in DNA extracts from HBV transgenic mice. HBV DNA replicative intermediates were detected in all liver samples except in the noninfected chimeric mouse. HBV DNA cccDNA copy number per infected hepatocyte was quantified by using quantitative real-time PCR. In the liver of a short-term-infected animal we detected 1.4 cccDNA molecules per HBcAg /H11001 human hepatocyte. In contrast, in the long-term-infected animal the amount of cccDNA molecules per HBV-infected human hepatocyte increased to 65.7 copies/cell. The liver of the FCH patient contained 6.8 cccDNA molecules per hepatocyte.

Northern blot analysis showed very high HBV gene expression in the long-term mouse liver but only low expression in the chimeric mouse liver after 21 days (Fig. 8). The preS1 mRNA was visible only in the liver extracts of the long-term-infected chimeric uPA-mouse and in the HBV transgenic mouse.

**DISCUSSION**

The hepatic injury during acute and chronic HBV infections is thought to be caused by the host’s immune response against the infected hepatocytes (6). However, in some severely immunosuppressed chronic HBV patients, excessive viremia and a very severe, sometimes fatal hepatic disease, known as FCH may occur. This suggests that severe liver pathology may arise in the absence of a functional immune system (5, 27, 39, 43). To examine the pathology induced by HBV in an immune deficient host, we have infected chimeric human liver-uPA-SCID mice with HBV.
At several moments after infection, the concentrations of HBsAg, HBeAg, and HBV DNA in mouse plasma were determined. In agreement with Degushi et al. (12) and previous observations of our lab (54), very strong linear correlations between HBsAg and HBeAg and HBV DNA were observed. Also, a linear correlation between the amount of HBeAg and HBV DNA was seen. Two groups that independently analyzed serum from chronic HBV patients were unable to find such a relationship before the start of antiviral treatment (4, 25). However, during antiviral treatment, these researchers found a correlation between HBeAg and HBV DNA levels and concluded that quantification of HBeAg in serum or plasma may be a valuable alternative to HBV DNA measurements to monitor the success of antiviral treatment.

Compared to noninfected human liver-uPA-SCID mice, HBV-infected animals have a decreased life expectancy and their premature death is caused by the progressive deterioration of the liver structure and function. Several observations are indicative of a direct cytopathic effect of the hepatitis B virus in our model. A large proportion of human hepatocytes in long-term-infected animals acquired a ground-glass appearance. This was not seen in noninfected or short-term-infected mice. This ground-glass appearance was caused by the presence of human hepatocytes in the liver, phenomena we also observed at a similar time point. Levels of circulating HBV DNA with values exceeding $10^9$ copies/ml are also common in immunosuppressed HBV patients (1, 28). Tsuge et al. recently described long-term HBV infection in uPA-SCID mice (57). Several weeks after infection, the concentration of human albumin decreased in these animals, albeit less dramatically than what we observed. It is not clear whether this is due to the HBV infection or to a spontaneous loss of chimerism. Obvious signs of a cytopathic effect were not reported, and a lethal effect is less likely in this setup because the low levels of human albumin suggest the presence of high amounts of regenerative red nodules. These would rescue the animals in case of graft failure.

uPA$^{+/−}$ RAG2$^{−/−}$ mice transplanted with tupaia hepatocytes have also been infected with HBV (10). In these animals the HBV infection persisted for 6 months, the experimental endpoint, and no signs of hepatocellular damage were reported. The main differences with the human liver-uPA model are that the onset of viremia was considerably delayed and that the plasma levels of HBV DNA were significantly lower. This may be due to the nature of the tupaia hepatocytes.

Similarly, hepatocytes from a woodchuck infected with the woodchuck hepatitis virus were transplanted in uPA-RAG2 mice. These animals experienced high viremia for more than 10 months without obvious lesions in the liver. It might be that the woodchuck hepatitis virus is less cytopathic than HBV.
Alternatively, this difference may also be related to the different nature of the woodchuck hepatocytes.

Our observations closely resemble those made by Lenhoff et al. in a model for the study of the duck hepatitis B virus (34). Three-day-old ducklings were infected with a duck hepatitis B virus strain that contained a mutation in the Pre-S region. This mutation induced elevated levels of viral cccDNA and viral capsid protein in the nucleus of the infected hepatocytes. In addition to high intracellular viral protein expression, we also observed a 47-fold increase in cccDNA copy number per infected hepatocyte when we compared the short- and long-term-infected animals.

The infected ducklings also displayed high viremia and hepato-cellular damage. Like in our model, this cell damage elicited an increase of mononuclear cells and regenerating bile ductule-like cells in the liver parenchyma. In biopsies of patients with chronic viral hepatitis, the number of hepatic progenitor cells is also correlated with the severity of hepatocyte damage and loss (18). As a consequence of the cytopathic effect, the ducklings experienced a marked growth retardation. In contrast to our study, this cytopathic effect lasted for only 23 days, and the recovery was associated with the appearance of a noncytopathic revertant virus (35). We did not observe any sequence variability during the 88-day infection period.

The mechanisms underlying the cytopathic effect of the virus have not yet been defined. Several in vitro and in vivo studies showed that the accumulation of the L protein leads to the appearance of ground-glass hepatocytes (7, 17) and can induce stress in the endoplasmic reticulum (58), as well as oxidative DNA damage and mutagenesis (26). The virus isolate we used here lacked the mutations in the pre-S region that have been shown to lead to the development of ground-glass hepatocytes in chronic carriers (16, 45, 61). It can be argued that the cytopathic effect seen in the present study is the result of the human hepatocytes being infected with an isolate that has mutations in the basic core promoter. However, these mutations have also be found in asymptomatic carriers, in patients experiencing only a mild form of chronic HBV infection (19, 31, 48, 55), and in immune compromised HBV patients with no or mild liver disease (23, 44, 50).

