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Contents

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Contents

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EDITORIAL

Nutritional modulation of the inflammatory response in inflammatory bowel disease- From the molecular to the integrative to the clinical

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Abstract

Nutrient deficiencies are common in patients with inflammatory bowel disease (IBD). Both total parenteral and enteral nutrition provide important supportive therapy for IBD patients, but in adults these are not useful for primary therapy. Dietary intervention with omega-3 polyunsaturated fatty acids contained in fish oil may be useful for the care of IBD patients, and recent studies have stressed the role of PPAR on NFκB activity on the potential beneficial effect of dietary lipids on intestinal function.

Key words: Crohn's disease; Ulcerative colitis; Enteral nutrition; Parenteral nutrition; Glutamine; Fiber; Long chain fatty acids

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INTRODUCTION

Inflammatory bowel disease (IBD) is a term used to denote inflammation of the gastrointestinal mucosa of unknown etiology. There are a number of hypotheses pertaining to the development and perpetuation of IBD. Three major theories emerge from the literature. The first implicates a persistent intestinal infection[1-3]; the second proposes that IBD is a consequence of a defective mucosal barrier to luminal antigens[4,5]; and the third suggests a dysregulated host immune response to ubiquitous antigens[4,6]. It is believed that IBD has both genetic and environmental components, and is immunologically mediated[4,7-9]. Data from IBD patients concerning cytokine profiles, permeability defects, response to treatment, and natural history of disease all indicate a heterogeneous group of illnesses that fall under the headings of ulcerative colitis (UC) and Crohn's disease (CD). Previous epidemiological data covering diet in UC and CD are conflicting, partly due to this heterogeneity of the diseases, difficulty in obtaining reliable data and publication bias such as negative structures of breastfeeding[10].

NUTRITION IN IBD

Specific antibody isotypes to major milk proteins are found in both UC and CD patients. In CD, the antibodies correlate with disease activity[11]. Although ethnic origin[12], and not the IBD disease state, appears to be the main determinant of lactose intolerance, the avoidance of dairy products by IBD patients is extensive[13]. Lack of breastfeeding in infancy has been associated with CD but not UC. In addition, increased carbohydrate consumption has been documented in CD[14]. Others have alluded to a lack of dietary fiber as a predisposing factor for IBD[15]. The development of UC has also been associated with high intakes of monounsaturated fatty acids (MUFA), n6 polyunsaturated fatty acids (n6 PUFA), sulphur-containing diets and vitamin B6[16].

Deficiencies

IBD is associated with a number of nutritional deficiencies including anemia, hypoalbuminemia, hypomagnesia, hypocalcemia and hypophosphatemia, as well as deficiencies in folic acid, niacin, Vitamins A, B12, C, and D as well as deficiencies of iron, zinc, copper[17]. It is not clear if low levels of micronutrients are one of the results of disease or one of primary importance. Plasma antioxidant concentrations are reduced in IBD patients, particularly those with active disease[18]. Antioxidant activity, assessed by measuring selenium concentrations and erythrocyte

glutathione peroxidase activity, is inversely correlated with inflammatory biomarkers, including TNFα[19]. Hyperhomocysteinemia is more common in patients with IBD, and is associated with reduced serum, concentrations of vitamin B12, folate and B6[20].

Several mechanisms contribute to the malnutrition observed in IBD patients. Firstly, there is a decrease in the oral intake of nutrients because of abdominal pain and anorexia. Secondly, the mucosal inflammation and associated diarrhea leads to a loss of protein, blood, minerals, electrolytes and trace elements. Thirdly, multiple resections or bacterial overgrowth may have an adverse nutritional effect; and lastly, the pharmacological therapies may also lead to malnutrition. For example, sulfasalazine reduces folic acid absorption, and corticosteroids decrease calcium absorption as well as negatively affecting protein metabolism[21]. Alterations in energy metabolism may result in increased resting energy expenditure and lipid oxidation in IBD patients[22,23]. The consequences of malnutrition are numerous, and include reductions in bone mineral density[24], as well as growth retardation and delayed sexual maturity in children[25]. Osteoporosis may also be implicated as a result of proinflammatory cytokine profiles.

Nutritional therapy can take on a number of forms which include Total Parenteral Nutrition (TPN) and Total Enteral Nutrition (TEN). The diets used are elemental, polymeric, and exclusion diets. Elemental diets contain nutrients reduced to their basic components: amino acids for proteins, glucose for carbohydrates, and short-chain triglycerides for fats. Polymeric formulas contain whole proteins for nitrogen, glucose polymers for carbohydrates and long-chain triglycerides for starch or fat, respectively[17].

Total parenteral nutrition (TPN)

The use of TPN for the management of IBD is based on certain theoretical advantages: bowel rest is beneficial because it diminishes motor and transport function of the diseased bowel[26,27]; a decrease in antigenic stimulation will eliminate the immunologic responses to food, especially in the presence of impaired intestinal permeability[28-30]; TPN fosters protein synthesis in the intestine which leads to cell renewal, healing, and reversal of impaired immunocompetence.

Ostro and co-workers[31] demonstrated remission rates of 63% to 89% with TPN in a large retrospective series of CD patients who were refractory to conventional medical management. However, Matuchansky *et al*[32] emphasized that there were high relapse rates (40%-62%) after 2 years. It has been suggested that TPN be used only in a nutritionally supportive role[33,34]. In UC, there is no evidence for better outcome with TPN[35,36]. Although remission rates of 9% to 80% have been reported, TPN given to patients with severe colitis appears to only be beneficial as perioperative nutritional support. In patients with mild disease, TPN is more effective but is not better than steroid therapy, and thus the invasiveness and cost of TPN are unjustified. Any benefits associated with TPN may be due to the administration of nutrients, and not bowel rest, as bowel rest alone has no effect on disease activity[37]. Therefore, although TPN has a role in patients with a non-functioning intestine or the short bowel syndrome due to excessive resections, TPN is of limited use as a primary therapy in IBD. This is not intended to be an extensive review of TPN, but it should be cautioned that even in expert centres, TPN is associated with complications such as sepsis and cholestatic liver disease.

Total enteral nutrition (TEN), elemental and defined formula diets

TEN excludes potential toxic dietary factors and antigenic exposure, since there are only amino acids, glucose or oligosaccharides and low lipid content. TEN is not associated with cholestasis, biliary sludge or gallstone formation, as is seen with TPN. Atrophy of the small intestinal mucosa has been observed in animal models receiving long-term TPN, but this atrophy is prevented with TEN. In addition, a 6-wk TPN treatment in dogs resulted in marked reduction in pancreatic weight, a decrease in small intestinal mass, and a decrease in intestinal disaccharidase activity in dogs[38]. For this reason, TEN is preferable to TPN.

The topic of nutrition in GI disorders occurring in IBD has been reviewed recently[39,40]. When compared to TPN, enteral nutrition yielded similar results of preventing and combating malnutrition[35,36,41]. Although Voitk *et al*[42] proposed that elemental diets may be an effective therapy for IBD, enteral nutrition as a primary therapy has failed to yield consistent results in numerous clinical trials. It is true that a number of trials have shown remission rates in CD patients receiving elemental diets, similar to the rates observed with steroid therapy[43-54]. However, it is noteworthy that significantly better remission rates were observed in patients receiving steroid treatment versus elemental diets when including all the diet group drop outs (i.e., on an intent-to-treat basis). The question remains as to the best way of analyzing the results when a large percentage of patients receiving diet therapy drop out because of unpalatibility or intolerance. Furthermore, some studies have shown no difference with elemental diets when compared to steroid therapy[48,52]. In children, elemental diets were associated with greater linear growth, while in adults these diets preserve nitrogen balance[55,56]. The role of nutritional therapy in the context of pediatric onset illness has been reviewed[57]. Thus, enteral nutrition is easier to use, is less expensive, and is a better alternative to TPN. Unfortunately, its unpalatability limits patient compliance, but with strong encouragement this may be partially overcome.

The fat composition of enteral diets may influence the results which are obtained in the various clinical trials. Elemental diets have a low fat content, while most polymeric diets generally contain more fat including more linoleic acid, which is a precursor for the synthesis of potentially proinflammatory eicosanoids[58].

Defined formula diets are usually more palatable and less expensive than are the elemental diets. While some investigators report no differences between elemental and defined formula diets in patients with acute CD[49,59,60], Giaffer *et al*[61] found elemental diets to be more effective

in active CD. A randomized double-blind trial in Crohn's patients demonstrated that elemental and polymeric (defined) diets, differing only in their source of nitrogen, were equally effective in reducing the Crohn's disease activity index (CDAI), and in inducing clinical remission[62]. Although defined formula diets provide less bowel rest, they have the potential advantage of exposing the GI tract to the usual dietary substrates, which allow thereby for the full expression of intestinal, biliary and pancreatic activity[63]. In animal studies, it has also been observed that luminal nutrition has trophic influences on the gut[64].

Is there a beneficial effect of supplementing polymeric formulas with TGF-β1[65]? In pediatric CD, reductions in proinflammatory cytokine concentrations and mRNA, paired with an up-regulation of TGF-β mRNA, was associated with improved macroscopic and microscopic mucosal inflammation. A meta-analysis and a Cochrane review have demonstrated that in adults, corticosteroids are more effective than enteral diet therapy[48-50]. It is unclear what is the role of nutritional therapy in adults with CD[51-53], although there is some evidence in Japan that enteral nutrition enjoys support as primary therapy[53]. In contrast to the generally agreed role in adults of enteral nutrition being useful to improve the patient's nutritional status as its main benefit, in children with CD enteral nutrition has a much clearer benefit to improve clinical, biochemical and growth parameters[55], and may as well have a steroid sparing effect[56,57].

Glutamine, fiber and fatty acids

Diets high in glutamine, an important source of energy for enterocytes and the preferred fuel of the small intestine[66,67], have been used with variable success. Glutamine probably exerts its trophic effects on the small intestine by increasing protein synthesis, and generating alanine as a substrate for enteric gluconeogenesis[68]. There is evidence that glutamine protects the small intestinal mucosa during critical illness[69,70]. However, oral glutamine supplements do not restore to normal the increased permeability seen in patients with CD, and these supplements do not beneficially affect the patients' CDAI or C-reactive protein (CRP) levels[71]. Similarly, a randomized controlled trial demonstrated that no benefit was associated with the intake of glutamine-enriched polymeric formulas in children with CD[72].

In animal studies, dietary fiber has been implicated in maintaining the integrity of the intestine, and in preventing the bacterial translocation from the gut to the mesenteric lymph nodes[73,74]. Short chain fatty acids (SCFA, C1 to C6 organic fatty acids), are produced by the fermentation of dietary polysaccharides by the normal anaerobic bacteria in the colon. These SCFA are a source of energy for the colonocytes, in addition to their enhancing sodium and water absorption and promoting blood flow[75,76]. Reduced levels of SCFA, particularly butyrate, and a defect in the oxidation of butyrate by colonocytes, have been proposed as a mechanism in the pathogenesis of IBD[77,78]. Evidence to support this hypothesis includes the observation that the oxidation of ^{13}C-labelled butyrate is lower in patients with active UC as compared to healthy controls[79]. However, Simpson and co-workers failed to demonstrate differences between UC patients and controls in the oxidation of rectally administered ^{13}C-labelled butyrate[80].

TPN supplemented with SCFA enhanced functional adaptation to intestinal resection in rats. It remains to be determined if patients with short bowel syndrome may benefit from SCFA[81].

Butyrate (C4 fatty acid) administered to UC patients resulted in remission rates comparable to corticosteroids and mesalamine[82]. In patients with CD, both intestinal biopsies and lamina propria cells cultured with butyrate had significantly reduced levels of inflammatory cytokines (TNF), possibly due to a reduction in NFκB activation and IκB degradation[83].

Eicosanoids are inflammatory mediators, and have been implicated in the pathogenesis of chronic inflammatory lesions in the bowel. Specimens from patients with IBD show increased eicosanoid formation[84]. High dietary intake of omega-6 polyunsaturated fatty acids (PUFAs), which reduces omega-3 intake, may contribute to IBD development[85]. The benefits of fish oil, which contain n3 fatty acids, have been shown in some inflammatory diseases such as psoriasis and rheumatoid arthritis. Epidemiological observations of the low prevalence of IBD in Japanese and Inuit populations consuming high n3 fatty acid fish provided a rationale for the use of n3 fatty acids in IBD. The n3 fatty acids are believed to compete with n6 fatty acids as precursors of eicosanoid synthesis. The n3 products are series 5 leukotrienes, which have less physiological activity than do the arachidonate- derived series 4 counterparts. Thus, fish oil may have an anti-inflammatory effect.

Rats fed with fish oil which had TNBS-induced inflammatory lesions in the bowel showed less prostaglandin- and leukotriene-mediated immune response[86]. Parenteral lipid emulsions enriched with n3 fatty acids reduce diarrhea, attenuate morphological changes, and decrease colonic concentrations of inflammatory mediators in an animal model of acetic acid induced colitis[87].

Loeschke *et al*[88] performed a placebo-controlled trial of n3 fatty acids in the prevention of relapse in UC. Patients in remission who received n3 fatty acids experienced fewer relapses than did those receiving placebo. Unfortunately, the beneficial results of this study did not persist throughout the length of the 2 year study, possibly due to decreased compliance over time. In a multicenter placebo controlled relapse prevention trial, Belluzzi *et al*[89] found a significant reduction in the relapse rate in CD patients given a special formulation designed to allow delayed ileal release of n3 fatty acids. A fish oil diet has been shown to increase eicosapentanoic and docosahexanoic acids in the intestinal mucosal lipids of IBD patients, as well as showing a decrease in arachadonic acid. An increase in the synthesis of leukotriene B5 as well as a 53% decrease of leukotriene B4 was shown in UC patients, whereas the fish oil treatment showed a nonsignificant trend to faster remission[90,91]. Fish oil supplementation results in clinical improvement of active mild to moderate disease, but was not associated with a significant decrease in leukotriene B4 production[84]. Thus, fish oil supplementation of the diet may provide some short-term benefit to patients with CD

or UC. The use of probiotics and prebiotics has received much attention; the interested reader is referred to recent reviews in this area[40].

Fatty acids and gene expression

The effect of fatty acids on gene expression was previously thought to result largely from alterations in membrane phospholipids or eicosanoid production. More recently, the discovery of nuclear receptors; such as peroxisome proliferator-activated receptors (PPARs), and their regulation by fatty acids, has changed this view. PPARs are ligand activated transcription factors that upon heterodimerization with the retinoic X receptor (RXR), recognize PPAR response elements in the promoter regions of various genes, and subsequently affect gene transcription. PPARs bind various ligands including nonsteroidal anti-inflammatory drugs (NSAIDS), thiazolidinediones (antidiabetic agents) as well as PUFAs and their metabolites[92-96]. Several subtypes of the receptor have been identified (α,δ,γ) and these are differentially expressed in a variety of tissues. PPARγ is expressed in intestinal tissue, with the highest abundance detected in the colon[97].

PPARγ has been implicated in the regulation of inflammation, and has become a potential therapeutic target in the treatment of inflammatory disorders, including IBD. It has been suggested that patients with UC have a mucosal deficit in PPARγ that may contribute to the development of their disease. Indeed, analysis of mRNA and protein from colonic biopsies shows reduced PPARγ in UC patients when compared to either Crohn's patients or healthy controls[98].

Using colon cancer lines, it has been shown that PPAR ligands attenuate cytokine gene expression by inhibiting NF-κB via an IκB dependent mechanism[99]. A number of other studies suggest that PPAR activators inhibit COX2 by interference with NF-κB[100-102]. PPARs inhibit the AP-1 signaling pathway[103], and interact with the Jun[104] and STAT signaling pathways[105].

Animal studies support the role of PPAR in intestinal inflammation. Thiazolidinedione ligands for PPAR markedly reduced colonic inflammation in a mouse model of IBD[99]. PPAR+/- and RXR +/- mice display enhanced susceptibility to TNBS-induced colitis[106]. The administration of PPAR and RXR agonists synergistically reduce TNBS-induced colitis, with improved macroscopic and histologic scores, reductions in TNFα and IL-1β mRNA, and decreased NF-κB DNA binding activity.

Although clinical data is limited, the results of an open label study using rosiglitazone, a PPARγ ligand as therapy for UC, showed that 27% of patients achieved remission after 12 wk of therapy[107]. Thus, PPARγ ligands may represent a novel therapy for UC, and double-blind, placebo-controlled, randomized trials are warranted.

Of considerable interest, the ability to modulate PPAR nutritionally has been studied. Dietary PUFA have dramatic effects on gene expression through the regulation of several transcription factors, including PPAR. Fatty acid regulation of PPAR was first noted by Gottlicher *et al*[108]. A diverse array of fatty acids, eicosanoids, and fatty acid metabolites have been shown to activate PPAR[94-96]. Both PPARα and PPARγ bind mono- and polyunsaturated fatty acids at levels, which are found in human serum[95]. Thus, the anti-inflammatory effects of n3 PUFA may involve PPAR and interference with NFκB, rather than simply alterations in eicosanoid synthesis.

Clinical Implications

It is widely accepted that nutritional deficiencies are common in patients with CD and UC, and these need to be anticipated, identified and treated. There are no specific diets which can be recommended for all patients with IBD; diet therapy needs to be individualized. TPN or TEN may be necessary to restore nutritional balance in selected IBD patients with malnutrition, but in adults these interventions do not provide a primary option to modify disease activity. The omega-3 PUFAs contained in fish oil may reduce disease activity in UC and CD when used in the short term in conjunction with standard medical therapy. Their mechanism of action may be to enhance the activity of the nuclear receptors PPAR (peroxisome proliferator-activated receptors) in the intestine, inhibiting the AP-1 signaling pathway and NF-κB, attenuating proinflammatory cytokine gene expression. Future research will focus on the identification and use of specific dietary lipids to reduce intestinal inflammatory activity and to maintain long-term disease remission.

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EDITORIAL

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Recent advances in hepatitis B virus research: A German point of view

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Abstract

More than 30 years after the discovery of human hepatitis B virus (HBV) this virus remains to be one of the major global health problems. In infected adolescents or adults, 5%-10% will lead to a chronic carrier state, whereas in infected neonates up to 90% develop chronicity. It is estimated that about 370 million people are chronic carriers of HBV worldwide. In many regions of the world, chronic HBV infection is still the major cause of liver cirrhosis and hepatocellular carcinoma. During the last 30 years, many steps of the viral life cycle have been unravelled, mainly due to cloning, sequencing and expression of the genomic DNA extracted from HBV virions. This has lead to the development of a safe and efficient vaccine and sensitive tests for HBV surface protein (HBsAg) allowing reliable diagnosis and screening of blood products. More recently, a growing number of reverse transcriptase inhibitors have been developed. However, together with these improvements new deficiencies in prevention and cure of HBV infections are becoming apparent. Although HBV is a DNA virus, it is highly variable under immunity or drug induced selection pressure, resulting in vaccine-related escape mutants and drug resistance. To overcome these challenging problems new antivirals and optimised vaccines have to be developed.

Key words: Hepatitis B virus; Molecular biology; Viral life cycle; Animal system; Antiviral therapy

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The discovery of the Australian antigen by Baruch S. Blumberg, which is now known as "hepatitis B virus surface antigen" (HBsAg), in the late 1960's opened the field of HBV research towards an understanding of an ancient scourge. During the following decades, many research groups all over the world worked successfully to unravel the life cycle of HBV. In Germany, the virologists Heinz Schaller, Peter H. Hofschneider, and Reiner Thomssen initiated HBV research and provided a profound impact on our understanding of the molecular biology of this virus. Among the clinicians; the names of Karl-Hermann Meyer zum Büschenfelde and Wolfgang Gerok are to be mentioned as founders of hepatitis B research. Moreover, in their labs, but also in other groups, students and scientists became inspired to work on this fascinating virus. Since then, an active and growing community of HBV virologists has been established in Germany. In this highlight topic a collection of reviews written by those scientists and clinicians is presented. The articles cover nearly all fields of hepadnaviral research, from attachment and entry to genome replication, maturation, pathology, animal models and antiviral therapy. The aim of this collection is to bring together recent findings in basic virological and clinical HBV research in order to provide a valuable reference for both hospital hepatologists and basic scientists.

Hepatitis B virus is the prototype member of the family *hepadnaviridae* that can be divided into the *orthohepadnaviruses* of mammals and the *avihepadnaviruses* of birds. To date, the *orthohepadnaviruses* are found only in humans and primates and in a special family of the *sciuridae*, namely North-American woodchucks and some squirrel species. Primate HBV were found in old world primates like chimpanzees, gorillas, gibbons, orangutans, and in one new world primate, the woolly monkey. In the review "Taxonomy and genotypes of Hepatitis B Virus", Stephan Schaefer reports that HBV sequences from old world primates are closely related and cluster in monophyletic groups, while the HBV sequence isolated from woolly monkeys (WMHBV) is more divergent[1]. Human HBV is further subdivided into several genotypes. To date, 8 genotypes (called A-H) that differ by definition in at least 8% of their complete genome have been found. The genotypes can be further divided into 24 sub-genotypes that differ by at least 4% from each other. Interestingly, no sub-genotypes have been described for genotype E and G. The fact that genotype E is common in West Africa, but absent in Americans of African origin from Venezuela and Brazil has led to the speculation of a recent genesis of genotype E. The geo-

graphic origin of genotype G is less clear, however a high prevalence of this genotype has been described in Mexico. Both, the genotypes and sub-genotypes show distinct virological and epidemiological properties. As an example, liver cirrhosis was detected in a greater number of patients chronically infected with HBV genotype C than genotype B in Japan. The same was also detected for the prevalence of hepatocellular carcinoma in these patients. Interestingly, detailed analysis of HBV genomes revealed recombination events between different genotypes. Hybrids between genotype B and C, A and D are very common in certain regions and distinct intergenomic recombination breakpoint hotspots were detected (e.g. the preS1/S2 region and the 3'-end of the surface gene). B/C recombinants occur in Japan and C/D recombinants in Tibet. However, the mechanisms underlying these potential recombination events are still unknown. In contrast to HIV or RNA viruses, recombination during replication is unlikely, because reverse transcription of only one RNA genome occurs within the capsid. Thus, it seems also possible that the described changes are the result of an adaptation to distinct genetic disposition in different human populations. Taking into consideration that a chronic HBV carrier can produce up to 10^{13} virions per day together with a high error rate of HBV reverse transcriptase, the HBV quasispecies can substitute every nucleotide of the small 3.2 kb genome within one patient every day. This favours very fast adaptation of the virus to a changing environment, and may result in modular adaptations within distinct segments of the genome.

Soon after the discovery of hepatitis B virus, it was shown that not only humans but also chimpanzees may test positive for this agent. Furthermore, artificial infection of chimpanzees, but also other apes like gibbons and orangutans with serum from HBV-infected patients induces acute and chronic infections related to human disease. Nevertheless, a strong species specificity of hepatitis B virus was observed, since it was not possible to induce HBV-infection in other mammals. Since then, many efforts have been made to establish a small non-primate animal model for HBV infection *in vivo* as described in the review "Viral and cellular determinants involved in hepadnaviral entry" in this topic highlight[2]. The finding that *Tupaia belangeri*, a small non-primate mammal from Southeast-Asia is susceptible to HBV and HDV raises doubt in the strict host specificity of HBV. Besides the *in vivo* infection, which is usually self-limiting and does not lead to chronic infection, the use of primary Tupaia hepatocyte cultures for *in vitro* infection has greatly improved the field. For many years, primary human hepatocyte cultures, obtained after surgical liver resection were the only possibilities to study infectivity of HBV *in vitro*. However, working with these scarcely available cell cultures is not easy and optimal HBV infection is highly dependent on artificial additives like dimethylsulfoxide (DMSO) and polyethyleneglycol (PEG) for optimal infection. Furthermore, susceptibility of these cultures varies strongly. Primary hepatocyte cultures from *Tupaia belangeri* have overcome these limitations both in availability and susceptibility. Furthermore, the infection is possible without the addition of DMSO and PEG. Most established hepatoma cell lines allow HBV production, but only after transfection of HBV DNA. Nevertheless, in 2001, a new hepatoma cell line called HepaRG was established that could be infected with HBV. However this feature is achieved only after prolonged cultivation in medium containing DMSO and other additives.

Experiments 20 years ago using synthetic peptides gave the first hint on the importance of the preS1 domain for viral infectivity. Later it was confirmed that the first 77 aminoacids of this domain are necessary for HBV infectivity, together with the N-terminal myristoylation at Glycin-2. Combining these important features, it turned out that these myristoylated preS1-peptides inhibited HBV infection at nanomolar concentrations *in vitro*. The exact role of myristoylation during the entry process of HBV is still not clear. It might enhance receptor recognition by insertion of the acyl chain into membranes of the receptor-complex. Interestingly, a so called myristoyl-switch is a well known element of viral entry mechanism used by non-enveloped viruses, like picornaviruses and reoviruses. Using a set of different myristoylated preS1-peptides the preS1 sequence responsible for this inhibition was further narrowed down to 10 aminoacids (NPLGFFPDHQ) in position 9-18 of preS1. Even single point mutations within this region abolish the inhibitory potential of preS1 peptides and when introduced into the viral surface proteins, also destroy infectivity of the virus completely.

The MHBs of HBV seem to be dispensable for infectivity of HBV and HDV. However, antibodies raised against the preS2 region that is an integral part of MHBs and LHBs could inhibit infection both *in vivo* and *in vitro*. Controversies exist about a role of a putative translocative motive (TLM) within the carboxyterminal part of preS2 and its importance for infectivity of HBV. However, no function of this sequence has been attributed to infection with HDV that uses HBV surface protein for infection.

The S-domain contributes to the main part of HBV surface proteins, but from the data obtained by use of myristoylated preS1 peptides, we know that the S-domain is not involved in initial binding to target cells. Nevertheless, antibodies raised against this domain are neutralising and contribute to protection against HBV infection in most cases. However, antibodies against the so-called antigenic domain (residues 100-160 that are also generated by the current S-containing vaccines) may not protect against naturally occurring escape-mutants that are frequently selected, e.g. by antiviral treatment. Since we now are aware that these antibodies will not counteract the binding of the virus to its target cells, the preS1 domain containing the minimal interaction site ought to be included into the vaccine. An unsolved problem is the identity of the still undetected high affinity receptor for HBV. An update of the still growing list of potential receptor candidates is listed in this reviews series, but none of them has ever been shown to be relevant for HBV infection.