In the context of the present study, an ultrastructural analysis on biopsies from a FCH liver has been performed for the first time. This evaluation revealed massive amounts of viral and subviral particles inside ballooned and damaged hepatocytes. Electron micrographs of human hepatocytes in the long-term-infected mice could not be distinguished from those of the FCH liver. We did not detect a significant increase in cccDNA copy number in the FCH patient, indicating that the cause of the cytopathic effect might be different at the molecular level.

The data and the clinicopathological observations made in other patients with FCH suggest that, in the absence of a functioning immune system as it occurs in SCID mice and severely immunosuppressed patients, HBV can replicate and express its proteins in an unrestrained fashion. This leads to excessive accumulation of viral proteins, cell damage, and ultimately cell death. Our mouse model can be useful for studying new therapies and strategies to control the viral replication during immunosuppression after organ transplantation in patients with chronic hepatitis B infection. It may also be useful for the evaluation and prediction of the pathogenic effect of the various genotypes of HBV and certain HBV mutants.

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Chapter 5: Human hepatocytes secrete soluble CD14, a process not directly influenced by HBV and HCV infection.


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Human hepatocytes secrete soluble CD14, a process not directly influenced by HBV and HCV infection

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Abstract

Background: Chronic hepatitis B (HBV) and hepatitis C (HCV) patients have elevated plasma levels of soluble CD14 (sCD14). We examined whether human hepatocytes produce sCD14 in vivo, and whether HBV or HCV infections influence this production.

Methods: uPA-SCID mice were transplanted with primary human hepatocytes and some animals were subsequently infected with HBV or HCV. Plasma from these mice was analyzed for the presence of human sCD14. The liver was examined via immunohistochemistry.

Results: A soluble form of human CD14 could be detected in the plasma from successfully transplanted mice, while it was completely absent in non-transplanted control animals. The isoform of this human sCD14 corresponded with the most abundant isoform found in human plasma. CD14 levels in circulation were not significantly different between non-infected, HBV infected and HCV infected animals.

Conclusions: Our data indicate that human hepatocytes produce sCD14 in vivo, and that liver cells might be the major source of sCD14 in normal human plasma. In addition we demonstrate that HBV and HCV infections have no direct influence on the production of sCD14 by human hepatocytes in this chimeric model.

Keywords: Hepatitis B; Hepatitis C; Endotoxemia; CD14; uPA-SCID; Human hepatocyte

1. Introduction

CD14 is a differentiation marker expressed on the surface of monocytes, macrophages and granulocytes [1]. Membrane bound CD14 (mCD14) has a molecular weight of 56 kDa and is attached to the cell surface via a glycosyl-phosphatidylinositol (GPI) anchor [2]. Binding of microbial lipopolysaccharide (LPS) to CD14 leads to activation of the cell via the co-receptor Toll-like receptor 4 (TLR-4), and the subsequent production of the cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) [3]. In addition to its function in LPS signal transduction, CD14 also recognizes apoptotic cells [4], mediates phagocytosis of Gram-negative bacteria [5], interacts with TLR-2 [6,7], is involved in monocyte–endothelial cell interactions [8] and binds recombinant hepatitis B surface antigen (HBsAg) [9].

Studies in mice and rats suggest that CD14 can also be expressed by hepatocytes. Fears et al. injected LPS in mice and observed a peak of CD14 mRNA expression in the liver after 8 to 16 h [10]. In situ hybridization showed that this CD14 mRNA was localized inside the hepatocytes. Administration of LPS to rats also induced expression of CD14 mRNA and protein in hepatocytes [11–13].

CD14 not only occurs on the membrane of certain cell types, but can also be found in a soluble form in human plasma. While normal mouse plasma is devoid of soluble
From non-parenchymal cells via three low-speed centrifugation steps (5 min, 50 g). Cell viability was measured using the Trypan Blue exclusion test and exceeded 90%.

2.2. Breeding and transplantation of uPA-SCID mice

uPA-SCID mice were generated by backcrossing B6SJL-TgN(Alb1Plau)144Bri mice (The Jackson Laboratories, Bar Harbor, Maine, USA) at least seven times on a SCID (CBySmm.CB17-Prkdcscid) background. The offspring were screened for the SCID phenotype based on the absence of mouse immunoglobulins, using an in-house Elisa. The uPA zygosity was determined by multiplex PCR as previously described [36]. Within two weeks after birth, homozygous uPA-SCID mice were transplanted with one million human hepatocytes via an intrasplenic injection. Five weeks after transplantation, some animals were infected with HBV or HCV by an intraperitoneal injection of plasma from patients infected with HBV or HCV, respectively.

All animals were bred and handled under specific pathogen free (SPF) conditions in the SCID mouse facility of the Department of Clinical Chemistry, Microbiology and Immunology, Ghent University.

2.3. Transplantation of NOD/SCID mice

NOD/SCID mice (NOD.CB17-Prkdcscid/J), originally purchased from The Jackson Laboratories, were bred and maintained in our animal facility. One day before cell transfer, all animals received a total body irradiation of 100 rad and were injected with 0.5 mg TM-β1 [37]. The next day, human PBMC were isolated from a buffy coat (BTC Oost-Vlaanderen, Gent, Belgium) via Ficoll-Hypaque (Nycomed Pharma, Oslo, Norway) density gradient centrifugation. Ten million PBMC were injected in the peritoneal cavity of the pretreated NOD/SCID mice. Three and 7 days after cell injection, plasma was taken and stored at −80 °C until further analysis. Some NOD/SCID mice were transplanted with one million human hepatocytes via intrasplenic injection.