Inspired by the presumption that the infection process of all hepadnaviruses may follow similar pathways, many labs used the more easy accessible duck hepatitis B virus (DHBV) infection system to study hepadnaviral entry *in vitro*. Although also restricted to primary hepatocyte cultures, this system is, compared to primary human hepatocyte cultures, relatively easy and accessible. Like in

HBV, the preS-domain of large surface protein of DHBV contributes to infection together with its N-terminal myristoylation. Furthermore, the preS-domain binds to a 180 kDa membrane protein and this binding can be inhibited by neutralizing preS-antibodies. Further work identified the gp180/p170 protein as carboxypeptidase D (CPD), a transmembrane protein with enzymatic activity. While substantial evidence supports the essential role of CPD for the DHBV infection process, expression of CPD in non-susceptible cells did not make these cells susceptible towards DHBV. Therefore, the first attachment partners for DHBV are still unknown. Furthermore, this molecule seems to be restricted to *avihepadnaviruses*, since the human homolog of CPD does not contribute to the HBV infection process. Therefore we have to be aware of the possible differences of entry mechanism of *avi-* and *orthohepadnaviruses*. Nevertheless with easy accessible *in vitro* infection systems for HBV available (primary Tupaia hepatocytes and the HepaRG cell line) it should now be possible to characterize cellular attachment factors and entry receptors for HBV.

After uptake, the viral genome has to be transported through the cytoplasm to the nucleus where active transcription can take place. As discussed by Kann *et al*[3]. evaluation of the transport modes of HBV from the plasma membrane to the nucleus is not easy since at least the early steps of attachment and entry are very species specific and usually need primary human or Tupaia hepatocytes. After uptake of the virus in a yet unknown compartment, the viral envelope proteins mediate fusion of viral and cellular membranes that results in delivery of the viral capsid into the cytoplasm. The capsid, harbouring the viral DNA, covalently linked to the viral polymerase must then be transported into the nucleus of the host cell. To study HBV capsid transport without an infection, isolated HBV capsids have to be either analysed within permeabilized cells or microinjected into *Xenopus laevis* oocytes. Both systems are very artificial and time-consuming. However, in the review of Kann *et al*, an easy *in vitro* system is described that allows analysis of intracellular transport after artificial entry of the capsids. The authors replaced the viral surface proteins of the virus by lipids, normally used for protein transfection. The lipids form an artificial membrane that allows fusion with the cellular plasma membrane and release of the viral capsid into the living cell. In fact, lipofection of HBV capsids isolated from virions allows "infection" of cells that are non-susceptible to the whole, complete virus. First, the core particles are transported *via* microtubuli towards the microtubule-organising centre, located at the perinuclear region of the cell. The viral capsid, containing a nuclear localisation signal interacts with the adaptor proteins importin alpha and beta. Importin beta facilitates contact with the nuclear pore and translocation into the nuclear pore. Interestingly, despite complete translocation, the core particles seem to be stuck at the end of their voyage through the nuclear pore, in a structure called "nuclear basket". Within the nuclear basket, the breakdown of the capsid and the release of the viral DNA into the nucleus should be facilitated. However, many questions are still unanswered, e.g. what determines the arrest of the core particle within the nuclear basket and genome release?

Once within the nucleus of the infected cells, the viral HBV genome is converted into a stable form that allows continuous production of progeny virus and is not lost during cell division. Jürgen Beck and Michael Nassal, give an overview of the hepadnaviral genome replication, starting from the conversion of the incoming viral genome with its circular, but only partially double stranded (rcDNA) to the very stable covalently closed circular DNA (cccDNA)[4]. The rc-DNA form of the HBV genome contains diverse modifications (e.g. the polymerase-protein covalently linked to the 5'end of the (-)-DNA strand) that have to be removed before generation of cccDNA that can serve as a matrix for proper transcription. Experimentally, this is difficult to investigate, since detection of cccDNA in the presence of high amounts of rcDNA is not trivial. Furthermore, cccDNA is present only in low amounts in infected hepatocytes (from 10-50 genomes per cells). Nevertheless, the episomal cccDNA is very stable (half-life > 30-60 d for DHBV infected ducks) and can therefore even persist during effective antiviral therapy. A detailed understanding of rc- to cccDNA conversion and its inhibition would therefore be desirable. The HBV cccDNA serves as the template for all viral RNAs that are transcribed by cellular RNA polymerase II. Interestingly, the reverse transcription step of HBV requires the specific packaging of the pregenomic HBV RNA together with the viral polymerase (containing reverse transcriptase, DNA polymerase and primase domains) into newly formed capsids. Therefore, the authors describe the newly formed capsid as a "dynamic replication machine" that puts the compartmentalization of genome amplification, known from other viruses, to its extreme. Besides the capsid, viral reverse transcriptase and pregenomic RNA, additional factors are essential for hepadnaviral replication. New methods that use *in vitro* reconstitution of purified components are beginning to reveal that cellular chaperones (e.g. heat shock proteins, hsps) are essential factors for viral polymerase-protein activation. Cell-free reconstitution systems will allow scientists to systematically study the factors necessary for hepadnaviral replication.

The review by Volker Bruss summarizes our current knowledge about hepatitis B virus morphogenesis[5]. Envelopment of mature core particles depends on the presence of HBV surface proteins that are synthesized at the endoplasmatic reticulum (ER). HBV envelope proteins contain three related co-carboxyterminal surface proteins. Common to all three proteins is the 226 aminoacids long small surface protein (SHBs). Aminoterminal addition of the 55 aminoacid long preS2-domain results in the middle surface protein (MHBs), while further aminoterminal addition of the preS1-domain (109 or 118 aminoacids, depending on the genotype) are found in large surface protein (LHBs). During synthesis at the ER, the SHBs builds a conformation that results in exposure of aminoacids around 99 to 169 to the ER lumen resulting in N-glycosylation of half of the S proteins at asparagine (asn) 146. After budding, this loop is located at the surface of virions and subviral particles and carries the major conformational epitope of the HBV surface proteins. The M-Protein, in addition to the

S-domain, is always N-glycosylated at asn-4 in preS2 and also present on the viral surface. The preS2-domain is also O-glycosylated at threonine (thr) 37, but interestingly only in genotypes B-H, since genotype A lacks thr-37. However, the overall function of this O-glycosylation for the viral life cycle is still unknown. Moreover, the function of the M-protein by itself is still not clear. Absence of the M-protein does neither suppress virion formation nor impede viral infectivity. However, the M-protein seems to have an evolutionary advantage for the virus, since it is conserved through all *orthohepadnaviruses*. The preS2-domain is a further integral building block of the large surface antigen (LHBs) by further aminoterminal addition of the preS1-domain to the preS2-domain. The preS1-domain has a dual function: the aminoterminal domain is necessary for attachment and entry of the virus, while the carboxyterminus together with aminoterminal part of preS2 is used for envelopment. Interestingly, the preS-domains of the LHBs are cytosolic during translation but half of the preS chains of LHBs are believed to translocate after translation. How the preS domains cross the membrane is still unknown, however cytosolic and ER-specific chaperones are believed to be involved in this process. In addition, the preS1-domain is myristoylated at Glycin-2, a modification that is not necessary for viral morphogenesis, but important for efficient viral infection. Even without involvement of the core particle, the surface proteins can bud from a post-ER, pre-Golgi compartment and build subviral particles that do not contain a viral capsid or DNA and are therefore non-infectious. In the case of HBV, they are built in the form of spheres and filaments and can reach concentrations that are 10 000-fold higher than the virions. Interestingly, the L-protein cannot build particles by itself, but needs S or M protein for proper segregation. Moreover, the LHBs can induce a dose-dependent inhibition of particle release even in the presence of SHBs. A massive storage of HBV envelope proteins in turn can lead to massive cell stress causing cell death or cancer. The significance of the secretion inhibition function of LHBs for the viral life cycle is still not clear. However, it seems to be dependent on the myristoylation of preS1 domain of LHBs since blocking of LHBs myristoylation abolishes the storage phenomenon of LHBs.

Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors world-wide. Behind lung and stomach cancer, HCC is the one of the leading cause of cancer death. In the review by Joachim Lupberger and Eberhard Hildt, titled "HBV Induced Oncogenesis", the authors discuss the relation of chronic HBV infections and HCC and describe the epidemiology of HBV-associated HCC[6]. Epidemiological data state that chronic HBV carriers have a more than 25 times higher risk of developing HCC, while the molecular mechanism underlying the development of HBV-associated HCC are still not clear. The authors distinguish between direct effects and indirect effects caused by the integration of HBV-DNA into the host genome. In contrast to retroviruses, integration of viral HBV-DNA is not necessary for the viral replication. However, almost all HBV-associated HCCs contain integrated HBV DNA. The integrated viral genomes are rearranged or contain deletions, and cannot lead to viable progeny. However, they may exert an effect on key regulators of the cell cycle. Of special interest in this respect is the frequent integration of woodchuck hepatitis B virus into the N-myc2 gene of the host cells of North-American woodchucks in WHV-related HCC. In HBV-associated HCC however, site-specific integration of HBV genome is a rare event. One indirect effect of integrated HBV-DNA is transcription of HBV open reading frames that remain conserved even after integration. Of special interest is the HBx protein, a small polypeptide that is produced at very low levels in HBV infected hepatocytes. A large number of possible functions have been ascribed to this still enigmatic protein. Initially described as a transcriptional activator, the authors discuss interference of HBx with different signal transduction cascades. Furthermore, HBx was found to interfere with DNA repair and might therefore account for an increase of critical cellular mutations that might increase the risk of developing HCC. Besides the HBx, a special form of the HBV surface protein was found in HBV-infected hepatocytes of HCC patients. The middle surface protein (MHBs) was found to be C-terminally deleted in its S-domain (MHBst) that in turn results in an altered topology of the aminoterminal preS2 domain. While the preS2-domain of wildtype MHBs is located in the lumen of the endoplasmatic reticulum (ER) and is glycosylated at asparagine 4, the preS2-domain of the truncated form is in the cytoplasm. This results in interaction of preS2 with protein kinase C (PKC) and permanent activation of Raf/MEK/ERK signal transduction cascades that might exert a tumor promoter-like function. In line with this, transgenic mice expressing MHBst develop liver tumors. However, a more detailed understanding of the molecular mechanism in HBV-associated HCC development is needed.

In general the infection with HBV can lead to a wide spectrum of clinical manifestations, e.g. self-limited acute or fulminant hepatitis, asymptomatic infection, or chronic hepatitis with progression to liver cirrhosis that can lead to hepatocellular carcinoma (HCC). In their review, Baumert *et al*[7] focus on the impact of virus-host interactions for the pathogenesis of HBV infection and associated liver disease. Both, viral factors and host immune response are discussed. One of the major viral factors are certain HBV mutants that are associated with distinct clinical manifestations, altering the natural course of the infection and confer resistance to antiviral agents, e.g. inhibitors of HBV reverse transcriptase. The authors focus mainly on pre-core (pre-C) stop codon mutations resulting in loss of hepatitis B e antigen (HBeAg) and core-promoter mutations that enhance viral replication. The pre-C stop codon mutation is clinically recognized mainly in patients with chronic and fulminant hepatitis but is also detected in asymptomatic HBV carriers or self-limiting hepatitis. In all these patients, HBeAg, a soluble and secreted form of the core-protein cannot be synthesized any more, despite active viral replication. Therefore the authors argue that HBeAg may play an important role for the interaction of the virus with the host immune system, e.g. HBeAg might have an immunomodulatory function. In line with this, HBeAg might predispose neonates born to HBV-

infected mothers to develop persistent HBV infection by establishing T-cell tolerance to HBeAg and HBcAg *in utero*. Core-promoter mutations are mainly found in patients with an aggressive course of disease like fulminant hepatitis B. Those mutations result in a viral phenotype that shows enhanced viral replication in cell culture and might alter viral kinetics and influence cellular immune response *in vivo*. This might result in more severe liver injuries and possibly fulminant hepatitis. Despite the effect of viral variants, the host immune response is the key player in the onset of liver disease. The authors describe that HBV does not induce strong immune response during the early onset of infection. At the onset of HBV clearance an influx of T cells into the liver occurs, but this is not typically associated with strong liver disease, suggesting non-cytopathic mechanisms for viral clearance that is CD8+ dependent and IFN gamma associated. Weeks later, final elimination of HBV-infected liver cells occurs, presumably by a strong T-cell response with associated liver disease during acute self-limited HBV infection. The absence of a strong T-cell response is detected in patients with chronic hepatitis B. The precise mechanism that contributes to the failure of virus-specific T cell response is still not clear and is discussed by the authors.

Hepatitis B virus is the prototype virus of the family of *hepadnaviridae* that is further divided into the *orthohepadnaviruses* of the mammals and the *avihepadnaviruses* of the birds. The review of Funk *et al*[8], describes avian hepatitis B viruses as an important animal model for the understanding of the life cycle of *hepadnaviridae*. Since the discovery of duck hepatitis B viruses (DHBV) in 1980 many other *avihepadnaviruses* have been characterized in various bird species including cranes and herons. The genomic and structural organization is very similar in human HBV and DHBV. Like HBV, DHBV replicate their DNA genome by reverse transcription of an RNA intermediate. Similar to HBV is their narrow host range, e.g. DHBV infects only distinct duck and goose species but not chicken. Both viruses infect mainly hepatocytes and share a similar life cycle. Nevertheless there are differences that have to be taken into consideration when comparing results obtained from HBV or DHBV systems. First of all, the transmission of infection in ducks occurs mainly vertically, while horizontal transmission is uncommon in contrast to human HBV. Although nearly all of DHBV infected ducks develop chronicity, none or very mild hepatitis is detected and no hepatocellular carcinoma occurs. Furthermore, the DHBV envelope is composed of only two surface proteins [large (L) and small (S) surface proteins] and lacks the middle surface protein (MHBs) found in HBV and other *orthohepadnaviruses*. Unlike the envelope proteins of HBV, DHBV L and S-proteins are not glycosylated, but the DHBV L-protein is phosphorylated, a modification that is absent in HBV L-protein. Another difference is the open reading frame (ORF) for the X-protein. For DHBV, only a cryptic X-like ORF has been described, but apparently lacks a functional role in DHBV *in vivo*. Despite these limitations, DHBV infection of ducks has been an important animal model for the understanding of the biology of hepadnaviral infections, due to the ready availability of susceptible ducks.

Because of the various limitations of the duck hepatitis B infection model like absence of a strong immune pathogenesis leading to the development of liver cirrhosis and HCC, infection of woodchucks (*Marmota monax*) with woodchuck hepatitis B virus (WHV) represents a well-accepted model for many aspects of pathogenesis of human HBV infection. In their review, the authors Stephan Menne and Paul J. Cote describe in detail the woodchuck animal model of orthohepadnaviral infections[9]. WHV, as HBV, is a member of the genus *orthohepadnavirus*. In nature, transmission of the virus occurs vertically and horizontally. Similar to HBV, infections of adult woodchucks with WHV results mainly in resolution and only 5% of infected animals progress to chronicity. Experimental infection of newborn woodchucks results in up to 75% to chronic infection, which is similar to the high properties of neonates born to HBV-infected mothers to develop persistent HBV infection. Chronic WHV infection is associated with life-long active viral replication and disease progression to chronic hepatitis and HCC in these animals. However, unlike chronic HBV infections, there is no naturally occurring e-antigen to anti-e seroconversion and step-down of viral replication. The high viral replication and the very high load of surface antigen and continuous presence of e-antigen in chronic WHV-infected woodchucks might therefore play a major role in disease progression that leads to a nearly 100% risk of these animals to acquire a HCC. However, even after recovery from acute WHV-infection a higher risk of HCC remains (up to 20%), when compared to uninfected woodchucks. Interestingly, the authors could show that WHV replication could be reactivated in serologically long-term resolved adult woodchucks by immunosuppression with cyclosporine A. These experiments support the hypothesis that replication-competent WHV (or HBV in humans) is able to persist years after recovery from acute hepatitis and is controlled by an intact host immune system. The observation in the woodchuck model is supported by recently described fatal reactivation of recovered HBV-infected persons during active immune suppression. The woodchuck could therefore be a very helpful model to study the mechanism of a possible long life persistence of HBV even after serological recovery from acute hepatitis. However, compared to humans, the immune system of woodchucks is not well characterized. Nevertheless, the woodchuck model has very much expanded our knowledge of immune pathogenesis of acute and chronic hepadnaviral infections. Chronically WHV-infected woodchucks have proven to be an invaluable tool for preclinical screening of antiviral drugs against chronic HBV infections of men. Of the many studies with nucleoside and nucleotide analogues tested so far in the woodchuck, telbivudine and clevudine are the most potent with a 8 log10/mL reduction in serum viremia after daily administration of 10 mg/kg for 4 wk, respectively. In addition, continuous treatment with clevudine also delayed the development of HCC significantly. The antiviral activity of tenofovir is much lower under same conditions with only 1.2 log10/mL reduction in serum viremia after 4 wk and is more or less comparable to those of lamivudine and adefovir in the woodchuck model. Furthermore, the authors describe in detail further immunological studies in neonatal

and adult WHV-infected woodchucks that might help to identify the factors responsible for the switch from acute to chronic infection. First results showed that under some conditions, a combination of long-term antiviral drug treatment and therapeutic vaccination can break humoral and cellular immune tolerance in chronic WHV-infected woodchucks, when compared with antiviral monotherapy. The woodchuck animal model of chronic HBV infection is therefore an invaluable tool to study viral pathogenesis and host immune responses.

Despite the existence of a HBV vaccine, more than 370 million people are chronically infected with HBV worldwide, leading to chronic liver disease and development of HCC in many cases. Therefore the overall goal of an antiviral therapy for these cases would be the cure of chronic HBV. However with the current antiviral drugs this might not be achievable in all cases within the near future. In the review by Hans L. Tillmann, the aims of today's antiviral therapy against chronic HBV are summarized[10]. That is, first of all, prevention of liver disease and development of HCC in these patients by control and suppression of HBV replication. The author discusses in detail the different types of chronic HBV and their treatment parameters. For antiviral therapy, only interferon alpha (IFN α) and inhibitors against the reverse transcriptase of HBV are currently available. With standard IFN α, seroconversion from HBe to anti-HBe is induced in 20%-40% of patients 24 wk post-treatment. In chronic HBV patients with a HBeAg negative phenotype, patients showed a good response under IFN α treatment for 6-12 mo but a sustained response was usually not observed in the majority of cases. Unfortunately, longer treatment is usually limited by strong side effects, e.g. flu-like symptoms. Pegylation of IFN α, leading to a longer half life of the modified interferon has similar efficacy, but in general the sustained response for both, standard and pegylated interferon seems to be dependent on the HBV genotype. In contrast to the side effects of interferons, inhibitors of HBV reverse transcriptase can be used for prolonged antiviral therapy. Many of the nucleotides or nucleosides analogues against HBV were developed and used in HIV therapy. Lamivudine, also called 3TC, inhibits HBV reverse transcriptase very efficiently and usually results in viral suppression of 5-6 log10 copies/mL after one year of treatment. Prolonged Lamivudine treatment may contribute to seroconversion of HBe to anti-HBe within 50% of patients after 4 years of therapy. The major problem with prolonged Lamivudine, but also other reverse transcriptase inhibitors is occurrence of drug resistance due to the selection of HBV mutants. The mutations occur mainly within the YMDD-motive of the C-domain of the viral polymerase, however other mutations are also described. The high variability of the HBV reverse transcriptase together with a high replication rate of HBV and the slow kinetics of viral clearance lead to rapid selection of mutants under drug selection pressure that results in development of resistance during continuous therapy. For Lamivudine, up to 70% resistance has been described after 4 years of treatment. This leads to viral breakthrough and progression of liver disease. The author notes that it is therefore necessary to perform early diagnostics of drug resistance and to adapt antiviral therapy prior to breakdown of liver function. Even more important is that the new antiviral in use must not show cross-resistance to the HBV mutants selected by the former antiviral, e.g. telbivudine and emtricitabine have been reported to show a similar resistance mutation profile like Lamivudine.

For the future, it will be necessary to have antivirals on hand that inhibit different steps of the viral life cycle. Rational targets might be inhibitors of attachment and entry, conversion from rcDNA to cccDNA, capsid assembly, envelopment and secretion of viral particles. For attachment and entry, it has been shown that acylated preS1 peptides block HBV infection *in vitro* and in animal models. Based on these results a potent inhibitor of viral infection (Myrcludex B) is currently being developed.

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TOPIC HIGHLIGHT

Dieter Glebe, PhD, *Series Editor*

Hepatitis B virus taxonomy and hepatitis B virus genotypes

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Abstract

Hepatitis B virus (HBV) is a member of the hepadnavirus family. Hepadnaviruses can be found in both mammals (orthohepadnaviruses) and birds (avihepadnaviruses). The genetic variability of HBV is very high. There are eight genotypes of HBV and three clades of HBV isolates from apes that appear to be additional genotypes of HBV. Most genotypes are now divided into subgenotypes with distinct virological and epidemiological properties. In addition, recombination among HBV genotypes increases the variability of HBV. This review summarises current knowledge of the epidemiology of genetic variability in hepadnaviruses and, due to rapid progress in the field, updates several recent reviews on HBV genotypes and subgenotypes.

Key words: Orthohepadnavirus; Avihepadnavirus; Hepatitis B virus; Genotype, Subgenotype; Recombination

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INTRODUCTION

Hepatitis B virus (HBV) is the prototype member of a steadily growing family of viruses called hepadnaviruses[1]. Hepadnaviruses can be found in both mammals (orthohepadnaviruses) and birds (avihepadnaviruses). HBV, the hepadnavirus infecting humans, is classified into eight genotypes today. HBV genotypes differ by at least 8%[2]. Since the first definition of the genotypes A, B, C and D[2], genotypes E[3], F[4], G[5] and H[6] have been detected. Due to the genetic diversity of HBV, numerous subgenotypes of HBV have been described[7] (Table 1). HBV subgenotypes differ by at least 4%[8].

HBV genotypes and most subgenotypes show a distinct geographic distribution. In Asia, where there is a high prevalence of HBV carriers, strong evidence suggests that HBV genotypes influence the course of disease. Several recent reviews have summarised knowledge on different aspects of HBV genotypes[7-12] and on hepadnaviruses that infect species other than homo sapiens[13-15]. This review will update recent developments in understanding HBV genotypes and taxonomy.

TAXONOMY

HBV is a partially double stranded virus that uses reverse transcriptase in its replication cycle. Thus, HBV is similar to many retroviruses found in animals and pararetroviruses in plants[16,17].

After cloning and sequencing the HBV genome[18], several related viruses were discovered in woodchucks (Marmota monax)[19], ground squirrels (Spermophilus beecheyi)[20] and pekin duck (Anas domesticus)[21]. Subsequently, numerous new viruses that are similar to HBV were found in mammals and birds and have been cloned (Tables 1 and 2). All these viruses are classified in the family of hepadnaviridae, including the genus orthohepdnavirus (mammals; Figure 1), and the genus avihepadnavirus (birds; Figure 2). In addition to the avihepadnaviruses listed in Table 2, five new hepadnaviruses were cloned from exotic duck and goose species; i.e., the Chiloe wigeon, mandarin duck, puna teal, Orinoco sheldgoose, and ashy-headed sheldgoose. Sequence comparisons revealed that 4 virus isolates were closely related to existing isolates of duck hepatitis B virus (DHBV), while the mandarin duck virus was closely related to Ross goose hepatitis B virus[22].

In chimpanzees, gorillas, orangutans and gibbons new putative members of hepadnaviridae were discovered and sequenced completely[14]. It is now widely accepted that primate hepadnaviruses are indigenous to their hosts. Because hepadnaviruses isolated from apes are grouped as HBV genotypes in phylogenetic analyses, it has been suggested that isolates from apes should be named following the nomenclature used for immune deficiency viruses[23] (Table 1), e.g. HBV found in chimpanzees should be called HBVcpz. With only 5% divergence from the chimpanzee HBV isolates, the HBV isolate from gorilla is categorized in the HBV genotype (Figure 3, unpublished

Table 1 Orthohepadnaviruses and their host

	Host	Ref.
Hepatitis B Virus	Man *Homo sapiens sapiens*	[75]
Chimpanzee Hepatitis B Virus	Chimpanzee *Pan troglodytes*	[76]
Gibbon Hepatitis B Virus	White handed gibbon *Hylobates lar*	[77]
Orangutan Hepatitis B Virus	Orangutan *Pongo pygmaeus pygmaeus*	[78]
Gorilla Hepatitis B Virus	Gorilla *Gorilla gorilla*	[79]
Woolly Monkey Hepatitis B Virus	Woolly monkey *Lagothrix lagotricha*	[80]
Woodchuck Hepatitis Virus	Woodchuck *Marmota monax*	[19]
Ground Squirrel Hepatitis Virus	Ground Squirrel *Spermophilus beecheyi*	[20]
Arctic Squirrel Hepatitis Virus	Arctic Squirrel *Spermophylus parryi kennicotti*	[81]

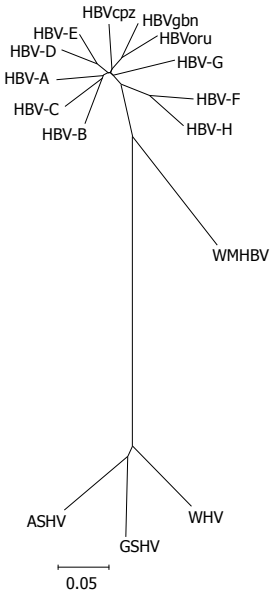

Figure 1 Phylogenetic tree of orthohepadnaviruses. Complete genomes of HBV genotypes A (X02763), B (D00330), C (M12906), D (V01460), E (X75657), F (X69798), G (AF160501) and H (AY090454); HBVcpz (D00220), HBVoru (NC 002168), and HBVgbn (U46935) were aligned using clustal w with orthohepadnavirus genomes from woolly monkey (AF046996) woodchuck (J02442), ground squirrel (K02715) and the tentative member from arctic squirrel (nc_001719). The alignment was tested with the neighbour-joining method.

Table 2 Avihepadnaviruses and their host

	Host	Ref.
Duck Hepatitis B Virus DHBV	Pekin duck *Anas domesticus*	[21]
Grey Teal Hepatitis B Virus (GTHBV)	Grey Teal *Anas gibberifrons gracilis*	[82]
Heron Hepatitis B Virus (HHBV)	Heron *Adrea cinerea*	[83]
Maned Duck Hepatitis B Virus (MDHBV)	Maned Duck *Chenonetta jubata*	[82]
Ross Goose Hepatitis Virus (RGHV)	Ross Goose *Anser rossi*	[4]
Snow Goose Hepatitis B Virus (SGHBV)	Snow Goose *Anser caerulescens*	[84]
Stork Hepatitis B Virus (STHBV)	White Stork *Ciconia ciconia*	[85]
Crane Hepatitis B Virus (CHBV)	Demoiselle cranes *Anthropoides virgo* Grey crowned cranes *Balearica regulorum*	[86]

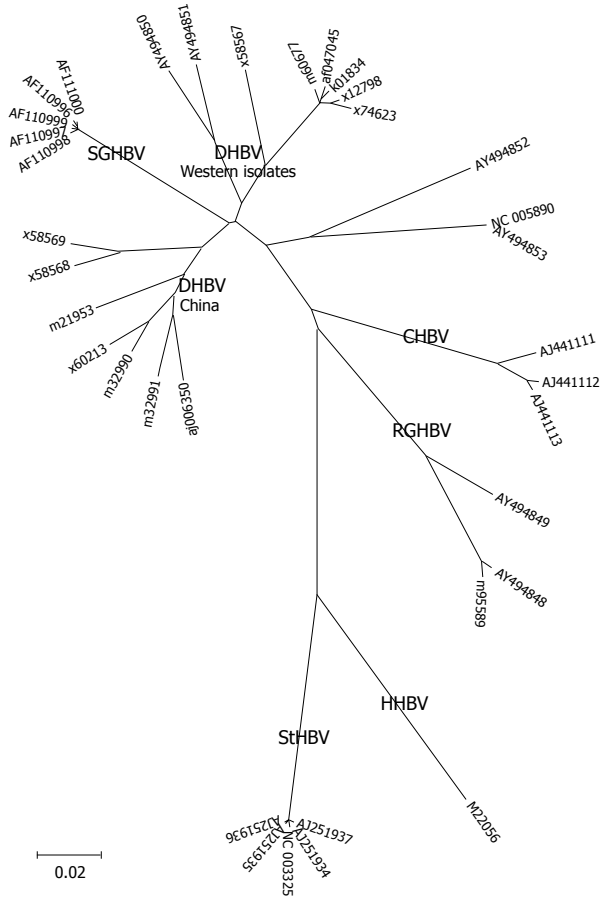

Figure 2 Phylogenetic tree of the genus avihepadnavirus.

results). Thus, three HBV genotypes from apes can now be differentiated. The chimpanzee and gorilla isolates from Africa are categorized as one genotype, i.e., HBVcpz. The isolates from the South-East-Asian apes, gibbon and orang-utan, are categorized into two genotypes, i.e., HBVgbn and HBVoru, respectively. These genotypes diverge by 8%. Within the gibbon genotype, distinct strains of HBV circulating in geographically separated populations have been described[24].

Avihepdnaviruses are the most distant relatives of HBV with a nucleic acid homology of only 40%. WHV and GSHV as mammalian hepadnaviruses are more closely related to HBV and differ by only 17%. Complete WHV and GSHV genomes from GenBank show a high degree of homology and only one genotype is listed[25-27]. However, using degenerate primers, several variant WHV isolates from wild-captured woodchucks were found that showed high divergence with sequencing of small parts of the genome[28]. DHBV has two genotypes, in contrast to WHV and GSHV, which have a narrow host range and geographical distribution[25,26], DHBV is found in different avian species with independent isolates in many countries

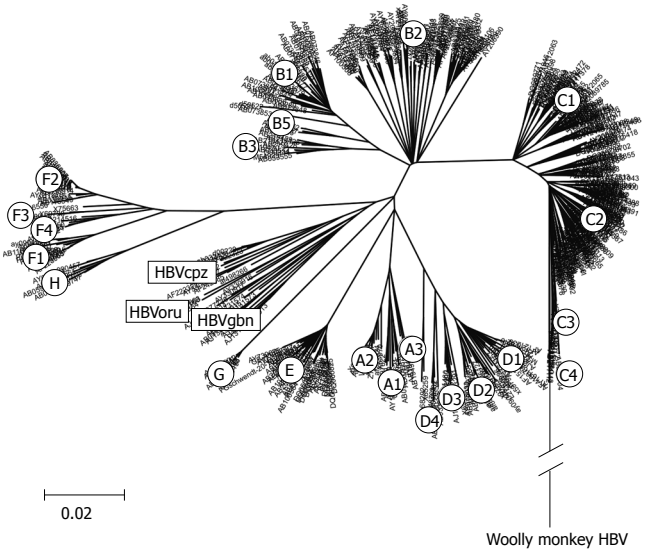

Figure 3 Phylogenetic tree of complete HBV genomes. An alignment of 601 complete HBV sequences was performed with Clustal X in the program DNAstar. The alignment was further analysed by boot-strapping using the Neighbourhood-Joining method contained in MEGA version 3.1[104].

Figure 4 Geographic distribution of HBV genotypes and subgenotypes.

around the world[29] (Figure 4).

Human HBV can be grouped into eight genotypes (based on more than 8% difference)[7,9-12]. Several attempts have been made to reconstruct the evolution of hepadnaviruses[30-34]. Estimating the rate of synonymous substitutions for HBV to be 4.57×10^{-5} per site per year, DHBV has been proposed to have diverged about 30 000 years ago from a common ancestor while GSHV and WHV should have diverged about 10 000 years ago from HBV and the HBV serotypes would be separated by about 3000 years[31]. However, as long as we are not able to accurately estimate the mutation rate of HBV over centuries or even millennia, it is not possible to calculate a time point for the separation of HBV genotypes or hepadnaviral species.

HBV GENOTYPES AND SUBGENOTYPES

HBV genotypes differ by more than 8%[2,3]. Phylogenetic analyses using alignments of whole genomes have shown that 8 genotypes, called A, B, C, D, E, F, G and H, of HBV can be distinguished[7,11,12,35] (Figure 1). In general, HBV isolates found in apes diverge similarly to HBV genotypes in phylogenetic analyses and have been named HBVcpz, -oru, -gor and -gbn for their host's, i.e. chimpanzee, orang-utan, gorilla and gibbon, respectively (Table 1)[23]. However, as elucidated above, the isolate from gorillas is always categorized into the chimpanzee clade.

A prototypic HBV genome may have a length of 3215 nt, as found in HBV genotypes B, C, F and H. Due to deletions and insertions (Table 3), the other HBV genotypes differ slightly in length of genome (Table 3). Thus HBV genotype G with 3248 nt. is 66 nt longer than genotype D with 3182 bp.

Extensive phylogenetic analyses have shown that HBV genotypes can be further subdivided into subgenotypes (Table 4). HBV subgenotypes differ by at least 4%[8]. In

Table 3 Fundamental properties of genomes and differences between HBV genotypes

Genotype	Genome length in bp	ORF-differences
A	3221	Insertion of aa 153 and 154 in HBc
B	3215	
C	3215	
D	3182	Deletion of aa 1-11 in preS1
E	3212	Deletion of aa 11 in preS1
F	3215	
G	3248	Insertion of 12 aa in HBc Deletion of aa 11 in preS1
H	3215	

Table 4 HBV subgenotypes and geographic prevalence

	Subgenotype	Synonyms	Geographic origin	Ref.
A	A1	Aa, A'	Africa, (Asia, South America)	[41,87]
	A2	Ae, A-A'	Europe	
	A3	Ac	Gabon, Cameroon	[88,89]
	(A4)		Mali	[59]
	(A5)		Nigeria	[59]
B	B1	Bj	Japan	[67,90]
	B2	Ba	Asia without Japan	
	B3		Indonesia, Philippines	[7]
	B4		Vietnam	[7]
	B5		Philippines	[91,92]
C	C1	Cs	South East Asia (Vietnam, Myanmar, Thailand, Southern China)	[37-39]
	C2	Ce	Far East (Korea, Japan, Northern China)	
	C3		Micronesia	[7]
	C4		Australia	[93]
	C5		Philippines, Vietnam	[92,94]
D	D1		Mongolia, Belarus, Europe?	
	D2		India?	
	D3		South Africa, East India, Serbia	[40,41]
	D4		Australia	[93]
	D5		East India	[40]
F	F1		South and Central America	[95,96]
	F2		South America	[4]
	F3		Bolivia	[97,98]
	F4		Argentina	[97,98]

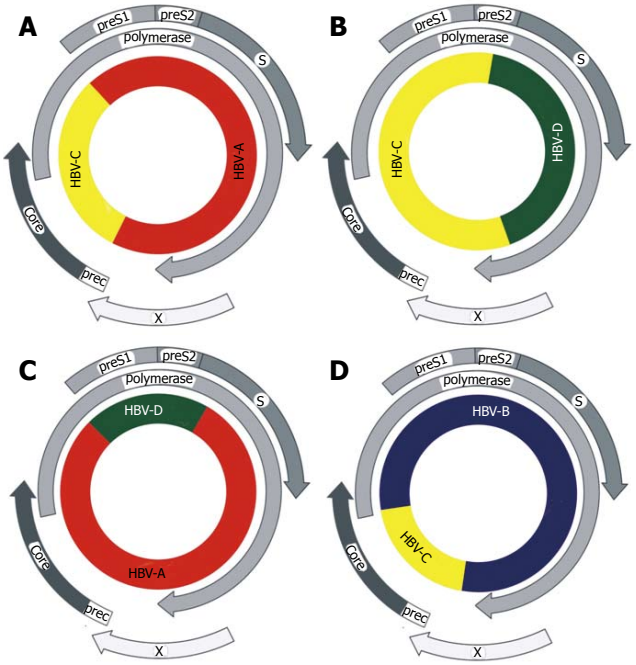

Figure 5 Schematic genome organisation of recombinants between HBV genotypes. HBV recombinants were described from materials sampled in **A**: Vietnam[99]; **B**: Tibet[68]; **C**: Africa[100] and **D**: Asia[67]. The ORF coding for the HBV proteins are shown as arrows, the inner circle represents the HBV genome.

Table 5 Examples for recombination events between of HBV genotypes

Genotype of		Recombination Breakpoint		No. in literature	Ref.
Backbone	Insert	5′	3′		
A	C	1801	2865	3	[99]
A	D	2895	327		
		2820	386-586	3	[100]
		?	670		
B	C	1740-1838	2443- 2485	41	[67,73,101,102]
B	C	3120	3171	1	[60]
		3060	3191	1	
		2910	2950	1	
C	B	1731-1838	2437- 2479	1	[102]
D	A	129	2339	3	[73,101,102]
		495	780		
		822	1775		
G	C	1860	2460	1	[103]
A	E	882	1060	1	[88]

genotypes A, B and C, epidemiological data show that the respective subgenotype pairs A1/A2 (formerly termed Aa/Ae)[36], B1/B2 (formerly Bj/Ba)[36] and C1/C2 (formerly Cs/Ce)[37-39] differ substantially in many virological and probably some clinical parameters. Subgenotypes also show distinct geographic distribution (Figure 3). However, this is not true for genotype D with subgenotypes D1, D2 and D3 being described as widespread in the world; e.g. D3 was found in Asia (East India)[40], South Africa[41] and Europe (Serbia) (Stanojevic *et al,* unpublished results).

Except for genotype E and G, all HBV genotypes can be divided into subgenotypes. The absence of subgenotypes in HBV genotype E has been assumed to be the consequence of a recent genesis for genotype E[42-45]. Furthermore, genotype E is not present in Americans of African origin from Venezuela and Brazil[46,47]. The case for HBV genotype G appears to be less clear. Genotype G was originally found in the USA, France[5] and Germany[48]. Later, partial sequencing of HBV genes pointed to a high prevalence of HBV genotype G in Mexico[49]. Nevertheless, the geographic origin of HBV genotype G remains unknown[50]. To date only a limited number of complete HBV genotype G sequences have been deposited in GenBank that are not classified into subgenotypes.

DOUBLE INFECTIONS AND RECOMBINANTS

Double infections with two different HBV genotypes have been known since typing was done serologically[51,52]. Subsequently, evidence of super infection with HBV isolates of the same or different genotype was described in chronic HBV patients[53]. Super infection was accompanied by acute exacerbation of the chronic disease. Additional observations came from patients treated with interferon. Before treatment, HBV genotype A was prevalent. After treatment and relapse, a switch of the genotype to HBV genotype D was described[54,55].

Using different methods for genotyping, several reports described high rates of double infection with two different HBV genotypes in all parts of the world. Using these methods double infections have been found in 4.4%[56], 10.9%[57], 12.5%[58], 14.1% (Kirschberg *et al,* unpublished results), 17.3%[59] and 17.5%[60] of HBV infected patients. Even triple infections with HBV of genotype A, B and C have been described in 0.9% of HBV infected intravenous drug users[60].

Infection with HBV of genotype G seems to be associated very often with an infection of HBV genotype A[61]. This was found in 4 individuals from the USA and in one patient from France[62].

Coinfection with two different HBV genotypes in one patient may lead to an exchange of genetic material between the two strains. However, with current knowledge of HBV replication, the mechanism for this supposed recombination remains enigmatic. No mechanism can be envisioned that would allow an exchange of genetic material between two hepadnaviral genomes at the level of transcription. Nevertheless, numerous authors described changes in the genome of HBV that appear to be the consequences of a recombinatorial event (Figure 5 and Table 5).

Two recent works have comprehensively analysed the prevalence of events in the HBV genome that are reminiscent of recombinations[63,64]. About 87% of the putative recombinants were B/C (120) and A/D (29) hybrids. The other recombinants comprised A/B/C, A/C, A/E, A/G, C/D, C/F, C/G, C/U (U for unknown genotype) and B/C/U hybrids. Genotypes A and C showed a higher recombination tendency than did

other genotypes. The results also demonstrated region priority and breakpoint hot spots in the intergenotype recombination. Recombination breakpoints were found to be concentrated mainly in the vicinity of the DR1 region (nt 1640-1900), the preS1/S2 region (nt 3150-100), the 3' -end of the Core gene (nt 2330-2450) and the 3'-end of the Surface gene (nt 650-830)[63,64].

Recombination events between human and chimpanzee[65]or gibbon[63] HBV sequences have also been described. Discrepant genotyping results from different parts of the genome are indicative of a recombination between genotype A and F[66]. Even mosaic genomes with sequences derived from three different genotypes have been described[59,64].

Some recombinants among HBV genotypes have become the dominant subgenotype prevalent in certain geographic regions. Recombination between genotypes B and C has led to the generation of two different strains with distinct geographic distribution[67]. Strains of genotype B without recombination are found in Japan (subgenotype B1), whereas strains with recombination between genotype B and C are found throughout Asia (subgenotype B2), sparing Japan[67]. Recombinants between HBV genotypes C and D are the leading HBV subgenotype in Tibet[68-70].

It remains open for discussion whether the observed exchanges are the consequence of direct genetic recombination taking place between two HBV strains or if they are the consequence of fast adaptation of HBV to a certain genetic and immunologic environment in different human populations in the world. The high replication capacity of HBV with a release of up to 10^{13} viral particles per day[71,72] and the high error rate of the viral polymerase, lead to the production of HBV genomes with all possible single mutations and double mutations of every nucleotide of the HBV genome every day[72]. Thus, a fast adaptation of HBV to a new environment is also a possibility.

A hypothetical mosaicism of the HBV genome has already been proposed by Bowyer and Sim[73]. This work and later works described most HBV genotypes as a modular genome[63] that represents a mixture of small segments coming from many different HBV genotypes. If we expand on this observation, the HBV genome may be made up of a number of allelic modules with different properties; e.g. different binding sites for transcription factors or antigenic epitopes. Thus, a certain combination of these modules would make up an HBV genotype. The findings of Fischer *et al*[74]are in support of this speculation. The authors described genotype specific activation or repression of HBV enhancer II, preCore-pregenomic promoter by the transcription factor COUP-TF1.

CONCLUSION

HBV has been recognised as a prototype member of a family of viruses infecting mammals and birds. Due to its high replication capacity and the high error rate of the viral reverse transcriptase, HBV is able to adapt to the host's environment. This adaptation has led to the emergence of eight genotypes in humans and three closely related genotypes in apes. The human genotypes have further diverged into at least 24 subgenotypes, with certainly many more to come, and a plethora of recombinants. From the analysis of recombinants there are indications that at least one more genotype remains to be detected.

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TOPIC HIGHLIGHT

Dieter Glebe, PhD, *Series Editor*

Viral and cellular determinants involved in hepadnaviral entry

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Abstract

Hepadnaviridae is a family of hepatotropic DNA viruses that is divided into the genera *orthohepadnavirus* of mammals and *avihepadnavirus* of birds. All members of this family can cause acute and chronic hepatic infection, which in the case of human hepatitis B virus (HBV) constitutes a major global health problem. Although our knowledge about the molecular biology of these highly liver-specific viruses has profoundly increased in the last two decades, the mechanisms of attachment and productive entrance into the differentiated host hepatocytes are still enigmatic. The difficulties in studying hepadnaviral entry were primarily caused by the lack of easily accessible *in vitro* infection systems. Thus, for more than twenty years, differentiated primary hepatocytes from the respective species were the only *in vitro* models for both *orthohepadnaviruses* (e.g. HBV) and *avihepadnaviruses* (e.g. duck hepatitis B virus [DHBV]). Two important discoveries have been made recently regarding HBV: (1) primary hepatocytes from tree-shrews; i.e., *Tupaia belangeri*, can be substituted for primary human hepatocytes, and (2) a human hepatoma cell line (HepaRG) was established that gains susceptibility for HBV infection upon induction of differentiation *in vitro*. A number of potential HBV receptor candidates have been described in the past, but none of them have been confirmed to function as a receptor. For DHBV and probably all other avian hepadnaviruses, carboxypeptidase D (CPD) has been shown to be indispensable for infection, although the exact role of this molecule is still under debate. While still restricted to the use of primary duck hepatocytes (PDH), investigations performed with DHBV provided important general concepts on the first steps of hepadnaviral infection. However, with emerging data obtained from the new HBV infection systems, the hope that DHBV utilizes the same mechanism as HBV only partially held true. Nevertheless, both HBV and DHBV *in vitro* infection systems will help to: (1) functionally dissect the hepadnaviral entry pathways, (2) perform reverse genetics (e.g. test the fitness of escape mutants), (3) titrate and map neutralizing antibodies, (4) improve current vaccines to combat acute and chronic infections of hepatitis B, and (5) develop entry inhibitors for future clinical applications.

Key words: Hepatitis B virus; Duck hepatitis B virus; Infection models; Receptor; Viral attachment; *Tupaia belangeri*; HepaRG; Carboxypeptidase D

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INTRODUCTION

The first step in virus infection is an energy independent attachment of the infectious particle to an accessible structure exposed at the host cell surface. Primary attachment, often characterized by low affinity and reversibility, is usually followed by the passage of the virion to a more specific receptor, which mediates further steps of entry. Both initial attachment and specific receptor recognition often contribute to host specificity and tissue tropism. For enveloped viruses, receptor binding is followed by fusion of the virus with either the plasma or an endosomal membrane. Fusion within intracellular vesicles is regularly triggered by acidification. The universal mechanism of membrane fusion requires conformational changes of virus-encoded fusion proteins leading to a physical approximation and finally merging of viral and cellular membranes[1]. A detailed understanding of receptor binding and membrane fusion is of general interest for molecular virologists and it also provides the basis for therapeutics that interfere with the early steps of infection, as has been successfully accomplished for HIV[2].

HBV and related animal viruses form the family *hepadnaviridae*, which are small, enveloped DNA viruses that cause acute and chronic liver infection. They are

divided into the *orthohepadnaviruses* of mammals and *avihepadnaviruses* of birds[3] (see also the review on HBV taxonomy and genotypes by S. Schaefer in this series). HBV is a serious global infectious diseases and it is assumed that 2 billion people have had contact with that virus[4]. The infection can lead to a chronic carrier state in 5%-10% of immunocompetent adults and up to 90% of infected neonates. Chronic HBV infection is the major cause of liver cirrhosis and hepatocellular carcinoma in numerous regions of the world[5]. While the viral life cycle is still not fully understood, a safe and efficient vaccine has been developed and sensitive tests for HBV surface protein (HBsAg) now allow for reliable diagnosis and screening of blood products. Present therapeutic regimens for HBV address either the host immune system (α-interferon [IFNα]) or inhibit reverse transcription of the viral pregenomic RNA by nucleoside inhibitors (Lamivudine, Adefovir, Entecavir). The latter provoke the selection of resistant or even cross-resistant mutants that will become increasingly problematic to therapeutic control in the future (see also review "Antiviral therapy and resistance of hepatitis B virus infection" by H.L. Tillmann in this series).

To overcome these challenges, antiviral substances that target different replication steps; e.g. inhibitors of viral entry or improved vaccines that counteract the current escape-mutants, are becoming increasingly important. In the past, however, the lack of feasible HBV *in vitro* infection systems hampered investigations aiming in this direction. The only immunocompetent *in vivo* model that could be used for studies related to the infectivity of the virus was based on primary human hepatocytes (PHH) and those of the chimpanzee, which were limited in availability.

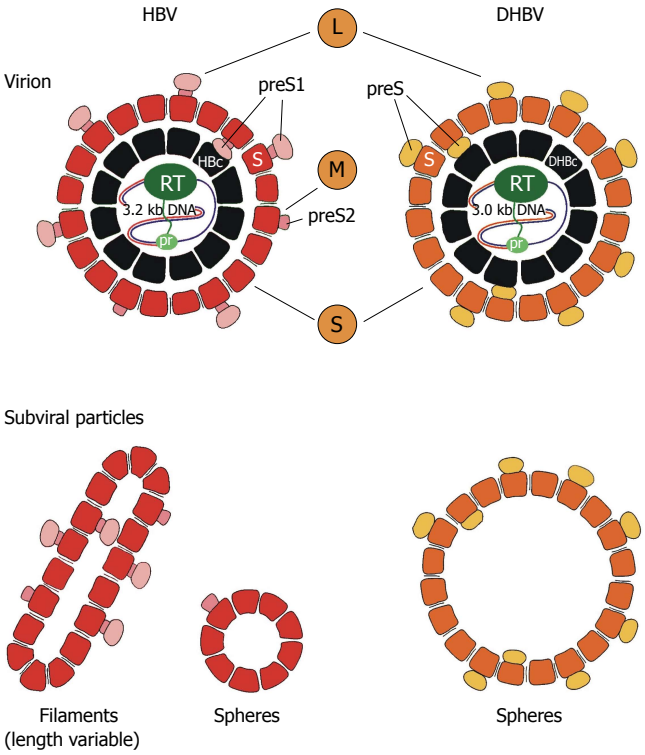

Figure 1 Schematic presentation of human (HBV) and duck (DHBV) hepatitis B virus. The viral DNA is drawn as a single or double line. The viral polymerase is depicted with the primer domain (pr) and the reverse transcription domain (RT). The nucleocapsid (core or HBc/DHBc) is shown in black. Reported encapsidated cellular proteins are omitted. For HBV the surface proteins L, M and S are shown with the S-domain, the preS2-domain and the preS1-domain, whereas for DHBV, L- and S-surface proteins with preS and S-domain are depicted. St, truncated form of DHBV S-surface protein. Non-infectious subviral particles of HBV are shown in filamentous and spherical form and in larger spheroids in the case of DHBV.

ORGANIZATION OF THE HEPADNAVIRAL ENVELOPE

A hallmark of hepadnaviral infection is the constitutive secretion of nucleocapsid-free subviral particles (SVP), mainly composed of the hepadnaviral envelope proteins (large and small in the case of DHBV and large, middle, and small for HBV). HBV-SVPs exist as 22 nm spheres or filaments of the same diameter but variable in length. The HB virions appear in electron microscopy after negative staining as spheres of 45 nm. Virions and SVP contain variable proportions of the three co-carboxyterminal surface (glyco)-proteins; i.e., the large (LHBs), middle (MHBs) and small (SHBs) surface proteins (Figure 1). The SHBs protein is the major component of the virion envelope and the subviral HBsAg particles, while virions and filaments contain more LHB proteins than spheres[6]. In contrast DHBV and DHBV-SVPs are similar in size and shape (55-60 nm)[7] and contain a similar ratio of both envelope proteins (L:S = 1:5). In addition DHBV incorporates a processed version of the DHBs protein consisting of only the two N-terminal transmembrane domains of S and are therefore called S^t[8]. The hepadnaviral surface proteins are products of a single open reading frame and distinguished by three (HBV) or two (DHBV) domains. HBV comprises: preS1 (108 or 119 aa depending on the genotype) only in LHBs, preS2 (55 aa) in LHBs and MHBs, and S (226 aa) common to all three HBs proteins (Figure 2A). All three proteins bear within the S-domain a potential N-glycosylation site (NG) at Asn-146, which is only partially utilised. The second N-glycosylation site at Asn-4 in the preS2-domain is modified in MHBs but not in LHBs[6]. In addition to N-glycans, the preS2 domain of most *orthohepadnaviruses* contains O-glycans[9]. The preS2 domain of both LHBs and MHBs contains a single mucin-type O-glycan (OG) at Thr-37 in genotypes B-H. O-glycans are absent in genotype A, because the O-glycosylation site at Thr-37 is exchanged to Asp in genotype A for an unknown reason[10]. Although potential N- and O-glycosylation sites within the preS1 domain are present, none of them are used due to the cytoplasmic exposition of preS1 during synthesis. In contrast, the two DHBV envelope proteins L (preS+S) and S remain unglycosylated during secretion, but DHBV-L becomes phosphorylated[11] (for more details see review "Avian hepatitis B viruses: molecular and cellular biology, phylogenesis and host tropism" by Funk *et al* in this series). The N-termini of probably all hepadnaviral L-proteins contain recognition sequences that lead to myristoylation at Gly-2[12] while, at least in HBV, more carboxyterminal parts serve as envelopment signals for cytoplasmic core particles[13,14]. Several lines of evidence indicate that hepadnaviral L-proteins adopt dual topologies with half of their preS-domains located inside the particle,

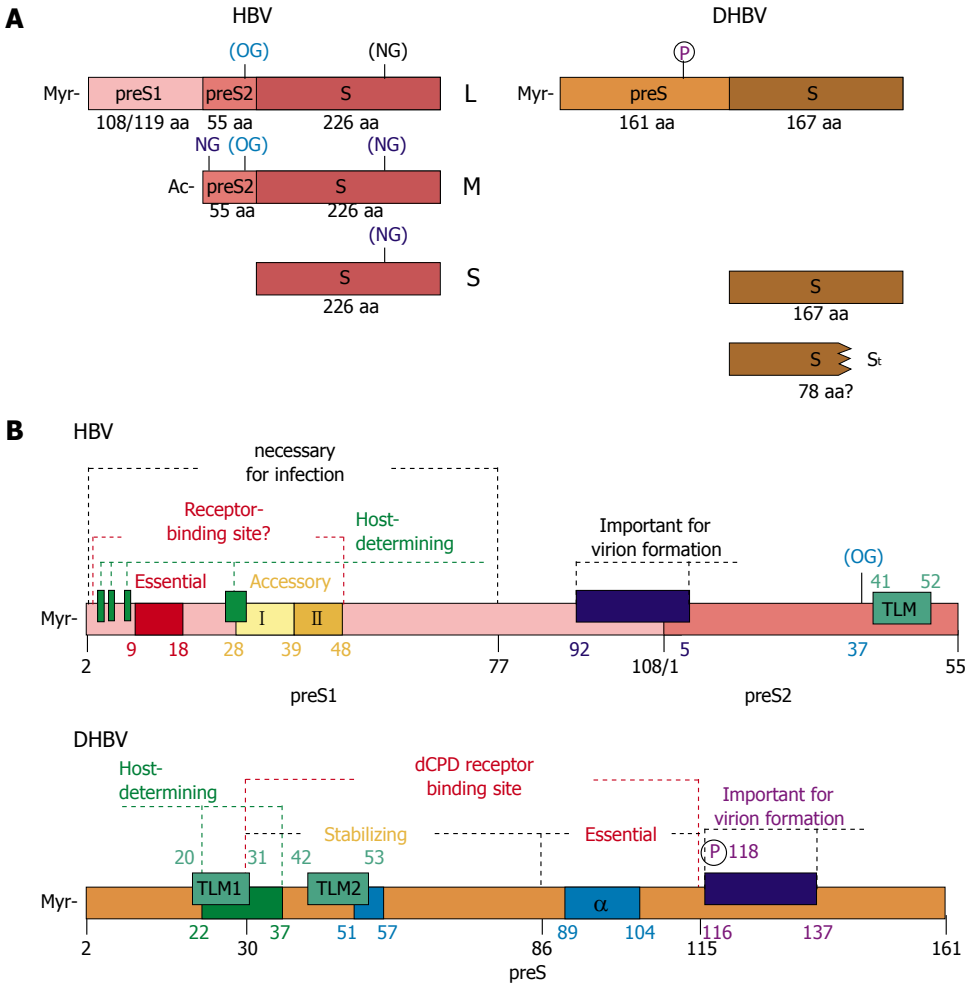

Figure 2 Surface proteins of HBV and DHBV. (**A**) Schematic organisation of the three surface proteins of HBV and the two surface proteins of DHBV. For HBV, used N- and O-glycosylation sites (NG and OG) are shown, (parenthesis indicate partial glycosylation). DHBV surface proteins are non-glycosylated, but the L-protein is phosphorylated at position 118 within its preS-domain. The L-protein of HBV and DHBV is myristoylated (Myr) at Gly-2 of the N-terminus of preS1 or preS, respectively. In case of HBV the preS1-domain encodes for 108 or 119 amino acids, depending on the genotype. The preS2-domain of MHBs is N-terminally acetylated (Ac) and is 55 amino acids long, while a preS2-domain and therefore a special M-protein is missing in DHBV. (**B**) Schematic presentation of preS-domains of HBV and DHBV important for virion formation and viral entry. The host-determining regions are depicted in green. The potential receptor binding site within the preS1-domain of HBV is shown with the essential domain (aa 9-18) and the two accessory domains (aa 28-39 and 39-48, respectively). For DHBV, the carboxypeptidase D (dCPD) binding site is depicted with the essential and stabilizing domains. Alpha helical domains are shown in blue (α). Reported trans-location motives (TLM) are marked in boxes. In DHBV preS, two TLMs are predicted (TLM1 and TLM2). TLM1 overlaps with the host-determining region, while TLM2 is located within the stabilizing sequence of the dCPD receptor binding site. In the case of HBV, the carboxyterminus of the 55 aa long preS2-domain was reported to contain a single TLM. The numbering of HBV preS1 is for genotype D (108 aa). P, phosphorylation site; OG, O-glycosylation; myr, myristoylation.

while the other half is located on the outside[15-18]. Virions and SVP particles bud to a lumen of a post-ER, pre-Golgi intermediate compartment. Therefore the particles contain lipids derived from intracellular compartments, rather than from the plasma membrane. The SHBs subunits of HBV and the subviral particles in the blood are highly cross-linked by disulfide-bonds and do not disassemble in the presence of detergents, unless the disulfide bonds are opened. Interestingly, this tight network of disulfide bridges is not found in DHBV particles. Those can easily be solubilised in mild detergents and both types of surface proteins do not form heterodimeric complexes (unpublished results).