2.4. Quantification of human albumin

The concentration of human albumin in chimeric mouse plasma was determined via a sandwich Elisa. Maxisorp ImmunoPlates (Nunc, Roskilde, Denmark) were coated with a goat-anti-human albumin antibody (Bethyl Laboratories Inc., Montgomery, TX, USA). After blocking, diluted samples were added and after incubation and washing, bound human albumin was revealed with a HRP conjugated goat-anti-human albumin antibody (Bethyl Lab.).

2.5. Detection and quantification of viral DNA/RNA

HBV DNA and HCV RNA levels in mouse EDTA plasma were quantified using the Cobas Amplicor HBV Monitor test
and the Cobas Amplicor HCV Monitor test v2.0 (Roche Diagnostics, Mannheim, Germany), respectively.

2.6. Quantification of sCD14

Human soluble CD14 levels in mouse plasma were determined using the sCD-14 EASIA kit (Biosource, Camarillo, CA, USA), according to the manufacturer’s instructions. This Elisa is specific for human sCD14 and is not cross-reactive to mouse sCD14.

2.7. Detection and quantification of HBsAg

HBs antigen levels in mouse plasma were quantified with a modified protocol [38] of the Axsym HBsAg V2 system (Abbott, Chicago, Illinois, USA). To determine the concentration of HBsAg, a curve containing six calibrators (5.867, 2.933, 1.467, 0.733, 0.367 and 0 IU/ml), standardized to the NIBSC HBsAg standard, was generated. The fluorescence rate of the unknown samples was compared to the calibration curve to calculate the actual HBsAg content.

2.8. Western blot analysis

Human and mouse EDTA plasma was diluted 1/100 in PBS. Ten microliters of sample was diluted in 2× loading buffer (Sigma-Aldrich, St. Louis, MO, USA) and loaded on an 8% SDS-PAGE after denaturation. Affinity purified human recombinant sCD14 (25 ng/lane) produced in CHO-cells [39] was used as a positive control. After separation, the samples were transferred to a nitrocellulose membrane (Schleicher and Schull, Dassel, Germany) that was then blocked with TBS–Tween 20 (0.1%) containing 5% Skim Milk (BD Difco, Franklin Lakes, NJ, USA). sCD14 was detected using a rabbit anti-CD14 antibody (dilution 1/5000; Biometec, Greifswald, Germany) in combination with a goat-anti-rabbit HRP labeled antibody (dilution 1/10,000; Dako, Denmark), both diluted in blocking buffer. After incubation for 1 h at room temperature, extensive washing and treatment with the ECL Advance Western Blotting Detection System (Amersham Biosciences, Freiburg, Germany) the signal was visualized on autoradiography film (Pierce, Rockford, IL, USA).

2.9. Immunohistochemistry

A part of each fresh mouse liver was snap-frozen in liquid nitrogen-cooled isopentane and stored at −80 °C until further use. The spleen and the remainder of the liver was fixed in B5-fixative or in 6% formalin. After fixation, the tissue was embedded in paraffin.

Four micrometer-thick sections from paraffin-embedded tissue were deparaffinized, rehydrated and incubated with the primary antibodies for 30 min at room temperature. The primary antibodies used to detect and study the different human cell types within the chimeric mouse were a polyclonal rabbit anti-human antibody against human albumin (dilution 1/200, Dako, Denmark), and mouse monoclonal antibodies against human cytokeratin 8 (CK8) (clone 35β1H11, dilution 1/100, Dako), CD14 (Biometec, Greifswald, Germany; Novocastra Laboratories Ltd., Newcastle, UK), CD45 (clone 2B11+PD7/26, dilution 1/50, Dako) and CD68 (clone KP1, dilution 1/75, Dako). CD45 is present on the surface of all leukocytes, while CD68 is a highly glycosylated lysosomal membrane protein that is strongly expressed in cytoplasmic granules, and weakly on the surface of macrophages, monocytes, neutrophils, basophils and NK-cells.

For all mouse monoclonal antibodies and the rabbit polyclonal anti-cytokeratin antibody, the secondary antibody was undiluted anti-mouse and anti-rabbit Envision (Dako), respectively. Sections wherein the incubation with the first and/or secondary antibodies were omitted and fully processed sections of liver tissue from untransplanted animals served as negative controls, while liver biopsies from the clinical routine served as positive controls.

2.10. Statistics

Statistical analysis was performed using GraphPad Prism v4. Before regression analysis, the normality of distribution of sCD14, HBsAg and albumin was assessed by the Kolmogorov–Smirnov test. Normalized sCD14 concentrations in non-infected, HBV infected and HCV infected uPA-SCID mice were compared using the Mann–Whitney U-test.

3. Results

3.1. Identification and quantification of sCD14 in chimeric uPA-SCID mouse plasma

Plasma from successfully transplanted uPA-SCID mice (n=23) was analyzed for the presence of human albumin and sCD14. The concentration of human albumin, which is a marker of hepatocyte graft take and survival, ranged from 1.1 to 8.5 mg/ml (median value = 2.60 mg/ml). All samples were also positive for sCD14 and ranged from 0.82 to 4.56 μg/ml (median value = 1.70 μg/ml). A linear correlation between the concentrations of human albumin and sCD14 was observed (r²=0.7395, P<0.0001; Fig. 1). The plasma of untransplanted control animals contained neither human albumin nor human sCD14.