CELL CULTURE SYSTEMS FOR VIRUS PRODUCTION AND ENTRY ANALYSIS

It is known that after artificial delivery of replication competent DNA constructs, later steps of the viral life cycle are not rigidly host-restricted. Transfection of hepatoma cell lines[19-21] or mouse liver cells[22] with replication-competent HBV/DHBV genomes results in the production and secretion of infectious virions. The same holds true for HBV transgenic mice[23]. Furthermore, lipofection of mature core particles, isolated from serum-derived virions into non-permissive hepatoma cells resulted in a full replication cycle of HBV[24]. This suggests that species-specificity of infection is determined at an early step; e.g. viral attachment, entry or fusion. Interestingly, productive infection not only depends on the species origin but also on the state of differentiation of the same cell line. This has been impressively demonstrated in HepaRG cells, which gain susceptibility towards HBV infection only several weeks after induction of differentiation[25]. In case of primary tupaia, human or duck hepatocyte cultures, susceptibility is achieved about one day after attachment

of the perfused and already differentiated hepatocytes, but is lost during prolonged culturing. Since binding and virus accumulation in HepaRG cells is similar in both, differentiated and undifferentiated cells (unpublished data) the restriction may not be caused by the bare presence or absence of a host entry molecule but might be complicated due to the polarisation state of the differentiated cell.

PRIMARY HEPATOCYTE CULTURES AND RELIABLE MARKERS FOR INFECTION

For many years, cultures of primary human hepatocytes (PHH), obtained by immediate perfusion of liver pieces after surgical resection, were the only cells to study viral infectivity[26]. PHH are not easy to handle, cannot be propagated *in vitro* and need particular growth factors for maintenance of their differentiated state[27]. Moreover, the efficiency of HBV infection of PHH *in vitro* was reported to be low leading to only a few percent of cells being infected and a negligible spread within the cell culture. Thus, virus amplification is not achievable. This can be counteracted to some extent by the addition of dimethylsulfoxide (DMSO) during cultivation and the use of 4% polyethylene glycol (PEG) during infection[28-31]. In contrast, infection of PDH with DHBV leads to a spread of infection to virtually every cell in the culture even when very low initial virus titers were supplied. A major drawback of PHH is their limited availability and the heterogeneity in quality with different liver cell preparations resulting in varying susceptibilities towards HBV infection. Furthermore, susceptibility is limited during culture (5-7 d) when DMSO or hydrocortisone is omitted[26,29,30]. Fortunately, primary hepatocyte cultures from *Tupaia belangeri* (PTH) can be infected with HBV as efficiently as PHH cultures of good quality. In contrast to PHH, PTH can also be infected with a primate hepadnavirus from woolly monkey (WMHBV)[32]. Nevertheless, the host range of PTH is restricted to human and primate HBV, since productive infection with rodent hepatitis B virus (woodchuck hepatitis B virus, WHV) is not possible[32].

Hepadnaviruses are non-cytopathic and do not induce conspicuous morphological changes of infected cells. Thus, detection and quantification of infectious virions cannot be achieved by simple virological methods; e.g. plaque test. In general, verification of *in vitro* HBV/DHBV infection should be done by quantification of different markers of an established HBV infection. Conversion of the relaxed circular (rc) DNA genomes of the incoming virus to covalently closed circular (ccc) DNA within the nucleus of an infected hepatocyte is the first marker of a productive HBV/DHBV infection, but is difficult to detect. The amount of cccDNA in just newly infected hepatocytes is very small compared to the large amounts of viral input DNA (as rcDNA), usually needed for efficient *in vitro* infection. Furthermore, DNA-containing particles are also taken up by cells that are not susceptible to infection. This is especially problematic when using embryonic duck hepatocyte preparations because they contain a high percentage of non-parenchymal cells (e.g. antigen-presenting liver sinusoidal endothelial cells). However, quantitative real-time PCR protocols are available that amplify specifically cccDNA, but not rcDNA. With these protocols, specific detection of small amounts of cccDNA is possible, even in the presence of a large excess of rcDNA within infected hepatocytes[24,33]. Verification of specific HBV infection can also be achieved by detection of viral mRNA extracted from cells by either Northern Blot hybridisation or quantitative RT-PCR, usually resulting in higher sensitivity. Less demanding is the detection of secreted viral antigens; i.e., HBeAg or HBsAg, 9-12 d post infection, which could be done by commercially available ELISAs[33,34]. Especially when using enriched or highly purified viral inocula, HBeAg should be the marker of choice, since it is not present in the viral input, in contrast to HBsAg. However, since HBsAg can be detected with higher sensitivity than HBeAg it can be used as a marker, provided that several medium exchanges have been performed prior to the measurement, optimally between day 9-12[33,35]. This is necessary to get rid of input HBsAg, which is released from cells even after removal of the inoculum. There are several reports of alleged infections that determine HBsAg 2 d post infection in the supernatant spuriously assuming that this is progeny viral antigen[36,37]. Using optimized methods, the current detection limit in the PTH-system for purified HBV from human plasma is one HBV particle per hepatocyte in culture plates with 10^5 cells[33,34]. Addition of PEG and DMSO during infection is not necessary to achieve optimal infectivity in PTH[38], as was reported to be beneficial to increase HBV infection of PHH[26,29]. Therefore, *Tupaia belangeri* represent a valuable tool to overcome the restrictions associated with PHH .

HEPATIC CELL LINES

To become independent in the utilization of primary hepatocyte cultures, many groups explored the potentiality of human hepatoma cell lines for infection experiments. HepG2 cells were employed extensively for binding and infection experiments. HepG2-cells exhibit some features of differentiated liver parenchymal cells; e.g. expression of serum albumin[39], and are successfully used for the production of virions after stable or transient transfection of HBV DNA[19,40]. Several studies reported specific binding and uptake of HBV by HepG2 cells[41-46], however, no productive infection was observed by these researchers. In contrast, Bchini *et al*[47] and Paran *et al*[48] reported successful detection of viral antigens upon infection of HepG2 cells that were cultivated with, inter alia, DMSO and 5-aza-2´-deoxycytidine. Unfortunately, these results could not be reproduced by others. In order to search for an explanation for the refractoriness of HepG2 cells towards HBV, Qiao *et al*[43] supposed an inability of the incoming HBV core particles to reach the nucleus, while overexpression of serine protease inhibitor Kazal (SPIK) was suggested by another group[49]. The discovery that the addition of 2% DMSO into the culture medium of primary rat hepatocytes upholds their differentiated state through maintenance of hepatocyte specific detoxification enzymes (e.g. cytochrome P450) was a milestone for hepatic pharmacology[50]. Since hepadnaviral infection depends on the differentiated state of the hepatocyte, this method was

Table 1 Described binding partners for HBV preS1-domain. Numbering of aminoacids (aa) are given for HBV genotype D

Domain	aa	Described interaction partners/binding factors for HBV	Ref.
preS1	10-36	Hepatoma cells bind to preS1 peptide. Inhibition by peptide-antisera	[41]
	19-25	Binding of recombinant subviral particles containing preS1 to human liver. Inhibition by anti-preS1 aa19-25	[142]
	10-36	PreS1-peptide binding not limited to liver cells, also on extrahepatic sites	[143]
	10-36	Partial homology of preS1 aa10-36 with IgA. HBV entry via IgA-binding receptor?	[144]
	18-25	PreS1 aa18-25 crossreact with IgA alpha-1 chain, IgA and HBV use related receptors?	[145]
	10-36	PreS1 aa10-36 binding to 31 kDa protein on HepG2 cells	[146]
	10-36	PreS1-peptide aa10-36 binds to Interleukin 6 (IL6) but not IL3, IL5 and IL7	[147]
	10-36	CHO cells transfected with IL6 cDNA acquire binding sites for preS1 peptide aa10-36	[148]
	10-36	Isolation of 44 kDa protein (HBV-BP) from HepG2 plasma membranes. Homology to SCCA1, human squamous cell carcinoma antigen 1 (human serpin)	[46]
	preS1-GST	Isolation of p80 binding protein from human hepatocytes. Needs preS1 aa12-20/82-90 for binding. p80 binding also to rat hepatocytes	[149]
	preS1	Anti-idiotypic antisera of antibody inhibiting binding of HBV to HepG2. 35kDa protein homology to Glycerinaldehyde-3-phoshate-dehydrgenase (GAPD)	[150,151]
	preS1/ preS2	50 kDa serum glycoprotein interferes with binding of preS1 and preS2 mabs. Soluble form of plasma membrane Protein on human hepatocytes	[152]
	preS1	Yeast two-hybrid assay I: preS1 domain and human liver cDNA library. Isolation of two unknown proteins.	[153]
	preS1	Yeast two-hybrid assay II: preS1 domain and human liver cDNA library. Isolation of cytoplasmic "nascent polypeptide-associated complex alpha polypeptide" (NACA)	[154]
	HBV particles	Asialoglycoproteinreceptor (ASGPR) Ⅰ: preS1-but not preS2-mabs inhibited binding of HBV to ASGPR. SHBs did not bind to ASGPR	[44]
	HBV particles	Asialoglycoproteinreceptor (ASGPR) Ⅱ: HBV uptake by HepG2 and HuH7 (ASGPR+), but not CHO (ASGPR-)	[45]
	HBV particles	Asialoglycoproteinreceptor (ASGPR) Ⅲ: Desialylated HBV only binds to HepG2. Uptake only by susceptible primary human hepatocytes	[155]
	HBV particles	Asialoglycoproteinreceptor (ASGPR) Ⅳ: Increased HBV uptake by HepG2 after desialyation results in HBV infection	[156]

also successfully used for HBV and DHBV infection after preparation of human and duck hepatocytes[26,29,51]. While the method of induced HBV-susceptibility of long-term DMSO-treated hepatoma cell lines was not successful in established hepatoma cell lines (e.g. HepG2 or HuH7), evidence for this principle has been shown for a new hepatoma cell line (HepaRG). This cell line, established from a liver tumour of a female patient suffering from hepatocarcinoma and chronic hepatitis C infection, was shown to become susceptible to HBV and HDV infection upon treatment with DMSO and hydrocortisone[25,52]. The necessity of long term induction of HepaRG differentiation by DMSO and hydrocortisone prior to infection is time consuming, however, it provides the opportunity to decipher cellular determinants of hepatocyte differentiation and their influence on HBV infection for the first time.

In summary, the optimal system for the study of hepadnaviral attachment and entry *in vitro* are primary hepatocyte cultures of Pekin ducks and humans, the latter being very limited in supply and heterogeneous in quality and susceptibility to HBV. Primary hepatocyte cultures from *Tupaia belangeri* and the newly established HepaRG cells can overcome these limitations and provide a nearly unlimited supply of HBV-susceptible hepatic cells for various experimental settings.

CELLULAR AND VIRAL BINDING FACTORS CRUCIAL FOR HBV INFECTIVITY

During the last 25 years, numerous reports on a variety of possible cellular HBV binding partners, involving all three HBV surface proteins, have been published (Tables 1 and 2). Many researchers tried to isolate HBV binding components from plasma membranes of either primary human hepatocytes or established hepatic cell lines (e.g. HepG2) with the help of HBV-peptides or complete (subviral-) particles. In contrast to the DHBV-model discussed later in this review, none of these potential HBV-binding factors has ever been convincingly shown to be essential in HBV entry[53].

FUNCTION OF THE PRES1 DOMAIN

In 1986, Neurath *et al*[41] reported that a synthetic preS1 peptide, comprising amino acids 21-47 and corresponding to amino acids 10-36 in genotype D, E and G binds HepG2 cells and also inhibits HBV binding to this cell line. Furthermore, antibodies directed against this peptide compete with binding of HBV particles to HepG2 cells. They could, however, not show the relevance of their findings for the infection process. These early data were consistent with functional studies of Le Seyec *et al*[54], demonstrating that preS1 amino acids 3-77 of the HBV L-protein are essential for infectivity. In two independent studies, it was further shown that acylation of glycine 2 of preS1 with myristic acid is necessary for efficient hepadnaviral infection[55,56]. Interestingly, a synthetic peptide representing this essential 77 preS1-amino acids, including the N-terminal myristic acid moiety, was able to block HBV infection when added to the medium during infection[25]. Similarly, DHBV infection was sensitive against N-terminal preS-peptides DHBVpreS2-41. Surprisingly the inhibitory activity could be drastically increased by N-terminal myristoylation[57]. The effect of myristoylation

Table 2 Described binding partners for HBV preS2 and S-domains

Domain	aa	Described interaction partners/binding factors for HBV	Ref.
preS2		Binds to polymerised human serum albumin (pHSA). pHSA has affinity to hepatocytes	[157]
		Preincubation of human liver membranes with pHSA induced binding of rec. HBsAg/preS2	[158]
		Natural pHSA only present in minor amount in serum	[159]
	3-16	Natural monomeric HSA binds to preS2 domain aa 3-16	[160]
	3-16	Binding site for pHSA	[161]
	2-24	Polyclonal antisera against preS2 peptide (2-24) neutralised HBV infection of chimpanzees	[71]
	14-32	Immunisation of chimpanzees with preS2 peptide (14-32) protected against infection	[162]
	3-16	PreS2 antisera against aa 3-16 inhibited infection completely, others only partially	[33]
S		Human apolipoprotein H (apo H) binds to small surface protein (SHBs)	[163,164]
		SHBs binds endonexin Ⅱ, now called annexin V	[165]
		Overexpression of annexin V in rat hepatoma cells supported HBV-infection	[166]
		Yeast-derived SHBs alone did not bind to HBV-susceptible hepatocytes	[34]

on the inhibitory potential of preS1 lipopeptides for human HBV[25] was also shown using PTHs[34], and HepaRG cells[58] confirming the fact that myristoylation of the preS-domain of hepadnaviral L-proteins is essential for infectivity, but also indicating that this modification seems to play a role in the context of extracellular inhibition by a peptide. Furthermore, it turned out that the inhibitory activity of the preS1 lipopeptides is dependent on the hydrophobicity of the N-terminal acyl residues. Stepwise increased inhibitory potential of the lipopeptides could be achieved by increasing the chain length from C5 (pentanoyl) to C14 (myristoyl), C16 (palmitoyl) and C18 (stearoyl)[34,58]. The role of the N-terminal myristoylation of preS1 during attachment and entry of HBV is still unclear. One explanation might be that the interaction of preS1 and its receptor might be enhanced by an insertion of myristic acid into the membranes or the receptor. Exposure of a myristoylated peptide or protein ("myristoyl-switch") after attachment is a known element of viral entry mechanisms for some nonenveloped viruses, such as picornaviruses[59] and reoviruses[60].

Using a set of myristoylated HBV preS1 peptides of variable length, the attachment site of HBV was further narrowed down[34]. The data obtained using PTH suggest that: (1) residues containing the first 8 amino acids of preS1 (19 in genotype A) are dispensable, (2) residues in aa 9-18 are essential, (3) residues within aa 19-28 are dispensable, whereas iv) residues of aa 29-48 enhance infection inhibition (Figure 2B). Similar results were obtained in HepaRG cells and PHH in the presence or absence of PEG[58]. Introducing an *E. coli*-based expression system for the production of myristoylated preS-fusion proteins, Engelke *et al*[35] verified that region 9-18 is essential for virion infectivity and identified single amino acids (aa 11, 12, 13 for geno-type D) within this region that are crucial for infection inhibition. Recombinant HBV particles carrying the same point mutations are not infectious. Interestingly, hepatitis delta virus (HDV), an RNA virus that replicates in HBV infected hepatocytes and packages its ribonucleoprotein into the HBV envelope (for a recent review see[61]) can be inhibited by acylated HBV preS-derived peptides with the same specificity[35]. Thus HDV uses at least one common step for entering hepatocytes and is therefore also suitable to study HBV entry events. Surprisingly, HBV-preS1 lipopeptides containing amino acids between aa 49 until aa 78 (the region that has been shown to be important for infectivity of virions[54]) did not further increase but weakened infection inhibition[34,58]. Thus this part of the L-protein may play a role in a different step of infection process. Segment 9-18 is highly conserved with only 3 exchanges in 330 positions of the eleven HBV-genotypes, while in the other segments of 10 aa between aa 1 and 48 there are 4-8 times more exchanges. The unexpected finding that internal deletions of the preS1 sequence 20-27, containing the epitope of the neutralizing monoclonal antibody (mab) MA18/7[6,62]. within lipopeptides 2-48, did not drastically affect infection-inhibition. PreS1-region 20-48 was speculated to contain major B-cell epitopes[63]. Indeed, mab KR359 neutralized HBV infectivity for PHH and binds to aa 19-26[64], whereas another neutralizing mab KR127 binds to aa 37-45. Furthermore, humanised mab KR127 inhibited HBV infection in chimpanzees[65]. Obviously, the binding of mabs to these epitopes hinders the attachment although the essential sequence element needed for infection is elsewhere. While the behaviour of the inhibitory preS1-peptides was very similar in the Tupaia system[34] and PHH/HepaRG cells[58] one point was worth mentioning: the preS1 sequence 20-27 was necessary for full inhibitory potency of the preS1 (2-48) peptide in HepaRG cells. A possible explanation might be that the receptor molecule(s) on Tupaia hepatocytes has binding sites for aa 9-18 and 28-48 comparable to the human receptor(s) but differs with the human binding site at aa 20-27. In a similar approach, Barrera *et al*[66] reported that the preS1-region involved in infection-inhibition of HDV spans residues 5-20 of preS1. However, they needed much higher concentrations of myristoylated preS1 peptides for inhibition in their study (> 5 μmol/L) than in the HBV studies using PTH[34], PHH (< 1 nmol/L)[58] and HepaRG[35]. However this discrepancy might be related to the peptide preparation the authors used, since myristoylated recombinant preS1-proteins obtained from a baculovirus expression system had much higher and comparable specific activities.

The remarkable potency of infection-interference, using acylated preS1-peptide 2-48 at (sub-) nanomolar concentrations, was demonstrated by the kinetics of the peptides using both PTH and HepaRG cells. Even short preincubation periods (30 min) of peptides (100 nmol/L) with the cells are sufficient to block subsequent infection and to induce non-susceptibility of the cells for hours[34,58]. Interestingly, infection could also be blocked by myristoylated peptides (100 nmol/L) after attachment of the viral inoculum at 4℃ had occurred[34]. These experiments supported the assumption that the peptides inhibited infection through binding to the hepatocytes (there possibly addressing a specific receptor), rather than with the virus, although we can presently not exclude the possibility that the peptide might address virions at a specific site on the cell. Using immunohistochemistry, we could demonstrate specific binding of HBV preS1 2-48 peptides to PTH, but not to primary rat hepatocytes or other hepatoma cell lines, such as mouse AML12[34]. The binding was considerably increased when using the respective acylated variants. The binding of the myristoylated HBV-preS-peptides could also prevent binding of highly purified HBV preS1-containing subviral particles, whereas preincubation with myristoylated preS peptides from avian hepadnaviruses did not[34]. However, inhibition of binding required micromolar concentrations of peptides, rather than nanomolar concentrations needed for inhibition of infection[34]. This discrepancy suggested presence of a more abundant low-affinity receptor for HBV on hepatocytes.

This low affinity receptor might be a sulphated glycan, because interaction of HBV and PHH is inhibited by heparin[67]. Furthermore, HBV binds to heparin *in vitro* and could be purified from the plasma of HBV-infected patients by heparin-sepharose columns[68]. Unfortunately, these reports could not clarify the relevance of this interaction for infection. Recently, we could show that HBV infection could be specifically blocked by preincubation of purified virus with heparin, or by treatment of PTH and HepaRG cells with heparinase (unpublished data). Since heparan sulphate proteoglycanes (HSPGs) are enriched in the liver within the space of Dissé, one may speculate that HBV is trapped by liver-specific HSPGs, serving as low-affinity receptors similar to the interaction and entry of apolipoprotein E lipoprotein remnants by liver HSPGs[69]. Specific entry of the virus may subsequently require passage to a yet undefined high affinity receptor(s), which can be blocked by the acylated preS1-derived peptides.

FUNCTION OF THE PRES2 DOMAIN

The M protein of HBV is not essential for infectivity[70], although antibodies against the N-terminal part of preS2 inhibited almost completely HBV infection in PTH cultures[33]. Similar results were reported for polyclonal antisera against HBV preS2 peptides (residue 1-24) *in vivo*[71]. The preS2-domain is present in both M- and L-protein (Figure 2A). However, due to the cytosolic orientation of the preS-domain in L-protein, N-glycosylation at Asn-4 of the preS2-domain occurs only in the M-protein[16,72]. In accordance with this, one of several preS2-antibodies (Q19/10), recognizing aa 1-6 in a glycan dependent manner, strongly bound to the N-terminal part of preS2, but showed the lowest neutralisation potential of all preS2-mabs used in the study[33]. Possibly, the preferential binding of mab Q19/10 to the preS2-domain of MHBs is responsible for the strongly reduced neutralisation potential, which is in agreement with the dispensability of MHBs for infectivity[70]. Furthermore, the HBV M-protein is not essential for infectivity of hepatitis delta virus[73]. A direct role of the N-terminal part of preS2 domain of L-protein for infectivity is still under debate. The carboxyterminal part contains a cell permeable translocation motf (TLM), which was suggested to be involved in HBV entry (Figure 2B)[74]. However, recombinant HBV variants with disturbed or lacking TLM sequence within the preS2 domain of LHBs are still infectious in PHH cultures[75]. The dispensability of the TLM sequence during the entry process of HBV was also detected using the hepatitis delta virus system that uses HBV surface proteins for viral entry. The infectivity of recombinant hepatitis delta virus, containing only the large and small HBV surface proteins, was not affected by the absence or presence of a TLM-sequence within the preS2-domain of the LHBs (Sureau *et al*, Taylor *et al*, personal communication).

FUNCTION OF THE S-DOMAIN

The observation that binding of HBV surface proteins to PTH cultures could be inhibited specifically by myristoylated preS1 peptides[34] argues against a predominant role of the S-domain in initial binding to hepatocytes. PreS1-rich subviral particles from human plasma bound specifically to more than 70% of cultured primary hepatocytes[33,34], while subviral particles containing only S-protein, did not[34]. Addition of the preS1-sequence 2-48 to the S-domain of these particles restored the binding to PTH up to wild-type levels[34]. The main function of the S-domain is morphogenesis, but it contains several elements that participate in entry. The main point is that antibodies against the S-domain (as generated by current S-containing vaccines) could neutralise infection *in vivo* and *in vitro*[33,76-78]. Furthermore, the presence of escape-mutants in HBV-infected patients positive for anti-HBs[79,80] demonstrates the importance of the antigenic domain (residue 100-170) of SHBs for viral spread *in vivo*. Since the S-domain does not contribute directly to binding, the question arises how these antibodies are able to neutralize infection. The S-domain contains 8 Cys residues within the antigenic loop that form inter- and intramolecular disulfide bonds[81,82], resulting in high molecular weight multiprotein complexes[83], while the preS-domain does not contain Cys and forms linear epitopes. We found that a mab recognizing a conformational S-epitope could completely neutralise infection of PTH, while a mab recognising a linear S-epitope failed to inhibit infection completely (90% inhibition)[33]. Therefore, distinct amino acids within the correctly folded antigenic loop of the S-domain might be essential for the uptake process of HBV leading to productive infection. Support for this

assumption comes from infection experiments with HDV, carrying mutant HBV S-proteins in their envelopes. Although short internal deletions, within the antigenic loop of the S-domain (residues 104 to 163), had no effect on HDV morphogenesis, virions with S-deletions between residues 118 and 129 showed reduced infectivity on PHH and HepaRG cells. Single amino acid exchanges within this domain revealed a sequence 119 to 124 (GPCRTC) to be most important for infectivity[52]. This domain contains a CXXC-motive, known to be the active site in protein-disulfide isomerase and related enzymes, involved in catalyzing disulfide-bridge exchanges. In murine leukaemia virus surface proteins, the receptor-binding subunit (SU) domain contains a CXXC motive that is activated after receptor-binding of the envelope transmembrane (TM) subunit. This leads to isomerisation of SU-TM disulfide-bonds and fusion-activation within the TM subunit[84,85]. Whether a CXXC-motive is actively involved in fusion in the case of HDV or HBV, or whether this region binds to a (co)-receptor, is currently unclear.

A clear function in entry can be ascribed to the first transmembrane sequence of the S-domain. It has sequence similarity to type 1 fusion peptides and replacement of the corresponding sequence in influenza virus hemagglutinin with HBV transmembrane sequences confers hemifusion activity of the resulting chimeric influenza virus hemagglutinin[86]. As recently proven for DHBV only the S-domain of the L-protein but not the S-protein itself provides the function of fusion[87]. This observation suggests that the topology of the S-domain in L-protein is different from that of the S-protein. For HBV, this difference is also recognizable in the glycosylation pattern. 50% of SHBs, 30% of MHBs, but 90% of LHBs are N-glycosylated in the S-domain[6].

EARLY EVENTS IN DHBV INFECTION

For more than 20 years, the duck hepatitis B virus model system was successfully used to study hepadnaviral replication. Until recently, one of the exclusive advantages of this system was the possibility to systematically investigate the early events of infection. Even though difficult, a routine preparation of PDH from newly hatched Pekin ducklings, or embryonic hepatocytes from fertilized eggs, has been successfully established in many laboratories to perform *in vitro* infection and infection inhibition studies[51,88]. Guided by the supposition that insights into the DHBV entry process also illuminate the HBV early infection events, a series of studies have been performed, however, only some of them provided satisfactory answers so far. In the following pages we would like to concentrate on the following major issues: (1) What are the roles of the two multifunctional viral envelope proteins, L and S, in entering the hepatocyte and which of their subdomains are involved? (2) Which cellular components have been described to be functionally implicated in these processes? (3) How and where does fusion of the viral and cellular membranes occur and which part of the viral envelope protein acts as a fusion promoter? (4) Which endocytic route is mandatory for the virus in order to productively deliver its nucleocapsid to the nucleus? (5) What determines host specificity of avian hepadnaviruses? (6) Why is the susceptibility of cells towards DHBV infection restricted to differentiated, resting hepatocytes? (7) Finally, taking into account the first functional insights into the HBV entry processes using the recently established *in vitro* systems (HepaRG cells and PTH), we would like to critically scrutinize the question whether there is justified hope that further insights into the DHBV entry processes are relevant for HBV, especially regarding the development of inhibitors for infection.

THE ROLE OF THE DIFFERENT ENVELOPE PROTEINS IN DHBV ENTRY

Soon after the discovery of DHBV and the possibility of replicating the virus *in vitro* using PDH[89,90], several groups characterized DHBV structural proteins biochemically and immunologically, including those that constitute the membranous envelope[91,92]. One approach was the recombinant expression of DHBV-preS fusion proteins to generate antisera. These sera detected two unglycosylated 35 and 37 kDa envelope proteins (the L-protein and its phosphorylated form), co-immunopreciptitated a 17 kDa protein under native conditions (which was identified as the DHBV S-protein by microsequencing) and were able to neutralize DHBV infection *in vitro*[92,93]. Further investigations concentrating on posttranslational modifications of L- and S-proteins and the possible functional implications for DHBV replication revealed, that the N-terminal Glycin-2 of the preS-domain of the DHBV L-protein becomes myristoylated during protein synthesis (Figure 2A)[94]. In addition to myristoylation the DHBV L-protein becomes partially phosphorylated preferentially at Serin 118 in its preS-domain (Figure 2B)[11,95,96]. Both modifications are not required for assembly and secretion of virions. However, while mutations in the phosphorylation sites did not interfere with infectivity of DHBV *in vitro* and *in vivo*[97] the prevention of myristoylation resulted in a loss of infectivity of the mutant in ducklings and a drastically reduced potential to infect PDH *in vitro*[94], (our unpublished results). These findings indicate that at least parts of the DHBV-preS domain, including its N-terminal modification by myristic acid, are involved in virus entry. Functional epitope mapping of mouse mabs obtained after immunization with DHBV particles or recombinant proteins supported this idea[93,98-104]. Three epitopes within the preS domain recognized by neutralizing antibodies were characterized, the first one covering a central part including amino acids 82-109, the second one including a more N-terminal part, amino acids 12-30, and a third part between amino acids 123-137.

The first direct evidence for the participation of the preS-domain of the DHBV L-protein for virus entry came from infection competition experiments using recombinant SVPs composed of only the DHBV L- or DHBV S-protein. These particles were purified from yeast and displayed significant differences in their ability to compete with DHBV infection of PDH with only the L-particles being active[105]. The investigators further showed that only the preS/S-containing particles bind

hepatocytes, supporting the view that the preS-domain as a part of DHBV L plays a pivotal role in attachment and infection of hepatocytes. Following this experimental strategy, Urban *et al* demonstrated that *E. coli*-derived preS-polypeptides, devoid of both the N-terminal myristoyl moiety and the hydrophobic TM-containing S-domain, specifically inhibit DHBV infection *in vitro* with IC_{50}s of about 800 nmol/L. Using a set of terminal and internal deletion mutants it became evident that an uninterrupted innermost preS-domain (amino acids 30-115), including epitopes recognized by neutralizing antibodies, is required for infection inhibition. Since the preS-polypeptide derived from the heron hepatitis B virus (HHBV) also competed with DHBV infection (despite an amino acid variation of 50%), it was evident that the addressed step cannot be responsible for the observed species specificity (see below) between these two avian hepadnaviruses. The striking correlation of the infection competition activity of DHBV-preS polypeptides with their ability to bind duck carboxypeptidase D (including the binding of HHBV preS to dCPD) suggested that it is this molecule which is addressed and inactivated at the surface of hepatocytes[106,107]. Interestingly a second peptide, consisting of the N-terminal 41 amino acids DHBV preS, devoid of CPD-binding and requiring myristoylation of Gly-2 for efficient inhibitory activity (IC_{50} = 200 nmol/L), was identified subsequently[57]. Pre-incubation experiments showed that the peptide addresses a cellular component. Antibodies raised against this peptide and recognizing amino acids 12-23 were able to efficiently block DHBV infection and immunoprecipitate particles indicating that this N-terminal preS-part is exposed on the particle surface and required for infection[108].

Until today, little is known about the role of the DHBV S-protein in DHBV entry. In contrast to the HBV S-protein, DHBV-S is smaller and does not include the antigenic loop, called *a* determinant, which bears epitopes involved in the protective immune response acquired upon vaccination against HBV. Indirect evidence for the involvement of DHBV-S in entry came from the observation that antibodies recognizing the DHBV S-protein, although rarely induced by immunization with DHBV particles[92], can neutralize DHBV-infection of PDH[100]. However, it is not clear if the antibodies directly prevent molecular contacts needed for infection or if they interfere with the formation of preS-dependent interactions by steric hinderance. Both single point mutations within TM-1 of S, resulting in a reduction of hydrophobicity, or the complete replacement of DHBV TM-1 by the HBV TM-1 had no effect on DHBV infectivity (in contrast to the effect the same mutations had when introduced in the TM-1 of L)[100]. It has recently been shown that, besides L- and S-proteins, the DHBV envelope contains a third 10 kDa membrane protein termed S_t[8]. It is a truncated version of the DHBV S-protein, consisting of TM-1, the internal cystein loop and a part of TM-2. S_t, however, seems to play a key role as a chaperone in L-protein translocation. Unfortunately, to date, no further systematic approaches aiming to identify S-specific amino acids that lead to non-infectious virions have been undertaken.

CELLULAR MOLECULES INVOLVED IN DHBV ENTRY

Based on accumulating evidence that the preS-domain plays crucial roles in DHBV infection and also possibly mediates binding to the hepatocyte, Kuroki *et al*[109] performed a biochemical approach for receptor identification and detected a 180 kDa membrane protein in ^{35}S-labeled duck hepatocyte extracts that co-immunoprecipitated with DHBV particles or recombinant envelope proteins. They showed that binding requires only the DHBV-preS part and can be inhibited by neutralizing preS-antibodies. Continuous work by this group and independent efforts by Tong *et al* using GST-preS fusion proteins for affinity purification, identified gp180 or p170 (as named by Tong and co-worker) as the prototype member of a new class of regulatory trans-Golgi network (TGN)-resident carboxypeptidases, soon afterwards termed carboxypeptidase D (CPD)[110,111]. Duck CPD (dCPD) like all other CPDs identified so far, consists of three luminal/extracellular carboxypeptidase E like domains of about 50 kDa each, one transmembrane domain and a highly conserved cytoplasmic tail required for accurate retrieval to the TGN[110,112]. While two of the three luminal/extracellular domains bind Zn^{2+}-ions and exhibit enzymatic carboxypeptidase activity towards yet unidentified cellular proteins that cross the secretory pathway[110,113], the membrane proximal C-domain of dCPD is enzymatically inactive and binds DHBV preS with very high affinity[110,114,115]. However, although the C-domains of human CPD and mouse CPD are homologous to each other and to the dCPD-C domain[116] they do not interact with DHBV preS. Chicken CPD, by comparison, displays only a very weak binding[117]. Interestingly Spangenberg *et al*[117] succeeded in rescuing the binding of DHBV preS to the human CPD C-domain by the introduction of a short dCPD-C domain-derived sequence (amino acids 920-949). Thus, since dCPD is essential for DHBV infection, species specificity could at least partially be explained by the potential of the viral preS-domain to bind CPD.

There is striking experimental evidence that dCPD serves a crucial role in DHBV infection: (1) recombinant DHBV-preS peptides, which are able to bind dCPD *in vitro,* are also active as inhibitors of DHBV infection in PDH[106,107]. (2) soluble dCPD as well as antibodies against dCPD block DHBV infection[115,118]. (3) adenoviral transfer of a dCPD mutant lacking the cytoplasmic TGN-retrieval signal into PDH, abolishes DHBV infection of the transduced cells[119]. (4) dCPD is greatly and selectively down regulated in DHBV infected duck livers and in infected PDH, which is a possible way to exclude superinfection[120], although there is evidence for a second dCPD-independent mechanism[121]. (5) a set of DHBV single point mutants that are deficient in dCPD binding lost their infectivity (unpublished data). However, despite this compelling evidence, it has not been possible to render non-susceptible cell lines that support replication of the viral genome (e.g. LMH cells) susceptible by expression of dCPD[106]. This indicates that either (an) additional factor(s) is/are missing in the dCPD-transduced cell line or that the remarkable and still enigmatic dependency of

hepadnaviral infections on a resting differentiated state of the hepatocyte provide additional constraints that must be overcome as well.

Following the identification of dCPD as a putative DHBV-receptor, extensive work from several groups addressed issues on the sequence requirements of the DHBV preS-domain in order to bind dCPD, as well as details in the mode of dCPD/DHBVpreS interaction[106,111,115,118,122]. Although the results of binding analyses are divergent to some extent, the variations are explainable by the dissimilar techniques that have been applied by the different authors. All findings, however, indicate that a central preS-sequence including amino acids 87-115 (containing major epitopes recognized by neutralizing antibodies) is indispensable for dCPD binding (Figure 2B). The disturbance of the integrity of this sequence abrogated binding entirely. Using quantitative real-time surface plasmon resonance spectroscopy, it became clear that sequences located N-terminal to this essential part (including amino acids 30-86) contribute to the complex stability in a sequence dependent manner, making the interaction of DHBV preS and the C-domain of dCPD to one of the strongest interactions between a viral ligand and a cellular protein[115]. Concerning the mode of interaction, two aspects are noteworthy: First, binding of preS induces conformational changes not only in the viral ligand but also in dCPD. Together with the unusual finding that preS binding to the dCPD C-domain occurs in close proximity to the cellular membrane, the preS-induced dCPD conformational changes indicate that dCPD may play an important role in the fusion of the viral and cellular membrane. If this holds true DHBV entry into hepatocytes would exemplify a novel type of a viral entry mechanism, involving the recruitment of a cellular protein to act as a fusion mediator. However, this hypothesis remains to be supported. Secondly, an extensive 2D NMR structural analysis of the DHBV preS-subdomain that binds dCPD (amino acids 30-115) revealed a mostly unstructured protein with only a short sequence within the essential binding site (amino acids 89-104,) exhibiting the tendency to form an alpha helix (Figure 2B). This is consistent with the observation that a DHBV preS-polypeptide can be treated repeatedly with denaturing agents without losing the ability to bind dCPD with an unaffected K_D of 1.5 nmol/L at 37℃[115]. Thus, the dCPD-binding domain of DHBV represents the first example of a viral protein belonging to the group of intrinsically unstructured/disordered proteins[123] and in that way differs from the well ordered structures found on the surfaces of other enveloped viruses; e.g. influenza virus hemaglutinin or HIV gp120. Structural analyses performed with the whole HBV preS1-polypeptide, as well as with myristoylated N-terminal preS1 peptide fragments, lead to similar results (unpublished data).

Immunization of mice using whole duck hepatocytes and subsequent screening of mabs with respect to their potential to inhibit DHBV binding to and infection of PDH, Guo and Pugh isolated two IgMs exhibiting both activities[124]. They immunoprecipitated a 55 kDa cellular protein that is also detectable in other tissues of ducks and in other birds. Unfortunately, this interesting observation has not been followed up and it therefore remains an open question if the 55 kDa protein represents a primary attachment factor that might be part of the viral entry machinery into hepatocytes.

Following the identification of dCPD as a putative receptor Li *et al*[125] identified a 120 kDa protein preferentially found in liver, pancreas and kidney that displayed binding activity only to some N-terminally and C-terminally truncated variants of GST-preS fusion proteins. Binding depends on the two crucial arginine residues at positions 101 and 102. Purification and mass spectroscopic analysis of p120 identified it as the P-subunit of glycin decarboxylase (GDC), which is an enzyme involved in mitochondrial amino acid metabolism[126]. Recombinant expressed GDC was located to some extent at the cell surface and bound truncated preS-fusion proteins with comparable specificity as the endogenous GDC. Downmodulation of GDC-levels in PDH, either by prolonged cultivation or expression of antisense RNA, resulted in a reduced susceptibility towards infection. Duck GDC has therefore been proposed to act as a co-factor in DHBV infection after proteolytic processing of the DHBV L-protein[127]. Although, the proposed concept of proteolytic activation of the L-protein during entry is attractive, the role of GDC in DHBV infection cannot be exclusive since a DHBV mutant carrying the point mutation R101H is fully infectious, although the respective mutation abolished binding to GDC[108].

MOLECULES INVOLVED IN MEMBRANE FUSION OF DHBV WITH THE HEPATOCYTE MEMBRANE

In contrast to viruses enclosing type 1 fusion proteins on their surface (e.g. HIV, Influenza, Ebola virus), hepadnaviruses do not encode a classical fusion peptide sequence, which becomes proteolytically released from an envelope protein precursor during secretion. It has therefore been hypothesized that instead they use the internally located hydrophobic transmembrane domain 1 (TM-1) as a fusion peptide, similar to the type 2 fusion proteins found in HCV, and alphaviruses[128]. Evidence for this assumption came from experiments with DHBV subviral particles that, upon low pH-treatment, expose hydrophobic domains on their surface, thereby increasing their ability to bind membranes[129]. An elegant subsequent analysis, including reverse genetics, demonstrated that lowering the hydrophobicity of TM-1 in the L- but not the S-protein through alanine substitutions resulted in a loss of DHBV infectivity[87]. Thus, TM-1 serves (a) distinct function(s) in DHBV L- when compared to the DHBV S-protein, with clear involvement in the fusion process on the part of the L-protein.

Subsequent to the observation that a short, possibly amphipatic helix in the C-terminal part of the HBV preS2-domain consisting of amino acids 41-52 is capable of translocating fused proteins, such as GFP or nucleic acids across cellular membranes[74], Stoeckl *et al* predicted two such structural motifs (called trans-location motifs, TLM) also in the N-terminal third of the DHBV preS-domain

(amino acids 20-31 and 42-53, Figure 2B). These two sequence elements are notably conserved among all avian hepadnaviruses. Amino acids 22-41 have previously been shown to be important for a dCPD-independent inhibition of infection mediated by myristoylated DHBV and HHBV preS-peptides[57]. Exchange of 4 highly conserved amino acids in the first motif (D1-mutant) or 3 conserved amino acids in the second motif (D2-mutant) or the concurrent exchange of 7 amino acids (D-1/D2-mutant) resulted in a reduced secretion (D1 mutant) and a loss of infectivity (all mutants) in embryonic hepatocytes, emphasizing the importance of both segments for infection. Interestingly, all mutants bind to and are internalized into cells but cannot be released from an endosomal compartment. This observation supports the preceding idea that the N-terminal preS-part functions at an event downstream of receptor binding and uptake. Based on the loss of the TLM-activity of the mutated sequence, the authors hypothesize that escape from the endosome does not follow a classical fusion mechanism but proceeds via direct translocation of the nucleocapsid and generalize this mechanism for all hepadnaviruses. Although attractive as a model, this hypothesis lacks direct evidence (e.g. that introduction of other TLMs, such as HIV-Tat, can replace the DHBV sequence) and is also not supported by the observation that mutated virions lacking the preS2-containing TLM of the HBV L-protein are infectious *in vitro*[75]. It is also difficult to explain how amino acids 20-53 can contribute to host discrimination between DHBV and HHBV if they provide only a functional TLM in both avian hepadnaviruses (Figure 2B)[130]. Moreover, it has been shown that amino acids 42-51 of TLM-2 as part of DHBV preS does not form an alpha-helix[115]. Thus, the inability of the described DHBV mutants to escape the endosome might be a consequence of the disruption of the interaction with the proposed co-factor and not the disturbance of a TLM-function.

ENDOCYTIC ROUTES USED BY DHBV

Although DHBV infection of PDH is efficient with respect to the percentage of cells that can be synchronously infected, our knowledge of the endocytic routes utilized by the viral particle is still rudimentary. This relates on the one hand to the fact that resting PDH cannot be efficiently transfected by routinely used protocols, making investigations with dominant negative mutants of the endocytic pathway complicated. On the other hand, the low percentage of virus particles that bind to hepatocytes, even when high multiplicities of genome equivalents "MGEs" are offered in the medium[105,131], requires very sensitive methods for a direct visualization of DHBV uptake in hepatocytes by fluorescence microscopy[87]. Consequently, most of our knowledge on the DHBV uptake route comes from results with chemical drugs that are known to interfere with specific intracellular events, which have previously been used to decipher entry routes of other viruses (e.g. binding to charged surface molecules, endosomal acidification, trafficking along microtubules, actin cytoskeleton integrity).

Regarding the question whether productive DHBV infection requires endocytosis and intracellular trafficking events, including acidification as a prerequisite for fusion, early experiments using the lysomotropic reagents ammonium chloride, chloroquine and monensin lead to contradictory results. While Offensperger *et al*[132] showed that infection was abolished with ammonium chloride and chloroquine, Rigg and Schaller reported the contrary[133]. Following DHBV particle uptake using confocal microscopy, Chojnacki *et al* convincingly demonstrated recently that DHBV particles co-localize with fluorescently labeled transferrin in an endosomal compartment 2 h after attachment. The addition of bafilomycin A1, which is a potent inhibitor of vacuolar proton ATPases, at different time points during/after infection clearly showed that transit to the late endosomal compartment is required for infection[87]. Within this compartment, the activation of the DHBV envelope into a fusion competent state is probably not solely triggered by a pH decrease, explaining to some extent the earlier conflicting results, but might include events like CPD-binding and proteolytic cleavage of viral surface proteins. This is consistent with the observation by Breiner and Schaller who, while successfully applying an adenoviral transduction system for PDH, demonstrated that recombinant expression of CPD-mutants lacking the complete TGN retrieval signal abrogated DHBV infection[119]. PH-independent fusion and the dependence of productive infection on endosomal trafficking events have also been confirmed by an independent study[134]. Although there is still some debate on whether the authentic DHBV uptake route into hepatocytes proceeds via dCPD or if dCPD acts at a later stage, these results allow little doubt that accurate vesicular trafficking towards the late endosome, where fusion is expected to occur, is a prerequisite for productive DHBV infection. Using a semiquantitative PCR-based binding assay and chemicals that are known to interfere either with infection of hepadnaviruses or the formation and maintenance of microtubuli and the actin cytoskeleton, Funk *et al* showed that suramin, which is a highly charged urea-derivative and well-known inhibitor of DHBV, RSV and interestingly also HDV infection[135], decreases binding of DHBV to hepatocytes. The authors estimated the number of DHBV binding sites on hepatocytes to be about 10^4/cell, which is remarkably low when compared to other viruses[131]. They further showed that infection at some post-entry step depends on microtubular integrity and that spread in cell culture proceeds via polar egress of new virions from the infected cell[136].

WHAT DETERMINES HOST SPECIFICITY OF AVIAN HEPADNAVIRUSES?

Hepadnaviruses are principally characterized by a narrow host range, restricting *in vivo* infections to only closely related species of their natural hosts. Well-known examples are the restriction of natural HBV infection to humans and chimpanzees. Similar observations have also been made for avian hepadnaviruses. As far as we know, productive DHBV infection exclusively occurs in Pekin

ducks. Related species, such as the Muscovy duck, do not support *in vivo* infection. This *in vivo* species specificity is to some extent reflected by the restricted susceptibility of the respective hepatocyte cultures (e.g., DHBV infects hepatocytes of Pekin ducks but not those from Muscovy ducks or chickens). Interestingly, this is not observed when replication competent viral genomes are artificially transferred into cell lines of different origin. This is best exemplified by the observation that infectious DHBV particles can be produced even in the human hepatoma cell line HuH7[137,138]. It has therefore been assumed that some early step in infection (e.g., attachment, entry, fusion) determines the host range of hepadnaviruses and that the liver specific factors needed for genome replication and virus assembly are not decisive. Comparing the binding of DHBV particles to hepatocytes from Pekin ducks with hepatocytes from Muscovy ducks or chicken hepatocytes and fibroblasts, Pugh *et al* provided evidence for this assumption showing that the difference in susceptibility corresponds to the ability to bind virions and subviral particles. The loss of susceptibility towards infection during prolonged cultivation correlated with a reduction of binding capacity of cells.

The discovery of the heron hepatitis B virus (HHBV) and its property to be not infectious for Pekin ducks and PDH[139], opened the way to investigate host specificity on a molecular level. In that line, Ishikawa and Ganem produced pseudotyped heron hepatitis B viruses (HHBV) with envelopes consisting of HHBV-S and chimeras of the DHBV and HHBV L-protein[130]. They showed that the replacement of the HHBV-preS domain with DHBV-preS rescued the infectivity of HHBV in PDH. This indicated that the preS-domain determines host range without the need for a species-specific "cross-talk" between L- and S-proteins. Further fine mapping revealed that a sequence element containing amino acids 22-37 is sufficient to overcome host restriction *in vitro*[130], (Ishikawa, personal communication). Similar experiments have also been performed with HBV particles that were pseudotyped with chimeric L-proteins carrying WMHBV preS-sequences. Chouteau *et al*[140] found that HBV pseudotyped with a WMHBV envelope lost their infectivity for PHH *in vitro*. However, substitution of only the first 30 amino acids of HBV preS1 could restore infectivity of the chimera, indicating that a short N-proximal region in the L-protein harbors a determinant that contributes to the species specificity of HBV.

Although these data accentuate host restriction of hepadnaviruses as a general theme of this virus family, some unexpected recent observations complicate our understanding. One observation identifies a new hepadnavirus isolated from crown cranes which, despite its close relation to HHBV, infects PDH[12]. Another observation demonstrates that primary hepatocytes from *Tupaia belangeri*, belonging to the order *Scandentia*, are susceptible for HBV infection *in vitro*[32]. Taken together, host specificity of hepadnaviruses is to some extent determined by an early step in infection involving the adaptation of the N-terminal preS-domain of the L-protein to an unknown cellular factor. Moreover, there might be additional viral and host determinants that are to be identified.

WHY IS SUSCEPTIBILITY TOWARDS DHBV INFECTION RESTRICTED TO DIFFERENTIATED, RESTING HEPATOCYTES?

Another hallmark of hepadnaviral infection is its restriction to differentiated resting hepatocytes. Although some attempts have been undertaken there is no proliferating cell line available that supports DHBV infection. The recently described HepaRG cell line, which is the first to support the full replication cycle of HBV, is also not susceptible in a non-differentiated state[25]. HepaRG cells become susceptible for infection only after prolonged treatment with hydrocortisone and DMSO (this process needs at least 2 wk). This induced susceptibility does not correlate with enhanced binding of HBV to differentiated cells. In fact, other hepatoma cell lines, although not susceptible towards infection, bind and accumulate HBV much better than HepaRG cells (unpublished data). This remarkable behavior is supplemented by the observation that initial amplification of cccDNA after *in vitro* infection of embryonic duck hepatocytes increases by the progression of the cell cycle[141]. Thus we have to assume that, in addition to the bare presence or absence of receptor molecules, unknown differentiation-specific and cell cycle-dependent factors of hepatic origin are important key players that are involved in early restriction events.

DO INSIGHTS INTO THE DHBV ENTRY PROCESSES HELP US TO UNDERSTAND HBV INFECTION?

Having now readily available *in vitro* systems to study HBV infection, important questions, such as the nature of the HBV receptor(s) or the characterization of the HBV entry pathway and its inhibition, can now be studied. These investigations are influenced by the results and concepts obtained from the DHBV studies. Just a few examples shall be mentioned: (1) The establishment of transduction systems for PTH, PHH and HepaRG cells will allow us to inspect the relevance of known endocytic pathways for HBV infection. (2) Using highly purified virus preparations, we may further be able to directly follow attachment and entry using sensitive microscopic techniques. (3) The application of microarray-based gene expression profiling will help us identify genes that are becoming up- or down-regulated during differentiation of HepaRG-cells and may therefore also be important regulators of the HBV replication cycle. (4) Having a set of well characterized preS-peptides that interfere with infection, it will be possible to identify the molecule(s) they address. However, it is of utmost importance to be aware of the possible differences in the uptake strategy that might have evolved in the two genera ortho- and avihepadnaviruses with their prototypic members HBV and DHBV, respectively. Two already known examples illustrate this. First, the discovery of dCPD as an important cellular factor for avihepadnavirus infection raised the

question of whether the human homologue plays a similar role in HBV infection. We have performed extensive studies related to that question (e.g., an infection inhibition experiment using soluble human CPD or anti-human CPD antibodies, investigations on whether transfection of human CPD promotes uptake of purified HBV, binding assays using HBV preS and human CPD *etc*.). None of these experiments gave any hint that this molecule is involved in HBV infection (unpublished data). Secondly, Chojnaki *et al*[87] provided unquestionable evidence that DHBV infection depends on the intracellular transport of virions from the early to the late endosome and is thereby blocked by bafilomycin A1. In contrast, HBV infection of HepaRG cells is not influenced by this drug (unpublished results) indicating, along with other evidence, that the two hepadnaviruses enter hepatocytes via different endocytic pathways.