To determine the isoform of the sCD14 produced by the human hepatocytes in the chimeric mice, plasma from a transplanted mouse was analyzed by electrophoresis and western blotting and this resulting pattern was compared with that of plasma from an untransplanted mouse, a human plasma sample and a specimen containing recombinant sCD14. Plasma from a successfully transplanted mouse contained human sCD14 (Fig. 2). The plasma from an untransplanted control animal was devoid of sCD14, which
were transplanted with 107 human PBMC. This cell number measured sCD14 in the plasma of NOD/SCID mice that cell graft could be the source of the sCD14 we next and expansion of the transplanted hepatocytes. disease which is an essential requirement for the survival immune deficient but do not suffer from a chronic liver be found. This is not surprising since hepatocytes do not was analyzed and neither human albumin nor sCD14 could suspension. Four weeks later the plasma of these animals transplanted with the liver cell suspension, we performed cytes, macrophages or other cells that could have been human liver-uPA-SCID mice was originating from mono- was produced in CHO cells, a difference in human and chimeric mouse plasma. Since this recombinant sCD14 was was visualized with a size slightly different from what is found in human plasma. Two forms of a recombinant sCD14 can be bands with a size higher than 75 kDa are also visible in all samples. confirms the Elisa results. The electrophoretic mobility of sCD14 in mouse plasma corresponded exactly to that of the most abundant isoform present in normal human plasma. The specimen with recombinant sCD14 contained two bands which differed slightly from the one found in the human and chimeric mouse plasma. Since this recombinant sCD14 was produced in CHO cells, a difference in glycosylation may be responsible for the different electrophoretic migration pattern [40].

3.2. Human hepatocyte-exclusive production of sCD14

To exclude the possibility that the sCD14 found in the human liver-uPA-SCID mice was originating from monocytes, macrophages or other cells that could have been transferred with the “liver cell” suspension, we performed the following experiments. Three NOD/SCID mice were injected with a “liver cell” suspension. Four weeks later the plasma of these animals was analyzed and neither human albumin nor sCD14 could be found. This is not surprising since hepatocytes do not survive in NOD/SCID mice. These mice are severely immune deficient but do not suffer from a chronic liver disease which is an essential requirement for the survival and expansion of the transplanted hepatocytes.

To examine whether passenger leukocytes in the “liver cell” graft could be the source of the sCD14 we next measured sCD14 in the plasma of NOD/SCID mice that were transplanted with 10^7 human PBMC. This cell number largely exceeds any possible leukocyte contamination of the liver cell graft. The plasma of the 5 transplanted mice was analyzed 3 and 7 days after PBMC transfer, but neither human albumin nor sCD14 could be detected.

Immunohistochemical analyses of the liver and spleen of successfully transplanted uPA-SCID mice unambiguously showed the presence of human hepatocytes in the liver, while lymphocytes, macrophages and other leukocytes could never be observed in the liver, or in the spleen (data not shown). These morphological data make it very unlikely that apart from the hepatocytes there is another cellular source of human sCD14 in our chimeric uPA-SCID mice.

3.3. Quantification of sCD14 in HBV and HCV infected chimeric mice

Since we previously observed that chronic HBV and HCV infections induced a rise in sCD14 levels in patients and since human hepatocytes were most likely the only source of the sCD14 in this animal model, we wanted to investigate whether HBV or HCV infections had a direct influence on the plasma levels of human sCD14 in the chimeric mice. Plasma from HBV infected animals contained at least 10^8 viral copies/ml, while in HCV-infected animals viral RNA levels exceeded 2.10^6 IU/ml. Histologic analysis of the liver confirmed these infections [35].

The linear correlation between the concentration of human albumin and sCD14 seen before (Fig. 1), suggested that both analytes are markers for the number and functional integrity of the human hepatocytes present in the chimeric animal. To demonstrate any effect of HBV or HCV infection on the production of sCD14 by human hepatocytes, the sCD14 values had to be normalized for the amount of human hepatocytes present in the chimeric liver. To this end a ‘normalized’ sCD14 concentration was calculated by dividing the sCD14 concentration by the human albumin concentration in each plasma sample. The ‘normalized’ sCD14 levels in the plasma of uninfected (n = 23), HBV-infected (n = 18) and HCV-infected (n = 11) chimeric animals (Fig. 3a) were then compared and no differences between the three groups of mice (uninfected vs. HBV infected mice: P = 0.6839; uninfected vs. HCV infected mice: P = 0.7966; and HBV vs. HCV infected mice: P = 0.7360) were observed.

In addition, the amount of HBsAg, HBeAg and HBV DNA in the plasma of the HBV infected chimeric uPA-
SCID mice was measured quantitatively and compared with the ‘normalized’ concentration of sCD14. Here as well no correlation could be found (Fig. 3b–d).

4. Discussion

Soluble CD14 found in human plasma is thought to originate from monocytes/macrophages. Recent studies in transgenic mice showed that also hepatocytes can produce sCD14 [18,41]. Administration of LPS to normal mice and rats induces the secretion of CD14 by hepatocytes [10,11,13]. Additionally, in vitro studies with HepG2 hepatoma cells [17,18] and primary hepatocytes [16] showed that human hepatocytes are able to secrete a soluble form of CD14. However, tumor cells may not be the best model cells to demonstrate the natural production of a protein and the release of sCD14 by primary hepatocytes in vitro may be a tissue culture artifact.