PERSPECTIVES

Since the cloning of the HBV genome, and the discovery of related viruses in the animal kingdom, many aspects of the hepadnaviral life cycle have been unravelled with the help of established hepatoma cell lines and the transfection of replication competent genomes. These cell lines were, however, not suitable for infection experiments, possibly due to the lack of one or more unknown factors required for infection. A huge and still growing list of binding partners for HBV and DHBV have been reported since then, however, none of them have been convincingly shown to be related to HBV infection, and only Carboxypeptidase D has been shown to play a crucial role for the infection of avihepadnaviruses. For over 20 years, primary human hepatocytes were the only possible *in vitro* system for studying HBV infections, which created strong limitations. These limitations have become obsolete with the discovery of the HepaRG cell line and the usability of PTH instead of PHH to study HBV infection in an accurate manner. Although both systems bear their specific difficulties (e.g., Tupaias have to be bred in captivity, and HepaRG cells require a laborious protocol in order to render them susceptible for infection) this should be manageable.

With these models it will be possible to characterise cellular attachment factors and entry receptors for HBV. It will further be possible to decipher the entry pathway(s) of HBV and thereby to relate this important pathogen to other viruses.

In light of the discovery of a crucial domain within the preS1 part of the L-protein, the available HBV vaccines have to be improved. Although the current vaccine has been shown to be safe and effective, it consists only of S-protein containing recombinant particles and relies solely on the generation of protective antibodies recognizing this part of the viral surface protein, which as we now know do not counteract binding of the virus to its target cells. This allows the emergence of escape mutants, frequently arising especially under antiviral therapy, with reverse transcriptase inhibitors (e.g. lamivudine). Inclusion of the preS1 sequences into vaccines should therefore directly protect against infection.

The discovery of HBV preS1-derived lipopeptides as potent inhibitors of HBV entry will not only stimulate further investigations aiming to decipher the early infection events, they also represent a novel antiviral approach for the treatment of acute and chronic hepatitis B and hepatitis delta, similar to the HIV-peptide entry-inhibitor T20 (also called *enfuvirtide* and *fuzeon*). However, compared to T-20, the most active HBV inhibitor (HBVpreS/2-48$^{\text{stearoyl}}$ and also called Myrcludex B) approximately displays a 1000 fold higher specific activity. This substance, which is presently under preclinical development, could be very useful for post-exposure prophylaxis or the inhibiting of re-infection after liver transplantation. Whether efficient entry inhibition will also be beneficial in the treatment of chronic HBV and HDV infections, alone or in combination with current therapies, is an interesting objective to be addressed in a clinical trial in the near future.

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TOPIC HIGHLIGHT

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Abstract

For genome multiplication hepadnaviruses use the transcriptional machinery of the cell that is found within the nucleus. Thus the viral genome has to be transported through the cytoplasm and nuclear pore. The intracytosolic translocation is facilitated by the viral capsid that surrounds the genome and that interacts with cellular microtubules. The subsequent passage through the nuclear pore complexes (NPC) is mediated by the nuclear transport receptors importin α and β. Importin α binds to the C-terminus of the capsid protein that comprises a nuclear localization signal (NLS). The exposure of the NLS is regulated and depends upon genome maturation and/or phosphorylation of the capsid protein. As for other karyophilic cargos using this pathway importin α interacts with importin β that facilitates docking of the import complex to the NPC and the passage through the pore. Being a unique strategy, the import of the viral capsid is incomplete in that it becomes arrested inside the nuclear basket, which is a cage-like structure on the karyoplasmic face of the NPC. Presumably only this compartment provides the factors that are required for capsid disassembly and genome release that is restricted to those capsids comprising a mature viral DNA genome.

Key words: Hepatitis B virus; Capsid; Intracellular transport; Microtubules; Nuclear pore; Importin; Nuclear localization signal; Nuclear basket

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INTRODUCTION

Eukaryotic cells are divided into different compartments and viruses have to get access to the compartment that provides the cellular machinery for replication. Unlike bacteriophages that "just" have to pass the bacterial wall and membrane all viruses that infect eukaryotic cells have to travel through the cell to reach the place of replication. Dependent upon the virus and the type of genome, the machinery of DNA replication, transcription and RNA processing may be required. Gaining access to the nucleus where these factors are found requires active transport since passive diffusion is ineffective.

Although summarized by the term "intracellular transport" one has to differentiate between the intracytoplasmic transport towards the nucleus and the passage through the nuclear envelope into the karyoplasm. Nucleic acids are not karyophilic *per se*. Thus proteins attached to the viral genome must interact with cellular factors that facilitate the different transport processes. As intracytosolic and nuclear transport are based on different mechanisms it is evident that the interacting domains on the viral proteins have to be exposed in a coordinated manner. Moreover an effective virus, meaning a virus with a good particle-infectious unit ratio, has to release its genome from surrounding proteins only after termination of the various transport steps preventing abortion of the infection process. It has to be considered that the analysis of the underlying principles is not only important for analysis of potential drug targets for treatment of individual viral infections but also for creation of efficient vectors in gene therapy.

OVERVIEW ON INTRACYTOSOLIC TRANSPORT PRINCIPLES

The need for active and directed intracytosolic transport results from the high viscosity of the cytoplasm. Protein concentrations of 170-350 mg/mL, RNA concentrations of 30 μg/mL[1] and micro compartmentalization evoke a viscosity 10 to 100 fold higher than the viscosity of water[2].

In consequence diffusion processes are enormously reduced so that only particles with diameters below 50 nm significantly diffuse[3]. Obviously such a passive movement is incompatible with efficient trafficking of organelles. Eukaryotic cells thus provide different active transport machineries that are not only used by large structures but even by small macromolecules as it was shown for the heat shock protein 90 (Hsp90)/glucocorticoid receptor β (GRβ) complex[4] and the human tumour suppressor protein p53[5]. It is thus likely that structures as the HBV capsid with a diameter of 36 nm (80 % of the capsids that show a T = 4 symmetry, 20 % exhibit a T = 3 symmetry and a diameter of 32 nm[6,7]) use the same active cellular transport pathways towards the nucleus.

Most investigations on intracytosolic transport of viruses are done by adding inhibitors to cells during infection. To prevent misinterpretations it is important to realize the variety of cellular processes that are affected. Eukaryotic cells provide two active cytoplasmic transport systems based on microfilaments (MF) and microtubules (MT). Microfilaments have diameters of 5-9 nm and are double-stranded helical polymers of the ATPase actin. They form linear bundles, 2D networks and 3D gels and are most highly concentrated underneath the plasma membrane. Microfilaments are dynamic polar structures with a fast-growing plus-end and a relatively inert, slow-growing minus-end. They stabilize the cell structure and are involved in cell movement e.g. by filopodia and lamellopodia. With a velocity of 2-6 mm/d transport via MF is slow[8,9] and generally thought to serve as the transport pathway for short distances.

Beside its role in transport actin is also involved in the internalization and/or formation of endocytic vesicles. In clathrin-mediated endocytosis a functional actin cytoskeleton enhances the internalization of the clathrin-coated vesicles but without being obligatory[10-12]. In phagocytosis and macropinocytosis local actin polymerization at the cytosolic site of the plasma membrane is required for vesicle formation as well as actin depolymerization[13-16]. Similarly, releasing of caveolin-coated vesicles into the cytoplasm depends on actin polymerization and depolymerization in caveolae-mediated endocytosis[17,18].

Microtubules are hollow cylinders of 11-13 protofilaments made of the GTPase α- and β-tubulin. MT are 25 nm in diameter and like MF they have a highly dynamic, fast-growing plus-end and a less dynamic minus-end, which is typically attached to a microtubule-organizing centre (MTOC). MT surround the nucleus and extend from the perinuclear MTOC to the cell periphery. They are thought to be the major long-range transport system[9] allowing a velocity of 3-5 mm per hour (HSV 1 capsids, retrograde)[19].

For both MT and MF two different mechanisms of transport exist (Figure 1) using polymerization (filament growth)/depolymerization (filament shrinkage) or motor proteins[20]. In the first mechanism the cargo binds dynamically *via* adapter proteins to one end of a growing or shortening filament and can be pushed or pulled in the given direction. Mitotic chromosomes for example bind at its kinetochor *via* a dynein/dynactin-complex to the plus-end of the kinetochor-MT[21-23]. Depolymerization at the plus-end as well as depolymerization at the minus-end directs the chromosomes to the minus-end that is fixed at the spindle pole. Phagosomes[24,25], macropinosomes[26,27] endosomes and lysosomes[27,28] are able to cross the cytosol by the help of a polymerizing actin tail comparable to the movement of the bacteria *listeria monocytogenes*[29-31] and the nuclear polyhedrosis virus (NPV) capsid[32-34].

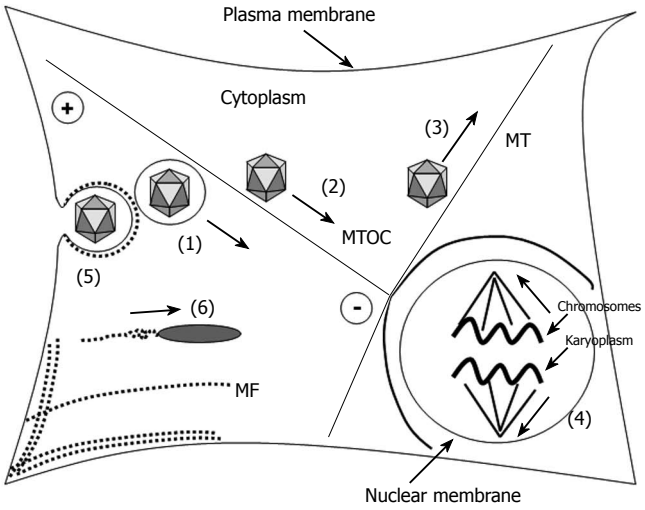

Figure 1 Participation of microtubules and microfilaments in transport processes. Transport processes are indicated as bold arrows. Microtubules (MT, bold lines) have a highly dynamic plus-end and a less dynamic minus-end that is located at the microtubule-organizing (MTOC). They participate in the transport of (1) organelles, e.g. endosomes, (2) direct retrograde transport of capsids via the dynein motor protein complex (adenovirus, HSV 1, parvoviruses), (3) direct anterograde transport of progeny HSV 1 capsids by conventional kinesin, and (4) participate in chromosome segregation upon mitosis. Microfilaments (MF), depicted as dotted lines participate (5) in separation of endocytotic vesicles from the plasma membrane and (6) *via* polymerization in transport of e.g. *Listeria* monocytogenes and nuclear polyhedrosis virus (NPV).

However the most commonly used transport strategy for macromolecules, mRNA, RNPs (ribonucleoprotein complexes), cellular organelles and vesicles along polar MF or MT involves motor protein complexes, namely myosins, kinesins and dyneins[20,35-38]. These cargo specific filament binding proteins exhibit a motor domain (ATPase) and move ATP-dependent and unidirectional along the stable filaments. Dependent upon the involved filament and transport direction different motor protein complexes are used. Class VI myosins are unconventional myosins which move towards the minus-end of MF[39]. They are involved in the transport of clathrin-coated vesicles from the plasma membrane into the inner cell[40,41]. Class I and class V myosins in contrast migrate towards the plus-end of MF[20,35]. Myosin I mediates transport of membranes whereas myosin V is responsible for the transport of organelles, as e.g. recycling endosomes towards the plasma membrane[20,42,43]. In addition myosin V can transport cargos along MT[44] thus linking both transport systems.

The MT specific kinesins can be distinguished upon the localization of the conserved motor domain into conventional kinesins, which have an N-terminal motor domain and that direct cargos in the anterograde direction

towards the plus-end of the MT (i.e. towards the cell periphery)[20,36]. They participate in the intracellular transport and localization of different cellular membrane organelles, as the extension of the ER (endoplasmatic reticulum) from the nucleus towards the cell periphery[45,46]. In addition, conventional kinesins are used in directing progeny capsids of herpes simplex virus 1 (HSV 1) in the anterograde direction[47].

Unconventional kinesins, which have a C-terminal motor domain move in contrast towards the minus-end of MT. They mediate axonal MT transport of vesicles and organelles[36].

The cytoplasmic dynein is commonly associated with the cofactor dynactin (dynactin-complex)[48] transporting cellular vesicles (endosomes and caveolin-negative vesicles[49-52] and macromolecules (e.g. tumor suppressor protein p53[5]) in the retrograde direction towards MT minus-end (the MTOC). Apparently the dynein-complex is frequently used in transport of viral capsids towards the nucleus as it was shown for adenoviruses[53-55], parvoviruses[56-59] and HSV 1 upon infection[47,60].

INTRACYTOSOLIC TRANSPORT OF HBV CAPSIDS

With the exception of NPV all viruses being analyzed so far make use of the MT transport system for their transport towards the nucleus[61]. This includes large viruses that have to travel long distances as the herpes simplex virus 1 (HSV 1;[47]) that has to be transported for centimeters between the axon end and the cell body and parvoviruses (18-26 nm)[56,58,62] that are below the diffusion limit inside the cytoplasm.

Evaluating the transport mode of hepatitis B virus is however not trivial as the entry mechanism is not fully understood. This is mainly caused by a lack of an appropriate and effective *in vitro* infection system that allows study of the early steps of the hepadnaviral life cycle. As described in "Viral and cellular determinants involved in hepadnaviral entry" hepadnaviruses enter the cells in vesicles[63] but do not need acidification for infection[64]. This step that normally occurs upon endocytosis has a major effect on viral structures as described for adeno- and parvoviruses[57,65,66]. The altered structure distinguishes capsids that have passed endocytosis from progeny capsids that are newly synthesized. The different exposed epitopes allow variant interactions so that incoming capsids are targeted to the nucleus while progeny capsids are not.

As such an acid-induced conformational change is missing in the hepadnaviral life cycle the viral capsids released from the endosomal pathway and the newly synthesized progeny capsids have the same structure. Consequently both types of capsid can participate in nuclear transport of the viral genome.

The restrictions of the experimental systems for analysis of hepadnaviral viral infections are most likely caused by an insufficient entry. It was thus a self suggested idea to replace the viral surface proteins by a lipid shell as it is done in protein transfection (lipofection)[67]. The lipids fuse with the plasma membrane and release the capsids into the cytoplasm. For studying the intracellular transport of the hepadnaviral capsid lipofection has the advantage that no cellular transport vesicles are involved.

In fact lipofection of hepatoma cells yielded in a highly productive HBV infection similar to the *in vivo* efficiency.

As lipofection is independent upon receptors high amounts of capsids could be loaded on the cells. This allows one to follow the fate of the capsids and of the viral genomes by microscopical techniques[67] showing that the capsids accumulated at the nuclear envelope after 15 min. As diffusion can be assumed to take 1 h (for calculation see[9]), these data imply a directed active capsid transport towards the nucleus. Released viral genomes occurred exclusively inside the karyoplasm suggesting site-specific disintegration at the nuclear envelope. The use of the MT depolymerizing drug nocodazole inhibits accumulation of capsids at the nuclear envelope and the release of genomes suggesting that genome liberation requires transport to the nucleus.

The microscopical finding of an active MT-mediated transport towards the nucleus was supported analysing the effect of nocodazole on the hepadnaviral life-cycle. It was demonstrated that MT are essential for formation of nuclear DNA (cccDNA that occurs only after repair of the partially double stranded DNA genome within the nucleus) and for amplification of viral DNA via synthesis of progeny mature capsids.

The use of the MT transport system was confirmed in another experimental system in which capsids were injected into the cytosol of *Xenopus laevis* oocytes. Due to the longer transport distances 30 min are required for the capsids to reach the nucleus upon injection at the pole of the oocyte opposed the nucleus. Assuming a distance of 0.5 mm that has to be bridged this period is consistent with the cytosolic transport of HSV 1 capsids that traverse the cytoplasm with 3-5 mm per hour (retrograde transport,[9]). As electron microscopy was used as read-out for capsid localization these data could show that the capsids did not bind to undefined sites of the nuclear envelope but to the nuclear pore complexes (NPC)[68]. However, when anti tubulin-antibodies were preinjected the arrival of the capsids at the NPCs was inhibited confirming that even in cells only distantly related to human hepatocytes the same transport system is used.

Although conclusive, it must be considered that all the observations described above were not done in the "authentic primary" cells and that a liver-specific factor may alter e.g. the place of genome release and lead to another transport model that does not involve the capsid. However, the generation of capsids with a translocation motif (TLM) fused to the N-terminus of the capsid protein recently gave further support[69]. These capsids were still capable of encapsidating the polymerase and the pregenome so that mature DNA capsids were generated being able to initiate an HBV infection in primary human hepatocytes. Although the mode of uptake by a TLM remains controversial-directly penetrating the plasma membrane or using transporters[70]-a clear uptake of the capsids could be observed, resulting in accumulation in the

perinuclear region. This uptake was not observed when wild-type capsids were used being contradictory to most recent results of others[71]. Irrespectively of this divergence the location of the TLM capsids at the perinuclear region where the MTOC is situated supports that the MT were used for transport towards the nucleus.

An open question not being answered for any cargo that uses MT transport for reaching the nucleus is derived from the polarity and arrangement of the MT. As their minus-end is not directly located at the NPC but attached to the MTOC the cargo must cross the distance between the MTOC and the nucleus. There are observations that even for this short gap passive diffusion is not likely: HSV 1 capsids show an equal distribution around the nucleus after infection and do not accumulate at those NPCs adjacent to the MTOC[47]. However, the mechanism of this translocation remains open.

GENERAL MECHANISM OF NUCLEAR TRANSPORT

Viruses that replicate in the nucleus of non-dividing cells have to traverse the nuclear envelope. For this reason nuclear proteins pass the nuclear pore complexes (NPCs). NPCs are large proteinaceous structures consisting of 30 different proteins[72], collectively termed nucleoporins (Nups). Nucleoporins exist in multiples copies, forming a complex of estimated 125 MD[73]. Many nucleoporins contain distinct domains of phenylalanine-glycine (FG) repeats, which mediate the main interaction between nucleoporins and soluble transport receptors. The NPC consists of a central ring-like framework with 8-fold symmetry, representing the part of the complex that is embedded in the nuclear envelope (NE) Attached to a cytoplasmic ring moiety 8 cytoplasmic filaments form an initial docking side for transport complexes. On the karyoplasmic face, 8 fibres form the cage-like structure of the nuclear basket[74]. The central framework is a ring-like assembly built around a central pore through which the exchange of macromolecules occurs. The dimension of the nuclear pore restricts complexes to a diameter of 39 nm including their shell of transport receptors[68]; a size that is exceeded by most viruses or subviral particles.

NPCs regulate the traffic of proteins and nucleic acids into and out of the nucleus[75]. Substrates smaller than roughly 9 nm in size, including ions, metabolites and proteins, travel through the NPC in a diffusion-controlled and energy independent manner[76].

Most nuclear cargos exhibit signals that interact with nuclear transport receptors of the importin β superfamily, comprising importins and transportins. All members of this family exhibit an N-terminal RanGTP-binding domain which is important for dissociating receptor and cargo (reviewed by[77]). There is a variety of different signals that are recognized as exemplified by the M9 domain, bound by transportin, polypeptides of basic amino acids that represent an importin β binding domain (IBB) and "classical" nuclear localization signals (NLSs) that show the consensus sequence K(K/R)X(K/R)[78]. The classical NLS does not directly bind to the transport-mediating receptor importin β (Imp β) but requires an adapter molecule, importin α (Imp α), which connects NLS and - *via* its IBB- Imp β.

The driving force of nuclear import and export is determined by the different concentrations of RanGTP in the nucleus versus the cytoplasm. RanGTP that is enriched in the karyoplasm, interacts with the transport receptors of the import complex, leading to dissociation of cargo and receptor. While the cargo diffuses deeper into the karyoplasm, the RanGTP-receptor complex becomes exported to the cytoplasm.

Hepadnaviral genomes have to enter the nucleoplasm for replication. As hepatocytes are terminally differentiated cells that do not divide they cannot wait until the cell undergoes mitosis as most retroviruses-excluding HIV-do. As karyophilic proteins the capsids use the nuclear pore complex to get for access to the nucleoplasm.

NUCLEAR IMPORT OF HEPADNAVIRAL GENOMES

Nucleic acids are not karyophilic *per se*. Therefore one or more proteins attached to the genome must interact with cellular nuclear import receptors. In case of the hepadnaviruses three models, each involving a different mediator, may play a crucial role in the nuclear import of the HBV genome: (1) The viral polymerase of the Hepatitis B virus. The enzyme is covalently attached to the viral genome and probably contains a hidden NLS. Expression of the polymerase in eukaryotic cells revealed that the protein stays cytoplasmic[79] but extraction of this complex from mature virions showed that it enters the nucleus[80]. However, the procedure of extraction requires harsh treatment and thus structure altering methods. (2) Some of the heat shock proteins as Hsc70 or Hsp90 activating the polymerase[81-84] and (3) the capsid proteins surrounding the viral replication complex.

The lipofection experiments described above, show that released viral DNA is exclusively present within the nucleus. The release is combined with the accumulation of capsids at the nuclear envelope. It thus has to be concluded that if the polymerase mediates nuclear import the release of the import complex must occur at the nuclear envelope, probably after docking of the capsids to the nuclear pore. Examples for such a pathway are the HSV 1 capsid that becomes opened upon the interaction of a penton with the NPC and adenovirus 2 that releases the complex of DNA and associated proteins at the pore[47,85-88].

The polymerase-associated heat shock proteins may act in a similar manner. For example, the interaction with Hsc70 that exhibits a nuclear transport capacity is established[84]. The capsid-nuclear envelope interaction was more extensively investigated in cell biological assays and in microinjection experiments using oocytes of *Xenopus laevis*. Another experimental design is based on Digitonin permeabilized cells most commonly used in studying nuclear import reactions in detail. Digitonin permeabilizes only cholesterol containing membranes as e.g. the plasma membrane and membranes of mitochondria. Other membranes as the nuclear and ER membrane remain

unaffected[89]. In general adhesive cells are analysed under conditions where they attach to the surface of a glass cover slip thus the Digitonin has to be removed by washing steps. The washing removes the soluble cytosolic proteins (and some small nuclear proteins that rapidly diffuse out of the nucleus) including the cellular nuclear transport factors. Consequently these factors have to be replaced either by addition of selected import factors or in form of a cytosolic extract. However, as the nuclear transport capacity is conserved this process can be transferred to the *in vivo* situation presupposed that the subjected cargo with its modification is physiological.

Microinjection in the cytoplasm is another established technique. For electron microscopy *Xenopus laevis* oocytes are frequently used as the huge dimensions of the nucleus allow the analysis of multiple sections. Since nuclear import is phylogenetically well conserved the results are transferable to other cell types as long as no embryogenesis related processes are affected.

These assays and biochemical analyses have been used to analyse the nuclear import of the hepadnaviral genome in more detail. Using permeabilized cells it was shown that the HBV capsid protein contains an NLS within its C-terminal domain. This domain is hidden in the lumen of RNA-containing capsids expressed in *E. coli* and in eukaryotically expressed capsids devoid of the polymerase[90,91]. These capsids failed to interact with nuclei of permeabilized cells. However, the exposure of the C-terminus on the capsid surface was shown to be linked to genome maturation as *in vitro* studies revealed[90]. Interestingly, the exposure could be initiated in RNA-containing capsids expressed in *E. coli* when the C-terminus was *in vitro* phosphorylated by protein kinase C[92] or protein kinase A[93]. These observations strengthen the idea that phosphorylation and genome maturation are linked. The impact of the exposed C-termini for nuclear pore complex association becomes evident by cleavage experiments in which the C-termini were removed from mature capsids followed by subjection of the capsids to Digitonin-permeabilized cells. According with the hypothesis of a capsid-mediated NPC docking the capsids and the necessity of exposed NLS to the digested capsids failed to interact with the nuclei. Consistently with the identification of an NLS on the capsid protein importin β was found to be the mediator of NPC interaction, requiring importin α as an adapter protein.

Most fascinating is the different import behaviour between capsids that have undergone genome maturation to a different extent. Capsids with an immature DNA genome interacted with the NPCs but remained associated with the pores while subjecting mature capsids to the permeabilized cells resulted in a nuclear capsid stain and released viral genomes within the nucleus.

Based on these data all three import models-polymerase, heat shock proteins or capsid mediation-could be true. The mature capsids could interact with the NPC releasing their genome followed by import of the genome mediated by the polymerase or heat shock proteins. The dissociated capsid subunits enter the nucleus as they apparently do in HBV infected individuals. The immature capsids could have been just more stable thus failing to disintegrate and to release the genome.

Alternatively, only the mature capsids that expose more NLS may have become surrounded by enough nuclear transport receptors to pass a hydrophobic mesh caused by the crosslinking of hydrophobe FxFG repeats of nucleoporins. Only these capsids interact with the factors required for genome release.

To differentiate between the models further investigations were initiated to follow the fate of the capsids at the NPC. Surprisingly, electron microscopy after microinjection into *Xenopus laevis* oocytes showed that not only the mature capsids passed the pore and entered the nuclear basket but also immature capsids that apparently failed to diffuse deeper into the karyoplasm. Consistent with the experiments in permeabilized cells, RNA containing capsids did not interact with the NPCs.

For the model of nuclear import these findings implied that apparently the capsids mediated the passage through the nuclear pore into the nuclear basket. Here only the mature capsids disintegrated while the immature capsid stay arrested. Hypothetically this arrest can increase efficiency of HBV infection, assuming that genome maturation proceeds in these capsids.

BEYOND NUCLEAR IMPORT

Hepadnaviral polymerases can only successfully synthesize the full length viral DNA when interacting with the capsid proteins. In fact, recent studies on the phosphorylation sites of the HBV capsid protein show that one serine residue (Ser 157) has to be phosphorylated for pregenome packaging while serine 164 is required for allowing DNA synthesis[94,95]. The highly efficient infections by hepadnaviruses thus imply that the genome release is well coordinated in that only capsids with a mature genome disintegrate. The factor however that apparently is only present within the nucleus remains unknown. Incubations of mature capsids with nuclear extracts failed to induce significant genome release while permeabilized hepatoma cells only require minutes to release thousands of HBV genomes per nucleus. Furthermore a liver-specific factor must be assumed as genome release is more efficient in permeabilized hepatoma- than in HeLa cells. *In vivo*, this conclusion is supported by observations of Untergasser *et al*[96], who observed that cccDNA generation does not occur upon infection of dendritic cells with chimera of HBV and adenovirus.

Another open question is raised by the high copy number of empty capsids found in the nuclei of HBV infected hepatocytes or in hepatocytes of mice that are transgenic for HBV. Apparently they are not derived from capsids that have transported the genome into the nucleus as the half-life of the genome-as different data in the literature are (ranging from 3 d[97], 55 d[98] to a non-relevant degradation[99])-do not explain their frequent abundance. However, the capsid protein is strongly over expressed with regard to the number of viruses and capsids that participate in nuclear entry of the viral genome. Apparently capsid proteins have a nuclear transport capacity by their NLS, which is not hidden as long as

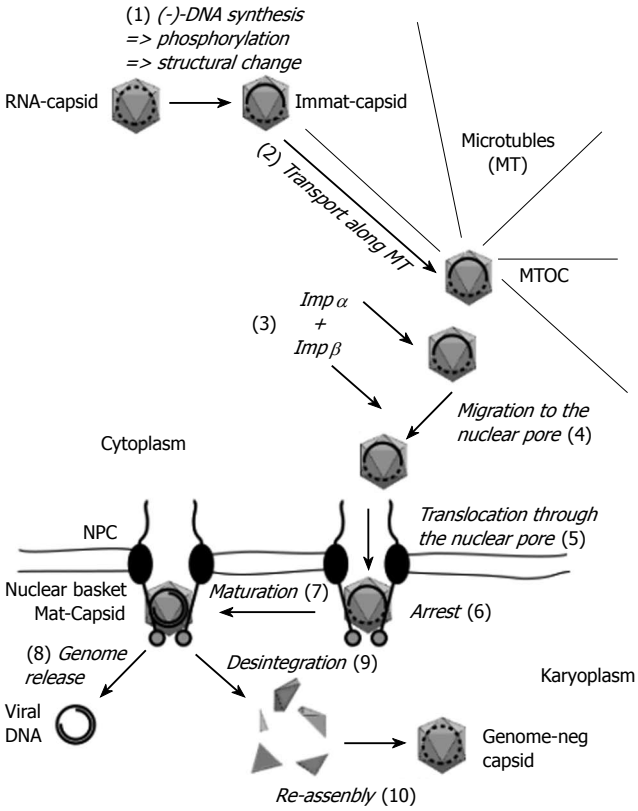

Figure 2 Hepadnaviral trafficking within the cell. Capsids are drawn as grey icosahedra. Immat-Capsid, immature capsid, Mat-Capsid, mature capsid. The nucleic acid found within the capsids is depicted as a dotted line (RNA) or a full line (DNA). The arrows present movements (2, 4, 5, 8) or changes of the capsid (1, 7, 9, 10). Further explanations are given in the text.

they are not assembled to particles. Based on the results described above one must thus conclude that not capsids but the supernumerous capsid proteins or their assembly intermediates are imported. In accordance with consistent biochemical data of all groups studying the assembly process, it must be proposed that the capsid proteins do not need any other protein for assembly. The only driving force is the affinity to each other being supported by their interaction with other components as e.g. RNA. It is thus likely that after import of high numbers of capsid proteins into the nucleus the threshold concentration is reached resulting in rapid assembly to particles.

Nonetheless, one has to ask why the capsid proteins do not become arrested in the nuclear basket as the immature capsids. One can assume that assembled capsids can interact with at least eight basket proteins, most likely the nucleoporin 153[100], that are arresting the capsid. Although the answer remains experimentally open a protein monomer in contrast is restricted to one interaction that is in competition with the thousands of proteins that pass the nuclear pore every second.

The meaning of the nuclear assembly is unsolved. It might be just a side effect caused by the intrinsic assembly ability of the capsid. However, as the capsid proteins interact preferentially with single stranded nucleic acids the assembly may prevent interference with the cellular transcription and RNA export machinery thus reducing toxicity of the virus and ensuring a long life of the infected cell for persistent virus production.

SUMMARIZING THE TRAFFICKING OF HEPADNAVIRAL CAPSID AND GENOME

The summary of the current knowledge on the nuclear import of the hepadnaviral genome is depicted in Figure 2. It must be considered however that there are several elements as e.g. the co-ordination of the transport processes that are yet unknown. (1) Upon genome maturation and phosphorylation the hepadnaviral capsids undergo a structural change that leads to exposure of increasing numbers of the C-termini that are part of the capsid protein. (2) The capsids are transported towards the microtubule-organizing centre (MTOC), which is located at the perinuclear region. (3) As the exposed C-terminal domain exhibits a nuclear localization signal the probability of an interaction with the adaptor protein importin α increases. As in the physiological import of karyophilic proteins this complex is bound by importin β. Whether this acquisition already occurs during the MT-mediated transport or (4) during the unknown passage from the MTOC to the nucleus remains open. (5) Importin β next facilitates docking to the cytosolic fibres of the nuclear pore and translocation of the complex into the nuclear basket. (6) After dissociation of the nuclear import receptors from the capsid the capsid most likely interacts with a protein of the basket. (7) While immature capsids stay arrested and may continue with genome maturation, (8) mature capsids that are less stable release the genome with the associated proteins into the nucleus where genome repair takes place. (9) Supernumerous capsid proteins that result from disintegration can diffuse deeper into the karyoplasm where (10) they re-assemble after the capsid protein concentration reaches the threshold concentration for assembly.

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TOPIC HIGHLIGHT

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Hepatitis B virus replication

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Abstract

Hepadnaviruses, including human hepatitis B virus (HBV), replicate through reverse transcription of an RNA intermediate, the pregenomic RNA (pgRNA). Despite this kinship to retroviruses, there are fundamental differences beyond the fact that hepadnavirions contain DNA instead of RNA. Most peculiar is the initiation of reverse transcription: it occurs by protein-priming, is strictly committed to using an RNA hairpin on the pgRNA, ε, as template, and depends on cellular chaperones; moreover, proper replication can apparently occur only in the specialized environment of intact nucleocapsids. This complexity has hampered an in-depth mechanistic understanding. The recent successful reconstitution in the test tube of active replication initiation complexes from purified components, for duck HBV (DHBV), now allows for the analysis of the biochemistry of hepadnaviral replication at the molecular level. Here we review the current state of knowledge at all steps of the hepadnaviral genome replication cycle, with emphasis on new insights that turned up by the use of such cell-free systems. At this time, they can, unfortunately, not be complemented by three-dimensional structural information on the involved components. However, at least for the ε RNA element such information is emerging, raising expectations that combining biophysics with biochemistry and genetics will soon provide a powerful integrated approach for solving the many outstanding questions. The ultimate, though most challenging goal, will be to visualize the hepadnaviral reverse transcriptase in the act of synthesizing DNA, which will also have strong implications for drug development.

Key words: Chaperone-mediated reverse transcription; HBV cccDNA; Hepadnavirus, P protein; Pregenomic RNA; Protein-priming; reverse transcriptase; RNA encapsidation signal

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INTRODUCTION

A hallmark of hepatitis B virus (HBV) replication is protein-primed reverse transcription, related to, but mechanistically distinct from, retroviral replication. This review will focus on the various genome transformations that occur during the replication cycle, with emphasis on the cis-elements on the one hand, and the trans-acting factors known or thought to be involved on the other. A general outline of the chain of events during hepadnaviral replication has been established, mainly using transfection of cloned HBV DNA into a few suitable cell lines, and has been the subject of several reviews[1-6]. Much less clear than what happens is, however, how these various steps are achieved and regulated.

Several features of HBV make resolving such mechanistic questions exquisitely difficult: Foremost, until recently, no *in vitro* systems were available to reconstitute individual replication steps under controlled conditions; conversely, there are no simple infection systems to follow the consequences of mutation-induced *in vitro* phenotypes in the context of authentic virus replication. Secondly, for various of the viral components and mechanisms no precedents exist such that drawing conclusions *in silico*, or by analogy to experimentally more tractable systems, is of limited value. Finally, there is a general lack of structural information on the involved viral factors, in particular on P protein, the viral reverse transcriptase. Fortunately, duck HBV (DHBV), the type member of the *avihepadnaviridae*, provides a valuable model system[7]. Although it differs from HBV in some details, the general features of genome replication are highly conserved; in fact, that hepadnaviruses replicate through reverse transcription has been established with DHBV[8]. Beyond allowing feasible *in vitro* and *in vivo* infection studies, the crucial initiation step of DHBV reverse transcription has recently been

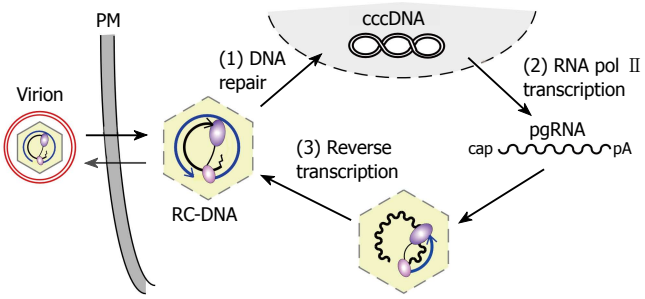

Figure 1 Replication cycle of the hepadnaviral genome. Enveloped virions infect the cell, releasing RC-DNA containing nucleocapsids into the cytoplasm. RC-DNA is transported to the nucleus, and repaired to form cccDNA (1). Transcription of cccDNA by RNA polymerase Ⅱ (2) produces, amongst other transcripts (not shown), pgRNA. pgRNA is encapsidated, together with P protein, and reverse transcribed inside the nucleocapsid (3). (+)-DNA synthesis from the (-)-DNA template generates new RC-DNA. New cycles lead to intracellular cccDNA amplification; alternatively, the RC-DNA containing nucleocapsids are enveloped and released as virions. PM, plasma membrane.

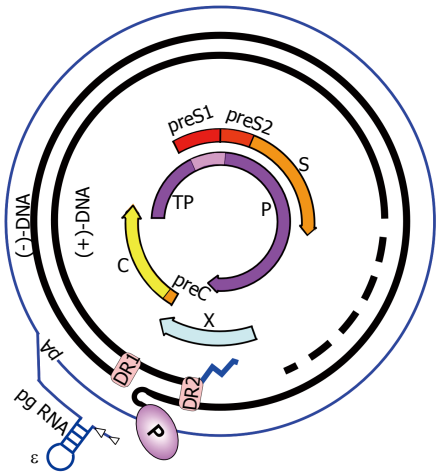

Figure 2 HBV genome organization. The partially double-stranded, circular RC-DNA is indicated by thick black lines, with P covalently linked to the 5′ end of the (-)-DNA, and the RNA primer (zigzag line) at the 5′ end of (+)-DNA. The dashed part symbolizes the heterogeneous lengths of the (+)-strands. DR1 and DR2 are the direct repeats. The outer circle symbolizes the terminally redundant pgRNA with ε close to the 5′ end, and the poly-A tail at the 3′ end. The precore mRNA is nearly identical, except it starts slightly upstream. The relative positions of the open reading frames for core (C), P, preS/S, and X are shown inside. TP, Terminal protein domain of P.

reconstituted in the test tube. On many occasions, we will therefore refer to data obtained with this model virus, and add what is available for human HBV.

OVERVIEW OVER THE HEPADNAVIRAL GENOME REPLICATION CYCLE

Replication of the hepadnaviral genome can broadly be divided into three phases (Figure 1): (1) Infectious virions contain in their inner icosahedral core the genome as a partially double-stranded, circular but not covalently closed DNA of about 3.2 kb in length (relaxed circular, or RC-DNA); (2) upon infection, the RC-DNA is converted, inside the host cell nucleus, into a plasmid-like covalently closed circular DNA (cccDNA); (3) from the cccDNA, several genomic and subgenomic RNAs are transcribed by cellular RNA polymerase Ⅱ; of these, the pregenomic RNA (pgRNA) is selectively packaged into progeny capsids and is reverse transcribed by the co-packaged P protein into new RC-DNA genomes. Matured RC-DNA containing-but not immature RNA containing-nucleocapsids can be used for intracellular cccDNA amplification, or be enveloped and released from the cell as progeny virions. Below we discuss these genome conversions, with emphasis on the reverse transcription step, and particularly its unique initiation mechanism.

RC-DNA TO cccDNA CONVERSION

Persistent viral infections require that the viral genome be present in the infected cell in a stable form that is not lost during cell division, and which therefore can be used for the continuous production of progeny genomes. Many DNA virus genomes harbor replication origins allowing them to directly exploit the cellular replication machinery for amplification; retroviruses integrate a terminally duplicated linear version of their DNA genome into the host genome, such that it is replicated along with the chromosomes. For hepadnaviruses, the genome persists, instead, as a nuclear, episomal covalently closed circle, i.e. the cccDNA. The circular form obviates the need for terminal redundancy in that, on the circle, the core promoter/enhancer Ⅱ is placed in front of the start sites for the genomic RNAs; conversely, in typical HBV expression vectors the cloned hepadnaviral DNA is interrupted by plasmid sequences, such that the control regions need to be duplicated[9].

Distinct features of the RC-DNA (Figure 2) are (1), only the (-)-DNA strand (with opposite polarity to the mRNAs) is complete whereas the (+)-strands comprise a cohort of less than full-length molecules; (2), the 5′ end of the (-)-DNA is covalently linked to P protein; (3) the 5′ end of the (+)-strand consists of an RNA oligonucleotide, derived from the pgRNA, which served as the primer for (+)-strand synthesis. For cccDNA formation, all these modifications need to be removed, and both strands need to be covalently ligated.

How this is achieved is not well understood, in particular because unambiguous cccDNA detection in the presence of excess RC-DNA is not technically trivial, not even by PCR approaches[10]. Also, whenever overlength HBV constructs are involved, e.g. upon transduction of cells with HBV carrying adeno-[11] or baculoviruses[12], caution is indicated because homologous recombination could provide for a virus replication independent mechanism of cccDNA formation.

Direct infection avoids this problem but as yet only limited data are available. Earlier evidence obtained with the DHBV-primary duck hepatocyte (PDH) infection system suggested that the activity of the viral P protein is not required for cccDNA generation[13]; however, more recent data using infection with HBV of primary tupaia hepatocytes[14,15] indicate that reverse transcriptase inhibitors can strongly reduce, though not completely block, cccDNA formation[16]. This would be in line with a

role for P protein in the process, probably the completion of the (+)-strands. However, the other steps towards cccDNA are likely to require cellular activities, as suggested by the apparent lack of cccDNA, despite formation of infectious virions, in non-authentic host cells such as hepatocytes from HBV transgenic mice[17]. However, low level cccDNA generation in mice has been reported in certain experimental settings[18,19].

Naturally hepadnavirus infected hepatocytes contain up to 50 or more copies of cccDNA[20], probably in the form of histone-containing minichromosomes[21]. This amplification occurs intracellularly[22,23] in that progeny RC-DNA genomes, like the initially infecting genome, undergo nuclear import and cccDNA conversion (Figure 1). Together with the long half-life (30 to 60 d for DHBV[20,24]), this ensures that cccDNA is not lost during cell division, and even persists during effective antiviral therapy[25]. Nuclear transport, and enveloped virion formation appear to be competing events, such that late in infection, when sufficient amounts of envelope proteins become available, further cccDNA amplification ceases[26]. A recent analysis on the single cell level revealed that the cccDNA copy number in DHBV infected PDH is not uniform and fluctuates over time; about 90% of the cells contained between 1 and 17 copies, the rest more than 17 copies[27,28]. Cells with only one copy may allow segregation of daughter cells containing no cccDNA. This could explain the apparent, though probably less than complete[29], disappearance of cccDNA during spontaneous clearance of HBV infection. However, therapeutic cccDNA elimination from the chronically infected liver remains a major issue even with the latest generation antivirals[30,31]; further investigations into the mechanism of cccDNA formation, and possibly breakdown, are clearly warranted.

FROM cccDNA TO PREGENOMIC RNA

All known hepadnaviral RNAs, i.e. the subgenomic RNAs as well as the greater-than-genome length pgRNA and precore RNA, are transcribed by cellular RNA polymerase Ⅱ (the enzyme responsible for cellular mRNA synthesis) using cccDNA as the template. All contain 5′ cap structures, all are 3′ terminally poly-adenylated at a common site, and all serve as mRNAs for viral gene products. Spliced transcripts do exist, and even can be packaged into progeny virions[32,33], yet their functional role is still obscure although for DHBV splice site mutants appear to have defects[34]. The extent of splicing appears to be controlled in DHBV by a long-range RNA secondary structure[35], and in HBV by the post-transcriptional regulatory element (PRE)[36].

The transcript relevant for virus replication is the pregenomic RNA (pgRNA), encompassing the entire genome length plus a terminal reduncancy of, in HBV, about 120 nt that contains a second copy each of the direct repeat 1 (DR1) and the ε signal, plus the poly-A tail (Figure 3A). The pgRNA starts immediately after the precore initiator codon. Its first essential role is that as mRNA for the core protein and the reverse transcriptase;

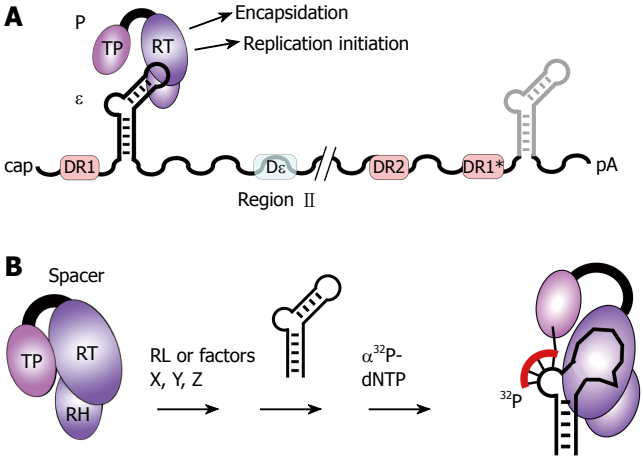

Figure 3 The pgRNA as substrate for P protein. **A**: pgRNA organization. The pgRNA is shown with some major cis-elements, i.e. ε (hairpin structure), DR1, DR2, and DR1*; DHBV, but not HBV, requires for encapsidation an additional cis-element (Dε region Ⅱ). Binding of P protein to 5′ ε, but not 3′ ε, initiates pgRNA encapsidation and replication; **B**: In vitro priming assay. P protein with its Terminal protein (TP), reverse transcriptase (RT), and RNase H (RH) domains can be activated by reticulocyte lysate (RL), or individual factors (X, Y, Z), to bind ε; ε may be supplied as small RNA covering just the hairpin structure. Upon addition of α^{32}P-dNTPs, P uses ε as template to copy 3 to 4 nt from the ε bulge region; by the covalent linkage of the 5′ nt to a tyrosine residue in TP, P protein becomes radioactively labeled, providing a sensitive assay for activity. In vitro priming does thus far not work with human HBV P protein and ε RNA.

unlike retroviral Gag-Pol proteins, P is expressed as a separate polypeptide by an unconventional mechanism[37]. Secondly, pgRNA is the template for generation of new DNA genomes by reverse transcription. The 5′ terminally extended precore RNA contains the initiator codon of the preC region and gives rise to the 25 kDa precore precursor protein of secreted 17 kDa HBeAg. It is unsuited as a pregenome, and is excluded from participating in replication on the level of encapsidation[38].

Of note, RNA polymerase Ⅱ transcription could contribute, in addition to reverse transcription, to hepadnaviral genome variability. Its extent is, however, unclear because the error rate of RNA polymerase Ⅱ is not firmly established and strongly affected by some of the subunits in the holo-enzyme complex[39,40].

CAPSID-ASSISTED REVERSE TRANSCRIPTION OF pgRNA

The next crucial step in hepadnaviral replication is the specific packaging of pgRNA, plus the reverse transcriptase, into newly forming capsids. Key actors are cis-elements on the pgRNA, most notably the encapsidation signal ε, and P protein which binds to ε. This interaction, in a still poorly understood fashion, mediates recruitment of core protein dimers and thus leads to packaging of the pgRNA-P complex. Remarkably, the precore RNA is not packaged although it contains all of the sequence comprising the pgRNA. Likely, active translation from the upstream precore ATG through the ε sequence prevents the P-ε interaction[38]. This implies, in turn, that P binding to ε on the pgRNA interferes with

translation of the core ORF, and evidence supporting this view has been forwarded for DHBV[41].

Once pgRNA and P protein are being encapsidated a second key function of the P-ε interaction is brought to bear, namely the initiation of reverse transcription. At this stage the first DNA nucleotide (nt) is covalently linked to P protein, extended into a complete (-) strand DNA, and (+) strand DNA synthesis ensues, giving rise to a new molecule of RC-DNA; the various immature DNA forms *in statu nascendi* are termed replicative intermediates, visible as a broad multiple band pattern in Southern blots from intracellular DNA.

Completion of reverse transcription before leaving the cell marks another fundamental difference to retroviruses which, except for foamyviruses[42], are secreted as virions containing two copies of RNA; an evolutionary rationale may be that DNA synthesis in the infected cell allows hepadnaviruses the intracellular genome amplification cycle as an alternative to proviral integration for stable virus genome propagation. Yet another difference is that hepadnaviral reverse transcription occurs largely, if not exclusively, in intact nucleocapsids rather than in retrovirus-like reverse transcription complexes which typically lack a continuous core shell[43,44].

Although compartmentalization of the genome amplification process is emerging as a common theme of several classes of RNA and reverse transcribing viruses[45], the hepadnaviral strategy appears as an extreme variation, considering the space restrictions imposed on the replicating complex inside the geometrically defined capsid lumen. Either the RT must slide along the entire 3 kb template, or the template must be pulled through the RT´s active site; at the same time the nucleic acid is most likely in contact with the Arg-rich C termini of the core protein subunits. In fact, capsids from core protein variants lacking part of this region still package pgRNA but appear unable to produce full-length RC-DNA; instead they preferentially reverse transcribe the fraction of spliced genomic RNAs[46,47]. Phosphorylation/dephosphorylation events at the S and T residues in the nucleic acid binding domain clearly accompany genome maturation[48], and mutations affecting the core phosphorylation status[46,49] can influence DNA synthesis in various ways. Outside capsids, or in the absence of core protein, apparently no full-length DNA can be formed.

Together these observations support the view of the capsid as a dynamic replication machine. A recent cryo electron microscopic comparison between recombinant, bacterial RNA containing capsids and authentic genome harboring nucleocapsids indeed showed some structural differences[50]; a complementary study on mutant recombinant cores revealed an enormous flexibility of the capsid structure[51]. Switching between different structural states could well be involved in supporting the progress of DNA synthesis.

The heterogeneous lengths of the (+)-strand DNAs generated by capsid-assisted reverse transcription may result from a non-identical supply of dNTPs inside individual nucleocapsids at the moment of their enclosure by the dNTP-impermeable envelope. This predicts that intracellular cores produced in the absence of envelopment should contain further extended (+)-DNAs. Alternatively, space restrictions in the capsid lumen could prevent (+)-strand DNA completion; in this view, further (+)-strand elongation after infection of a new cell might destabilize the nucleocapsid and thus be involved in genome uncoating.

CIS-ELEMENTS AND TRANSACTING FACTORS ESSENTIAL FOR HEPADNAVIRAL REPLICATION

The absolute requirements for replication are a template nucleic acid, plus an enzyme that is able to read the template information and use it for synthesis of a complementary nucleic acid. Clearly, these basic components are the pgRNA (and later the (-)-strand DNA) and P protein. However, generation of a functional genome also depends critically on precise start and end points, provided by cis-elements on the template (Figure 3A); a further specialty of P protein is its strict dependence, for activity, on cellular factors, namely heat shock proteins (Hsp´s) or chaperones (see below).

Most initial knowledge on the cis- and trans-factors involved in hepadnaviral replication was derived by transient transfection of mutant viral genomes into stable hepatoma cell lines. This system faithfully mimics several of the authentic replication steps, however its complexity precluded elucidation of many mechanistic details. The recent establishment of *in vitro* systems, culminating in the complete *in vitro* reconstitution of active DHBV replication initiation complexes from purified components[52-54], overcame these restrictions (see below). It should be noted, however, that such minimal systems have their own limitations; for instance, the crucial role of the proper capsid environment for RC-DNA formation has not yet been modeled *in vitro*, and HBV P protein has thus far proven refractory to *in vitro* reconstitution of DNA synthesis activity. Hence which of the two approaches is more useful depends on the question addressed, and where ever possible they should be combined.

STRUCTURE OF THE RNA ENCAPSIDATION SIGNAL ε

The best understood cis-element on the hepdnaviral pgRNA is ε (Figure 4), a stem-loop structure initially defined as the sequence from the 5´ end of HBV pgRNA that P-dependently mediated encapsidation of pgRNA, and also of heterologous transcripts to which it was fused[55]; later, the P-ε interaction was found to constitute the first step in initiation of reverse transcription[56-58]; hence ε also acts as the replication origin (Figure 3A).

The hairpin structure of ε (Figure 4A) was confirmed by secondary structure analyses[59,60], and its importance was established by following the effects of site-directed mutants on the packaging efficacy in transfected cells[59-63]. Furthermore, the ε sequence is highly conserved in other mammalian hepadnaviruses, as well as between different

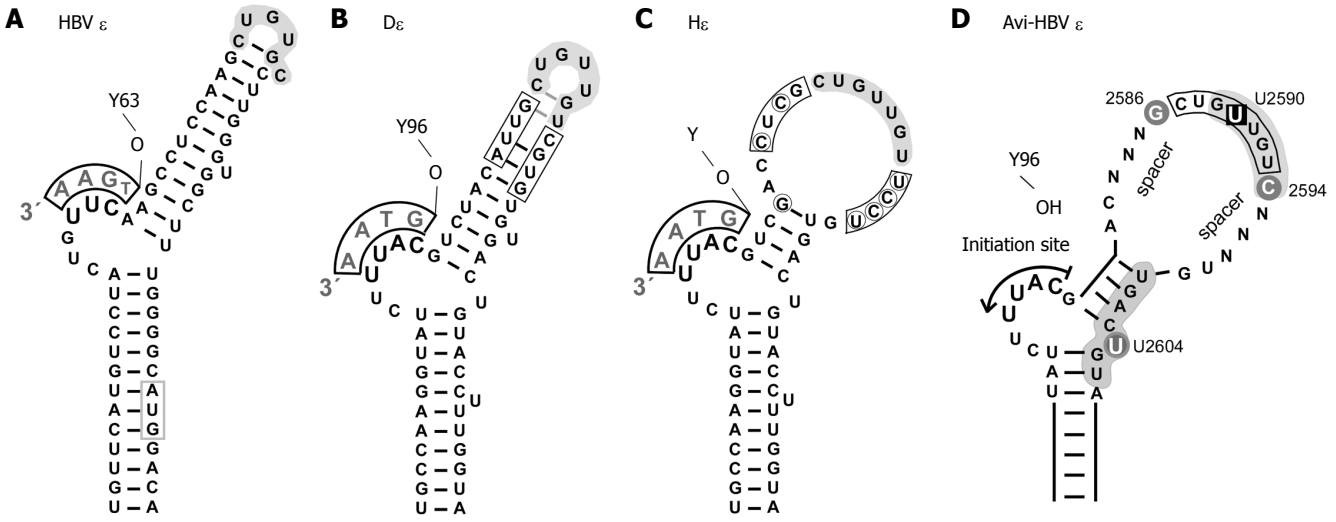

Figure 4 Secondary structures of hepadnaviral ε signals. **A**: HBV ε. The entire hairpin, including in the upper stem region, is stably base-paired; formation of a stable tri-loop, as indicated, is confirmed by direct NMR analysis[68]. The conventional annotation for the loop sequence is high-lighted by grey shading. The bulge-templated DNA oligonucleotide and the priming Y63 are indicated; **B**: DHBV ε. The overall 2D structure is similar to that of HBV ε, including a largely base-paired upper stem[69], as confirmed by preliminary NMR data. The boxed positions were randomized in a SELEX approach, and P binding individuals were selected from the corresponding RNA library[71]; **C**: HHBV ε (Hε). Hε shows substantial sequence variation to **D**: (encircled nt), leading to a largely open upper stem structure; **D**: Generalized secondary structure of an avihepadnaviral ε signal. The scheme summarizes major determinants for productive interaction with P protein[71]. Grey background indicates nt positions that are probably in contact with protein. The bulge and its immediate vicinity, particularly the tip of the lower stem, are essential for P binding whereas the loop appears critically involved in the transition to a productive initiation complex[109]. U2590 > C or U2604 > G mutations abrogate, or strongly reduce, P binding whereas G2586 and C2594 do not affect binding but are important for priming. The major role of the nt termed N may be to provide a proper spacing to the bulge element; their sequence is not important as long as formation of highly stable base-pairing is prevented.