Here we investigated the in vivo production of sCD14 by human hepatocytes in chimeric uPA-SCID mice [35] at several time points following transplantation of human hepatocytes. The plasma of these chimeric mice contained human sCD14 concentrations comparable to those found in healthy humans [42]. A direct correlation was found between plasma sCD14 levels and the amounts of human albumin in the mouse plasma. Since the concentration of human albumin in chimeric mouse plasma is an excellent marker of the engraftment and function of the human hepatocytes, the significant correlation between albumin and sCD14 levels is highly suggestive for a hepatocytic origin of sCD14.

To exclude the possibility that other, non-hepatocytic, human cells present in the “liver cell” graft were contributing to the sCD14 production, we transplanted NOD/SCID mice with human peripheral blood mononuclear cells (PBMC). Although more than 10% of these cells express CD14, no sCD14 could be detected in the plasma of these transplanted mice. Since our “liver cell” suspension contained only a fraction of contaminating cells capable of producing sCD14, it is most unlikely that these contaminants are responsible for or contribute to the sCD14 production in the chimeric uPA-SCID mice. Despite careful microscopic analysis we could never detect human monocytes, macrophages or other leukocytes in the chimeric mice. Transplantation of NOD/SCID mice with a “liver cell” suspension did not lead to detectable human albumin or sCD14 secretion in the mouse plasma. This is not surprising since hepatocytes do not survive in NOD/SCID mice, which do not provide the environment that favors hepatocyte engraftment. The absence of sCD14 observed in the latter experiment is yet another indication that the human hepatocytes are the origin of the CD14 in the circulation of the chimeric uPA-SCID mice.

Despite the use of two different anti-CD14 antibody clones, we could not visualize CD14 protein inside the...
human hepatocytes via immunohistochemistry. This was anticipated since we (unpublished data) and others have shown that in rat and human liver biopsies only Kupffer cells stain for CD14 [43,44]. Intracellular CD14 protein has only been detected inside hepatoma cells [17] and in cultured human hepatocytes [16]. This suggests that the intracellular CD14 expression is low and that, once synthesized, the protein is quickly secreted into the circulation.

Depending on the production pathway, two forms of sCD14 with different molecular masses can be identified [14,15]. The size of the human sCD14 found in the mouse plasma corresponded exactly to that of the isoform which is most abundant in normal human plasma [40,45] and in human milk [45]. This isoform is the result from a direct secretion from intracellular compartments into the plasma, while the smaller form originates from membrane-bound CD14 that is released after proteolytic cleavage [15].

We recently observed increased sCD14 concentrations in the plasma of patients suffering from chronic HBV and HCV infections in comparison with healthy subjects [30]. In the chimeric mouse model, infections with HBV or HCV had no effect on the plasma sCD14 levels. This suggests that infections of human hepatocytes with HBV or HCV have no direct effect on the production of sCD14 by these cells. The increased sCD14 concentration in the plasma of chronically infected patients could be due to an increased production by activated monocytes. It is known that the plasma of patients suffering from chronic liver diseases contains more lipopolysaccharide (LPS) than healthy controls [46]. This was also observed by Sozinov et al. in patients with chronic viral hepatitis [47]. This peripheral endotoxia in chronic liver disease may be the result of a decreased detoxification of endotoxin-rich portal blood by the diseased liver, an increased intestinal permeability for bacterial toxins [48], or a combination of both. This endotoxia may activate monocytes and increase the production of sCD14.

We previously observed that sCD14 concentrations in plasma of patients with chronic HBV infections were inversely correlated with the amounts of circulating HBsAg [30]. Therefore we quantified HBsAg in the plasma of our HBV-infected chimeric uPA-SCID mice and compared these values with the concentrations of sCD14. No correlation could be found. This may be due to the absence of human macrophages in our model and the possible role of these cells in the production of sCD14 during liver disease.

Here we show for the first time in vivo that human hepatocytes are able to secrete sCD14 into the circulation. Since the transplanted human hepatocytes are the only human cells that survive for extended periods in the recipient mice, we can be certain that hepatocytes are the sole source of the human sCD14. We dare to suggest that a still undefined, but possibly large fraction of the sCD14 in human plasma originates from the liver. This is not surprising since the liver produces a series of acute-phase proteins in response to inflammation. The production of sCD14 by the liver may be a powerful mechanism to rapidly clear the blood from LPS and another mechanism of host defense and control of systemic inflammation. The hepatic production of sCD14 may also play a role in the transport and transfer of phospholipids [23]. Finally, we have demonstrated that HBV and HCV infections have no direct influence on the production of sCD14 by hepatocytes. The elevated sCD14 levels observed in chronic HBV and HCV patients are therefore most likely of non-hepatic origin.

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References


A small animal model that is suitable for the study of HBV and HCV infections would greatly promote our understanding of these infections and the development of preventative and therapeutic strategies. Until now, these viruses have been primarily studied in chimpanzees but the use of these animals is not only very expensive but also increasingly questionable for ethical and ecological reasons. In addition, the natural course of chronic HBV and HCV infections in chimpanzees differs significantly from that observed in humans.

Because mouse hepatocytes are not permissive for HBV or HCV infections a mouse must harbor human hepatocytes before it can be infected with HBV or HCV. The development of a mouse model for HBV and HCV therefore necessitates the transplantation of human hepatocytes. Two major obstacles impede the successful transplantation of human hepatocytes in any recipient mouse: 1) xenogeneic hepatocytes transplanted into immune competent mice will be rejected and 2) xenogeneic hepatocytes will not thrive and expand in a “healthy” mouse liver due to lack of “space” and absence of appropriate “growth support”. A mouse that will accept a human hepatocyte graft and support its survival and expansion therefore needs to have the following qualities: 1) be immune deficient and not reject xenografts and 2) suffer from a severe and chronic liver disease that provides the “supportive niche” for the transplanted, healthy, human hepatocytes. An animal with these qualities does not exist as such but can be created by combining, through cross-breeding, the qualities of two existing mouse strains. The severe combined immunodeficient mouse (SCID, CBySmn.CB17-Prkdc<sup>scid</sup>/J) (1, 2) brings in the immune deficiency and thus the “acceptance” of the xenograft. The urokinase-type plasminogen activator (uPA) transgenic mouse (B6SJLTgN(Alb1Plau)144Bri) (3) suffers from a severe and chronic liver disease through the liver specific overexpression of uPA and thereby provides the required “niche” for healthy donor hepatocytes.

In 2000 we started breeding SCID mice (available in the lab) with uPA<sup>+/−</sup> transgenic mice (obtained from The Jackson Laboratories) and kept crossing the uPA<sup>+</sup> offspring with “pure” SCID mice. After seven generations more than 99% of the mouse background is expected to be of the SCID genotype. For this backcrossing program it sufficed to distinguish uPA<sup>+</sup> from uPA<sup>−</sup> animals. However as we started transplanting human hepatocytes in the uPA-SCID mouse recipients we realized the need to discriminate uPA<sup>+/+</sup> (homozygous) from uPA<sup>−/−</sup> (heterozygous) mice. This
distinction is very important since heterozygous animals only allow limited and transient repopulation due to spontaneous deletion of the uPA transgene and consequent regeneration of the diseased liver by “revertant, healthy” mouse hepatocytes (3). Existing screening methods were based on a semi-quantitative analysis of the copy number of the uPA transgene using Southern blotting (4). However, this methodology has some weaknesses: it is time consuming and the results are sometimes difficult to interpret.

Therefore we designed a method that would allow for the discrimination between heterozygous and homozygous uPA-SCID mice in a rapid, robust and unambiguous manner. We took advantage of the fact that the introduction of the uPA-transgene cassette in the mouse genome had induced a small deletion of the target chromosome (5). This region was picked-up in a plasmid that we have sequenced. We then selected a combination of three PCR primer pairs that unambiguously allowed for the identification of homozygous uPA-SCID mice within a working day (6). The speed of the method is of the utmost importance since homozygous uPA-SCID mice should ideally be transplanted within two weeks after birth. Our screening method is currently being used by the two other groups that use the uPA mouse for human liver cell transplantation (7) (Kneteman N., personal communication).

Additionally, we also needed an easy, sensitive and non-invasive method to assess the success of our transplantation experiments. We chose for an Elisa based method using antibodies that detect human albumin, a protein of hepatic origin, in a sensitive and specific manner. The concentration of human albumin in the mouse plasma is a reliable “in vivo” marker of the extent and functionality of the human liver cells graft.

Once we had developed these crucial instruments to select homozygous uPA-SCID mice and to measure human albumin in a mouse matrix, we transplanted uPA+/−-SCID mice with fresh human hepatocytes. These cells were injected in the spleen from where they migrated to the liver via the splenic and portal veins. This injection route is superior to direct injection into the liver since the latter is more traumatic and would only result in a focal localization of the injected hepatocytes. In contrast, intrasplenic injection results in a more dispersed distribution of the injected cells.

At different time points after transplantation, we evaluated the integrity and morphology of the human hepatocytes in our mice (8). Plasma analysis showed that the human hepatocytes secreted at least 22 human proteins. Most of these proteins are classical human plasma proteins, but others are known to be localized in the membrane or in the nucleus of the cell. This is not surprising since the applied proteome method is very sensitive and able to pick up less abundant proteins (9). Using comparable methods, such atypical plasma
proteins can also be found in plasma from healthy human blood donors (10, 11). Other groups that have developed chimeric human liver mouse models have not performed such a systematic analysis of the human proteome in the chimeric mouse. A Japanese group has reported the expression and induction of human cytochromes P450 enzymes in the chimeric liver (12-14). These data are an additional proof that the human hepatocytes retain their normal functional characteristics also in a xenogeneic environment.

Histological analysis of our chimeric mouse livers showed that up to 87% of the liver parenchyma can be repopulated by healthy human hepatocytes. The repopulation process occurs in a gradual and organized fashion. The human hepatocytes clearly communicate with the mouse environment, as illustrated by the connections made by bile canaliculi formed by human hepatocytes with canaliculi of mouse origin and even functional chimeric bile canaliculi can be observed.

Although the general appearance of the human hepatocytes closely resembles that in human liver, there are some minor differences. The human hepatocytes tend to accumulate glycogen, comparable to what is seen in glycogen storage diseases (15). Since the chimeric mice are hypoglycemic (Meuleman et al., unpublished data) it is very likely, but not yet proven, that certain mouse signals (not yet determined) fail to interact adequately with their receptors on the human hepatocytes. The chimeric animals are relatively small (10 to 12 g) in comparison to healthy SCID mice of the same age (20 to 25 g). Homozygous animals transplanted with mouse hepatocytes do not experience this pronounced failure to thrive and 6 to 8 weeks after liver cell transfer these cannot be distinguished form their healthy SCID counterparts. This growth retardation is likely the result of the glycogen accumulation, but other types of “dysmetabolism” can also be involved. It is intriguing that although up to 87% of liver parenchyma is occupied by human hepatocytes, plasma human albumin levels are only 20% of normal values. These phenomena deserve further investigation since similar abnormalities may arise when whole livers of xenogeneic origin (e.g. pig or baboon) will be transplanted in humans, an approach that is being considered. Other investigators working with similar chimeric animal models have not described the glycogen storage. The glycogenosis is however also present in the human hepatocytes of their chimeric mice, but they have overlooked this phenomenon because they have not enjoyed the same anatomopathological support and expertise as we did (Kneteman N., personal communication).

A remarkable finding was the fact that not only mature hepatocytes were involved in the repopulation process, but that also hepatic progenitor cell (HPCs) participated in this process. Immunohistochemical analysis revealed HPCs that were differentiating towards...
mature hepatocytes via intermediate hepatocyte-like cells. These HPCs were generally observed at the interface between human and mouse regions in the chimeric liver. This observation has a major impact and may shed a new light on certain qualities attributed to hepatocytes. Using liver cell suspensions comparable to ours, other investigators have observed that these may give rise to biliary epithelial cells and concluded from this that “mature hepatocytes” were able to transdifferentiate towards cholangiocytes (16, 17). Other groups used such cell suspensions to prove that mature hepatocytes have an extensive repopulative capacity (18, 19). The presence of HPCs in the suspensions used represents an alternative explanation for these phenomena.

Successfully transplanted animals could be easily infected with HBV and HCV. In both infections the viremia was considerably higher than what is routinely observed in chronically infected humans (20-22). On retrospect this is not so surprising. After all, the chimeric animals are severely immune suppressed and the viremia in these animals should not be compared with that observed in immune competent “chronic patients” but rather with that observed in chronically infected patients undergoing immune suppressive therapy after organ transplantation (23-25). Using electron microscopy, complete HBV particles (Dane particles) and subviral particles (spheres and rods) could be easily visualized in the HBV-infected hepatocytes. This was not the case for HCV-infected chimeric livers in which no HCV could be observed despite very careful examination. HCV particles are also hard to identify inside HCV infected human liver biopsies (26). Considering the size of a human hepatocyte (25 - 30 µm), the ultra-thin sections used for EM analysis (50 nm), the size of a HCV virion (56 - 60 nm) and the fact that hepatocytes only produce between 10 and 100 complete virions per day (27), this is not unexpected. An indirect proof of HCV replication and the production of intact, infectious HCV particles is given by the fact that HCV infection can be transferred from an infected chimeric animal to an uninfected one with minute (<5 µl) amounts of infected mouse plasma. This procedure resulted in a 10⁹-fold amplification of the virus.

While we did not observe any obvious effect of the infection on the morphology of the HCV-infected hepatocytes, infection of chimeric mice with HBV resulted in severe hepatocellular damage and cell death, which ultimately led to the death of the animals. High plasma concentrations of HBV virions and viral proteins could be detected up to 5 weeks after infection. Thereafter all viral markers (HBsAg, HBeAg and HBV-DNA) as well as the human albumin levels rapidly decreased. This decrease coincided with the clinical deterioration and these animals survived the infection on average for only 50 days. Histologic and electron
microscopic analysis of the livers of clinically terminal mice revealed several signs of hepatocyte malfunction and degeneration. The infected human hepatocytes were completely filled with viral and subviral particles. Molecular analysis showed very high replication in these long-term infected human hepatocytes. Like in patients with chronic HBV (28) and in a model for the study of duck hepatitis B virus (DHBV) (29), we also observed an activation of the hepatic progenitor cell compartment which is another indirect sign of hepatocellular damage. Sequence analysis of the HBV inoculum employed for these experiments did not reveal any mutations that were known to be cytopathic. Therefore, the only possible explanation is that HBV has endogenous cytopathic capacities that only prevail under conditions of severe immune suppression. This is in contrast to the generally accepted opinion that HBV is a non-cytopathic virus and that the hepatocellular damage in chronic HBV patients is caused by the host’s immune response towards the infected hepatocytes (30). On the other hand, a condition known as fibrosing cholestatic hepatitis (FCH) can occur in chronic HBV patients whose immune system is severely suppressed (31-35). The serology and the liver histology in this, often fatal, syndrome closely resembles those observed in our chimeric animals. These observations support our theory that also in chronic HBV patients the immune system, despite its inability to completely eradicate the viral infection, plays a role in the control of the infection by preventing the massive viral replication and protein expression that leads to the destruction of the infected cell. Nowadays, FCH is rather uncommon in HBV infected transplant patients due to more effective antiviral treatment and passive administration of anti-HBsAg immunoglobulins (36-38).

We (39) and others (40) have described that chronic HBV and HCV patients have elevated soluble CD14 (sCD14) levels in their plasma. sCD14 is involved in LPS-induced responses by cells that do not express CD14 on their surface (41-43), but can also prevent activation of CD14 bearing cells by LPS (44). In addition, sCD14 forms a protein pair with lipopolysaccharide (LPS) binding protein (LPB) that is involved in the transport and exchange of phospholipids (45). Using our animal model we investigated whether this increase in sCD14 was directly caused by the virus. The unique nature of our model allowed us to prove that human hepatocytes are able to produce and secrete sCD14 in vivo (46). Monocytes, another important source of sCD14 in humans, are completely absent in our chimeric animals. The levels of sCD14 found in our animals were comparable to those measured in healthy humans, indicating that hepatocytes may be a major source of sCD14. Infections of chimeric mice with HBV or HCV did not induce significant changes in the sCD14 levels measured in the plasma of the mice.
This finding suggests that HBV or HCV infections have no direct effect on sCD14 production by the hepatocytes (46). The elevated sCD14 levels observed in patients suffering from chronic viral hepatitis can be explained by the fact that patients with (viral) liver diseases also have elevated levels of LPS in their plasma (47, 48). These higher than normal levels of LPS might in turn activate monocytes to secrete sCD14. In addition, chronically infected livers are also known to be less efficient in clearing numerous serum constituents, amongst which endogenous proteins.

The development of the uPA-SCID model is a major breakthrough in the study of viral hepatitis, but the model has some drawbacks that deserve to be discussed. First of all, large numbers of uPA-SCID breeding pairs are needed to generate a constant and sufficient output of homozygous animals suitable for transplantation. Secondly, the transplantation of these homozygous animals is a complex and difficult task. Due to the high uPA expression and consequent bleeding disorder, perioperative complications are not uncommon even in skilled hands. A third and probably the most important limitation is the availability of human hepatocytes of excellent quality. These cells are instrumental for success and due to the short window in which the animals need to be transplanted, a good liaison with liver surgeons is of utmost importance. Cryopreserved human hepatocytes are an alternative but the possible use of frozen cells needs to be further studied. We have shortly explored the usefulness of these cells, however with limited success (unpublished data). Finally, since the chimeric human liver in uPA-SCID mice are devoid of an immune system, these animals cannot be used to study the “host” immune response towards HBV or HCV. The direct evaluation of new prophylactic or therapeutic vaccines is therefore impossible in the present model. However, the neutralizing capacity of polyclonal or monoclonal antibodies against these viruses can be examined by passive administration to the chimera prior to the injection of the viral inoculum. The “perfect” model that would allow the study of innate and adaptive antiviral immune responses against HBV or HCV requires the presence of an intact and functional human immune system in addition to the successful human hepatocytes graft in the uPA-SCID mouse. The model could be adapted by several ways to achieve this goal. First, the infected chimeric animals can be transplanted with lymphocytes derived from the same donor from whom the hepatocytes were isolated. Alternatively, lymphocytes from an HLA-matched donor could be used. In both set-ups a method has to be devised to overcome the occurrence of a graft-versus-host reaction which will lead to the premature death (2 to 4 weeks after cell transplantation) of the chimeric animals. Second, the chimeric animals could be transplanted with selected cell populations,
like T-cell clones, NK-cells, etc. A third method could be the transplantation of human cord blood cells or other haematopoietic stem cells. However, to generate a functional immune system, here again the HLA restriction must not be overlooked.

Despite these concerns which are essentially of a logistic and technical nature, the human liver uPA-SCID mouse remains the best available alternative for the chimpanzee. We will use this model for the in vivo evaluation of neutralizing anti-HCV antibodies. Several reports already described the neutralizing activity of monoclonal and polyclonal antibodies against HCV in the HCV pseudo-particle system (49-51). However, it remains to be determined whether these antibodies will demonstrate neutralization in vivo where the properties of authentic virus and target cells may be different from the in vitro systems. The advantage of our small animal model is that much lower quantities of antibodies are required than in chimpanzees. Recently, successful HCV infection and replication were established in vitro (52-54). This in vitro culture system resembles the real situation more closely than the pseudo-particle system, but does not match an in vivo challenge. The market for antibodies that are able to neutralize a HCV infection in vivo must be huge. Indeed, chronic HCV infections and their complications represent the major indication for liver transplantation in Western countries. Infection of the transplanted liver with HCV is an almost certain event that cannot be prevented, nor treated (55). Even worse, because of the immune suppression the allograft recipient is undergoing, this infection evolves more rapidly towards end-stage liver disease than the original one. The effectiveness of passive immunization to prevent infection of the liver graft following transplantation has been established in HBV-infected patients (36-38). This clinical experience suggests that passive immunization may equally be the best approach for transplanted HCV patients.

In collaboration with other groups, we will also use our chimeric mouse model for the evaluation of novel antiviral drugs against HBV and HCV. Candidate antiviral compounds are now only evaluated in the subgenomic replicon system (56) before human trials can commence. If the chimeric mouse proves to be a good system to evaluate antivirals, regulatory agencies will certainly require its use before a compound will be allowed to go into clinical testing. Although such a measure seems to make the procedure of drug development heavier it will be most useful in preventing the loss of time and money required for clinical tests (phase I and II).

Besides its use as an animal model for the study of HBV and HCV, the human liver uPA-SCID mouse has also potential in other research areas. Any microorganism that targets the liver or needs human hepatocytes at a specific stage of its life
cycle can be studied in this model. One such example is malaria, where the sporozoites of the *Plasmodium falciparum* mature inside the hepatocyte before they move into the red blood cells (57).

Applications of the uPA-SCID model reach far beyond the limits of microbiology. The model is not only useful to examine biotransformation of drugs by human hepatocytes in vivo but may also be employed to study the potential liver toxicity of new compounds (12-14).

Finally, the uPA-SCID mouse can be used for basic research on stem cells and liver regeneration. We have already shown that human HPCs are involved in the liver repopulation process (8). To what extent progenitor cells and mature hepatocytes each contribute to the liver repair is currently unknown. Transplantation of selected immature subpopulations and purified mature liver cells may help elucidate this question. In addition, the potential of other stem cells of different origin to (trans)differentiate towards hepatocytes can also be evaluated in this model (58, 59). The disease state of the uPA-SCID liver mimics the situation of chronic liver disease more closely than the currently used *in vitro* systems which employ all different kinds of artificial matrices in combination with a cocktail of growth factors. We have already evaluated the capacity of different stem cells from haematopoietic origin to repopulate the sick livers of our uPA-SCID mice. None of these “pluripotent” stem cells were able to engraft and expand in the uPA-SCID liver, despite their promising and multipotential qualities *in vitro* (unpublished data).
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