HBV isolates[64,65]. An illustrating example are the HBV precore variants in which HBeAg synthesis is prevented by stop mutations in the ε overlapping precore region. The only mutations causing this phenotype found in nature are those which maintain a stable ε secondary structure[66,67].

It should be noted that RNA secondary structure (2D) analysis provides a mere description of the base-pairing pattern. Hence a true mechanistic understanding requires knowledge of the three-dimensional (3D) structure. The structure of the HBV ε upper stem, recently solved by nuclear magnetic resonance (NMR) techniques[68], revealed that the apical loop consists actually of only 3 nt (as in Figure 4A). This analysis is currently extended to determining the 3D structure of the entire stem-loop, which seems to form a nearly contiguous double-helix (S. Flodell, M. Petersen, F. Girard, J. Zdunek, K. Kidd-Ljunggren, J. Schleucher and S. Wijmenga; submitted); of particular interest will be the structure of the bulge region which is the template for the first few nt of (-)-DNA (see below). Thermodynamic calculations as well as experimental melting curves indicate that the entire HBV ε structure, including the upper stem, is highly stable. Notably, however, in DHBV rearranging this structure is necessary for the RNA to act as a template (see below). Determining the ε RNA structure in the complex with P protein is therefore the ultimate, yet demanding, goal.

Despite limited sequence homology to HBV ε, DHBV ε (Dε) has a similar secondary structure[69] with a bulge and an apical loop (Figure 4B), which suggested that this structure is a common trait of all hepadnaviruses. Surprisingly, the corresponding ε signal (Hε) from the related heron HBV (HHBV) has much reduced base-pairing in the upper stem region (Figure 4C), yet it functionally interacts with DHBV P protein *in vitro* whereas HBV ε does not[70].

Selection, from a library of RNAs with partially randomized upper stems for individuals able to bind to *in vitro* translated DHBV P protein (see Cell-free reconstitution of hepadnaviral replication initiation) revealed the absence of base-pairing in the upper stem region as a common theme (Figure 4D)[71]. Some of the selected P-binding RNAs supported *in vitro* priming while others did not, confirming that a productive interaction, leading to DNA synthesis, requires more than mere physical binding (see below). Hence for avihepadnaviral ε signals an open upper stem structure is beneficial for both physical and productive binding to P; in fact, deliberate Dε stabilization strongly reduces P binding[71]. This is one line of evidence that structural reshaping of the upper stem is a crucial event for initiation of DNA synthesis. Independent support comes from preliminary NMR and melting curve data for wt Dε RNA, according to which the upper stem is the least stable region (F. Girard, O. Ottink, M. Tessari and S. Wijmenga, to be submitted). This marked difference to the highly stable HBV ε structure may be related to the *in vitro* inactivity of the HBV P protein[72].

The functional consequences of mutations affecting ε and other recognized cis-elements are discussed in more detail below. Of note is, however, whereas the HBV ε stem-loop alone is sufficient to mediate encapsidation of heterologous RNAs[62,73], DHBV pgRNA encapsidation requires additional elements. "Region Ⅱ"[74], several hundred nt downstream of 5′ ε, and the intervening sequence[75] may be operative *via* long-range RNA

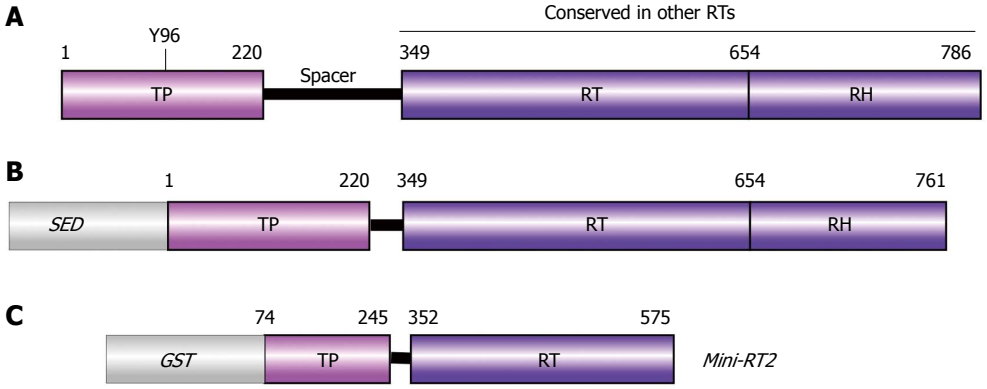

Figure 5 Domain structure of P protein. **A**: Authentic DHBV P protein. Numbers are aa positions for DHBV P protein. The priming Tyr residue Y96 is indicated; **B**: Typical recombinant P protein construct. For solubility, a heterologous solubility enhancing domain (*SED*) such as NusA, GrpE, or *GST* is required, and a short stretch of C terminal aa must be removed. Deletion of the spacer has no negative effects on *in vitro* activity; **C**: Mini-RT2. This heavily truncated recombinant DHBV P protein requires mild detergent, but no chaperones for priming activity.

interactions[76]; why a similar element is dispensable for HBV pgRNA packaging is unclear.

Even HBV ε, however, does not act as a completely autonomous encapsidation element. The 3´ copy of ε cannot substitute for 5´ ε in the context of otherwise authentic pgRNA[77] (although its DHBV counterpart is useable for DHBV P interaction in *in vitro* translation;[78]). Furthermore, 5´ ε mediates encapsidation of heterologous RNAs only up to a limited distance from the 5´ end (about 65 nt), and seems to require the 5´ cap structure[79]. Hence the 5´ cap and factors bound to it appear to have a role in the process, possibly in concert with the 3´ poly-A tail and its associated cellular proteins[80]; this may explain why attempts to reconstitute encapsidation by simultaneous *in vitro* translation of P and core protein from uncapped, non-poly-adenylated RNAs in reticulocyte lysate (RL) were thus far unsuccessful.

An unexplored issue is whether the ε hairpin, or other base-paired regions on the pgRNA, would be substrates for cellular dsRNA recognizing systems such as Toll-like receptor 3 (TLR3) or retinoic acid inducible gene I (RIG-I) which play important roles in innate immune responses against viral infection[81,82], or for enzymes involved in processing of cellular hairpin RNAs such as the microRNA precursors[83]. Direct screens identified a 65 kDa nuclear protein[84] of unknown sequence and, more recently, a novel large RNA-binding ubiquitin ligase, hRUL138[85], as potential cellular ε RNA interaction partners. However, their roles in the viral life-cycle are not known. Also, any cellular ε-binding factor would have to cope with the physical sequestration of pgRNA into nucleocapsids.

P PROTEIN STRUCTURE

The P ORF, covering nearly 80% of the hepadnaviral genome (Figure 2), has a coding capacity of about 830 aa for HBV, and about 790 aa for DHBV (Figure 5). There is no indication for downstream processing of the primary translation products. In further contrast to retroviruses, hepadnaviral virions contain probably just one P protein molecule per particle[86], in line with the covalent linkage to the genome. In transfected cells, P appears to be produced in excess, such that most molecules are not capsid associated[87-89]; they have a short half-life, and are bound to large cytoplasmic structures; their function, if any, is unclear.

Bioinformatic[90] and genetic analyses showed the presence in all P proteins, of two conserved domains, namely the polymerase/reverse transcriptase (RT) domain, and the C-terminal RNase H (RH) domain. Both are necessary as structural components for pgRNA encapsidation[73]. An absolutely hepadnavirus-specific feature is, however, the Terminal Protein (TP) domain at the N terminus, separated from the RT domain by a highly variable, and dispensable, spacer[91]. TP was first identified as the (-)-DNA linked protein[92] and later was shown to provide a specific Y residue to which the first nt of the (-)-DNA becomes covalently linked (Y96 in DHBV TP;[93,94]; Y63 in HBV TP[95]).

At present, no direct structural information on any hepadnaviral P protein is available although homology-based models for the RT[96] and RH domain[97] have been proposed. The RT model is in accord with drug resistance data, and it is supported by mutational analysis of the putative dNTP pocket of DHBV P protein where a single aromatic residue (F451) was shown to have a homologous role in dNTP versus rNTP discrimination as Y115 in HIV-1 RT[98]; replacement of the bulky F451 by smaller residues conferred to the protein a low but clearly detectable RNA polymerase activity. However, outside the active site the accuracy of the modeled structure is unknown. Hence direct structure determination of the RT and RH domains remains a major objective.

This holds even more for the TP domain, which shares no significant sequence similarity to any other protein in the data base, not even to the few other terminal proteins involved in viral genome replication, such as the VPg in picornaviruses[99], or the terminal proteins of adenovirus[100] and the bacteriophage ϕ29[101]; moreover, those TP proteins are not covalently linked to their polymerases.

Structure determination requires a source for sufficient amounts of pure, soluble protein which proved to be immensely difficult for P protein. Eventually, this problem was partly overcome by slight modifications in the primary sequence of DHBV P and particularly by adding solubility mediating fusion partners such as GrpE, NusA, or GST[53,102,103]. However, although such fusions (Figure 5B) display activity (see below) they appear to be present as "soluble aggregates" which are unsuited for crystallization.

Particularly TP, when expressed in *E. coli*, is completely insoluble on its own, mostly due to a hydrophobic region

in the C terminal part. By selection from a pool of TP variants with random mutations we could isolate several TP variants, harboring fewer hydrophobic residues in this region, as monodispersely soluble proteins (J. Beck and M. Nassal, unpublished data). Interestingly, the same region was recently implicated to contain a molecular contact site, possibly for the RT domain[104], as suggested by the ability of separate TP and RT/RH domains to trans-complement each other[53,95,105]. Since replacing several conserved hydrophobic regions at a time may affect TP function the challenge will be to find, for crystallization, mutants that combine solubility with functional activity. However, being at the heart of hepadnaviral replication, solving the structure of TP is one of the big current challenges.

CELL-FREE RECONSTITUTION OF HEPADNAVIRAL REPLICATION INITIATION

Cell-free systems are inherently much more manipulatable than intact cells. The first such system to investigate the mechanism of hepadnaviral reverse transcription was based on the observation that DHBV P protein, *in vitro* translated in rabbit RL, became radioactively labeled when the translation reaction was supplied with α^{32}P-dNTPs[78]- as expected if the initial step of reverse transcription, i.e. covalent attachment of the first nucleotide to P protein, had occurred ("*in vitro* priming"; Figure 3B). In fact, the 3´ copy of Dε present on the P protein mRNA was shown to be the template for limited elongation; the role of ε, though only the 5´ copy, as authentic genome replication origin was confirmed for DHBV[56,57], and finally also HBV[58].

The *in vitro* translation system has been used extensively to functionally analyze P protein as well as Dε mutants, particularly because Dε can be added as a separate short RNA covering just the hairpin structure ("trans-priming"). Human HBV P protein and ε, however, show no enzymatic activity in this setting.

THE P-ε COMPLEX: DETERMINANTS FOR BINDING, PRIMING AND ENCAPSIDATION

A first - though not the only-requirement for a productive P-ε interaction is specific binding. In many RNA-protein interactions, structural diversity and hence specificity is achieved by deviations from a fully base-paired double-helical structure[106], e.g. by interspersed single-stranded bulges and loops. Indeed, the Dε bulge structure (but not its actual sequence, unless it affects structure) is absolutely necessary for P binding. Mutants in the upper stem (see Figure 4B) which favor stable non-bulged structures do not bind to P[70]. Similarly critical is the sequence and structure at the junction between the lower stem and the bulge: Mutation of the unpaired U2604 opposite the bulge to G substantially reduced binding (and nearly abolished priming)[71]. At the tip base-pair of the lower stem, base identity of G2605, but not base-pairing itself, is important; in addition, most ribose residues in Dε could be replaced by deoxyribose residues, except in the two top base pairs of the lower stem and in the bulge residue templating the first nt of (-)-DNA[107]. Hence this small region contains an essential base- and backbone dependent determinant for P-interaction (Figure 4D), likely because it forms, together with the bulge, a distinct three-dimensional recognition surface for P protein.

In the apical loop, various mutations had no drastic effects on P binding, suggesting the loop is not principally required for complex formation (though probably involved in forming a priming competent structure; see below). An exception is U2590 replacement of which by C abrogated binding[70,108]. However, deletion of this residue did not affect binding, and the negative effect of the U2590C mutation was partially rescued by an additional G to A mutation at the neighboring position 2589[61]. A direct 3D structure comparison between wild-type Dε, currently underway (S.S. Wijmenga, personal comm.), and the U2590C mutant RNA may help to explain this complex phenotype.

The existence of Dε RNA variants which bind P but have a much reduced or no template activity indicated that a productive P-ε interaction requires more than binding[61,70,71]. 2D structure comparisons of free versus P bound RNAs provide compelling evidence that the ability of an RNA to undergo a specific conformational shift is such a decisive additional feature[109]. Several base-paired nt in the upper stem of Dε become highly accessible to nucleases once bound to P; similar changes are not seen with priming-deficient variants. Hence it is likely that the RNA, after an initial binding step, must experience an induced-fit alteration into a new structure, and that only this is usable as a template. Non-productive complexes, by contrast, appear to be trapped at the initial binding stage. Foot-printing analysis further revealed that in productive, but not non-productive, complexes the 3' half of the loop plus 3´ adjacent nt are protein bound, as are the unpaired U opposite the bulge and the nt at the tip of the lower stem (Figure 4D). Hence although the loop nucleotides may not be strictly required for initial binding, they probably provide a protein binding site that becomes crucial in the transition to a priming-active complex.

Also P protein undergoes structural alterations in this process. Proteolysis of *in vitro* translated DHBV P protein yielded a distinct proteolytic fragment only in the presence of priming-competent but not priming-inactive RNA variants[108,110]. Hence RNA and P protein mutually alter each others conformation, likely to properly arrange the ε template region and the priming Y residue of TP in the active site of the RT domain.

Notably, of the various P binding RNAs only those that are priming-active also support pgRNA encapsidation. Hence the abilities to initiate reverse transcription, to package pgRNA, and to adopt a distinct RNA-P protein complex conformation appear strictly coupled. In effect, this represents a quality control mechanism ensuring that only RNAs suitable as templates for reverse transcription are packaged. The current methods to analyze these replication-relevant structural changes have a very limited resolution; ultimately, biophysical examination of a priming-active complex will be required, and its

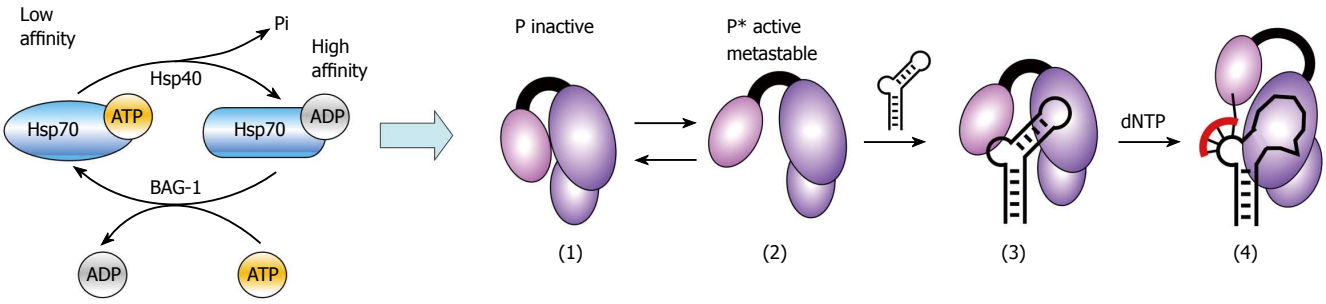

Figure 6 Model for Hsp40/Hsp70 mediated in vitro activation of P protein. The low ATPase activity of Hsp70 is stimulated by Hsp40, yielding high substrate affinity Hsp70/ADP. A new cycle of substrate release and folding requires exchange of the ADP for new ATP which is stimulated by nucleotide exchange factors such as BAG-1. This ATP-dependent Hsp70 cycling applies to the chaperone´s global folding activities but likely also to P activation: in the inactive state of P (1), the ε RNA binding pocket is inaccessible; Hsp40/Hsp70 activation creates active P* (2) which is able to bind ε RNA (3); P* is metastable, and decays to the inactive state (1) within minutes. Maintaining a steady-state level of P* requires constant re-activation by Hsp40/Hsp70 and thus a continuous supply of fresh ATP. Complexes containing priming-competent ε RNAs (3) undergo induced-fit rearrangements in the RNA and the protein, enabling them to initiate DNA synthesis upon dNTP addition (4). Several Dε variants bind P but do not act as template; most likely they are trapped at stage (3). The same may hold for human HBV P protein complexes with HBV ε RNA.

comparison with non-productive complexes should be most revealing. However, several significant obstacles will have to be overcome for this approach, not the least being the strict dependence of DHBV P protein activity on additional cellular factors (see below).

This holds even more for active initiation complexes of human HBV. Insect cell expressed HBV P exerted a low but clearly detectable polymerase activity, with part of the DNA products covalently linked to TP as expected from authentic initiation[111]. The system revealed some important features, such as the priming role of Y63 in HBV TP[95], trans-complementation between TP and RT/RH domains[95], and even some nucleocapsid formation by co-expression of core protein[112]. Disturbingly, however, various of these activities were not strictly ε dependent, and they could not be reconstituted after (partial) purification of the P protein. Whether insect cells contain some RNA that can substitute for ε has not been clarified. *In vitro* translated HBV P protein shows no priming activity in reticulocyte lysate and not even in lysates from Huh7 cells (J. Beck and M. Nassal, unpublished data) which support replication when transfected with HBV. Recently, however, Hu *et al*[72] reported a partial but important progress in that they were able to demonstrate specific binding-though not priming-for HBV P and ε in an *in vitro* reconstitution system (see next paragraph).

IN VITRO RECONSTITUTION OF REPLICATION INITIATION FROM PURIFIED COMPONENTS

One aspect for *in vitro* translation of P protein was, at the time, the lack of alternative ways to recombinantly produce the protein. Surprisingly, wheat germ extract versus rabbit RL translated DHBV P protein had a much reduced priming activity, suggesting the mammalian RL provided additional essential factors[113]. These turned out to be cellular chaperones, which are abundantly present in RL. Apart from Hsp60 chaperonins[114] (GroEL in bacteria), Hsp70 (DnaK in bacteria) and Hsp90 constitute the major chaperone systems. Hsp70 assists folding of many newly synthesized polypeptides, refolding of misfolded proteins resulting, e.g., from heat-shock, and protein translocation through membranes[115-117]. Hsp90 has also broad but more specialized folding functions[118], usually if not exclusively in concert with Hsp70; the two systems are linked *via* the Hsp70/Hsp90 organizing protein Hop. By analogy to the activation of nuclear hormone receptors[119] it was proposed that Hsp90 plus its small co-chaperone p23 are the essential factors for P protein activation[113,120]. However, the complex overall composition and the high chaperone content of RL compared to the minute amounts of P protein precluded a clear-cut distinction.

This was overcome when DHBV P protein became accessible in larger quantities by expressing, in *E. coli*, slightly modified variants[53,102], in particular as fusions with solubility enhancing heterologous domains (Figure 5B). Such recombinant P proteins could now be added to the RL system and showed activity. Finally, because the translation function of RL was not required any longer, it became possible to systematically analyze the chaperone requirements for P protein activation with purified individual components.

In this way we could demonstrate that DHBV P can efficiently be activated *in vitro* by Hsp40 and Hsp70 plus ATP as an energy source, without the need for Hsp90 or other cofactors[54]. The primary reaction product is an RNA binding-competent form of P protein (P*) that decays quickly in the absence of ε RNA but, in its presence, accumulates in an initiation-competent form (Figure 6).

Maintaining P in its activated P* form requires a constant supply of ATP[54], and the same holds for the general folding activity of Hsp70[117]. This suggested that P activation represents a special form of Hsp70 mediated folding in which the chaperone, rather than helping P from an unfolded into a stable folded state, affects the equilibrium between the inactive P ground state and the metastable activated P* form. Of note, bacterial DnaK has been shown to interact, physiologically, with a few folded, as opposed to misfolded, proteins such as the transcription factor σ32[121,122].

Hsp70 chaperoning is a cyclic ATP-driven process[117].

Hsp70 binds ATP and in this form exerts low affinity to folding substrates (Figure 6, left). ATP hydrolysis then generates the high affinity Hsp70/ADP form. In the presence of substrate, the weak Hsp70 ATPase activity is stimulated up to 1000 fold by Hsp40 and related J-domain proteins (named after the prototypic *E. coli* Hsp40, DnaJ;[123]), explaining the important role of Hsp40. Initiating a new folding cycle requires reconversion of Hsp70/ADP into Hsp70/ATP, i.e. replacement of the bound ADP by fresh ATP; otherwise Hsp70 would be trapped and not be available for acting on new substrate molecules. Spontaneous nt exchange is slow but strongly enhanced by nucleotide exchange factors (NEFs). This predicted that addition of BAG-1, an established NEF of Hsp70[124], to the minimal *in vitro* reconstitution system should enhance the formation of P* molecules. Indeed, we observed a strong BAG-1 dependent increase in priming-active P molecules, but not with a BAG-1 mutant unable to interact with Hsp70. Hence ADP-ATP exchange on Hsp70 is the rate limiting step in the *in vitro* priming reaction (M. Stahl, M. Retzlaff, M. Nassal and J. Beck, submitted). A working model for the Hsp40/Hsp70 activation of DHBV P protein is shown in Figure 6.

Though these data are clear-cut, Hu and colleagues, using a similar experimental set-up though a different (i.e. GST) DHBV P fusion protein, reported that in their *in vitro* system P activation was strictly dependent on the additional presence of Hsp90 and the Hsp70/Hsp90 adaptor protein Hop, with the Hsp90 co-chaperone p23 enhancing the reaction rate[52].

In our system, Hop and Hsp90 do have a stimulatory- but not an essential-effect (in a similar range as BAG-1), particularly at low P protein concentrations. In addition, the specific nature of the Hsp40 used can affect activation efficiency. Eukaryotic cells contain numerous Hsp40-like (or J-domain) proteins[125]; all contain the about 70 aa long J-domain which mediates interaction with Hsp70. Hsp70 activation of P protein appears to proceed selectively with the Hdj1 variant of Hsp40 but not Hdj2 or its yeast homolog Ydj1. With Ydj1, Hop and Hsp90 may become essential for detectable activation. Why different Hsp40s have different effects is currently unclear because all Hsp40s can stimulate the Hsp70 ATPase activity; however, there are different ways of how a substrate can enter the folding complex: either it is bound directly by Hsp70, or it is presented to Hsp70 by the Hsp40-like protein[123]. Therefore, the various additional domains present in different J-domain proteins could effect the formation of P* *via* different pathways. Hence in summary, P activation *in vitro* is fundamentally dependent on Hsp70/Hsp40 but can be enhanced by additional factors, including Hop/Hsp90.

Interestingly, the strict chaperone dependence of DHBV P protein activity was relieved by an extensive C terminal truncation that removed the entire RH domain and some 75 aa from the RT domain; the only requirement for priming activity (though restricted to the very first DNA primer nt) of this truncated Mini-RT 2 protein (Figure 5C) was the presence of mild detergent[126]. This suggests that in full-length P protein C terminal parts somehow block the ε RNA binding site, and that this occlusion is removed by the chaperone action[102,127].

Where exactly the chaperones bind to P and which conformational rearrangements they induce is unclear. Using *in vitro* translated DHBV P protein in RL Tavis *et al*[110] noted the ε-dependent generation of a papain- and trypsin-resistant fragment covering mainly the RT domain. Taking advantage of the simple, defined composition of the *in vitro* reconstitution system we could directly investigate the effects of Hsp40 and Hsp70 on P protein conformation. Limited V8 protease digestion revealed specific chaperone- and ATP-dependent cleavages in the C terminal part of TP (between aa 164 and 199). Hence this TP region is inaccessible in non-activated P protein but becomes exposed in P* (M. Stahl, J. Beck and M. Nassal, unpublished data). The functional relevance of this conformational alteration is supported by its correlation with the ability of P to bind ε RNA, and the shielding of the same region from protease attack as long as the Dε RNA is bound; furthermore, the larger fragment encompasses two residues, K182 and R183, which are essential for RNA binding. These data support the model shown in Figure 6. Inclusion of Hop and Hsp90 in this assay should now allow one to monitor whether these, or additional factors, have differential effects on P conformation, or whether they mainly stabilize the changes already established by Hsp40/Hsp70.

Extending the scope of the *in vitro* reconstitution system, Hu and colleagues recently demonstrated that specific binding to ε by the human HBV P protein also appears to be controlled by cellular chaperones[72,128]. On the ε RNA side, the region immediately surrounding the bulge was essential but the apical loop was not. This was surprising given that the loop is essential for pgRNA encapsidation and initiation of replication in intact cells. However, in light of the above described DHBV data, it may just be a drastic manifestation that mere physical binding is not sufficient for a productive interaction; this is further supported in that only about half the P protein sequence was required for RNA binding, with even the catalytic YMDD motif being dispensable. Apparently, the crucial second step, which in Dε involves rearranging the upper stem and the loop, does not occur in the reconstituted HBV P protein-ε complexes, possibly due to the high stability of this region in HBV ε. Finding conditions under which HBV P protein exerts authentic ε-dependent *in vitro* priming-activity remains therefore another major challenge in HBV biology.

IMPORTANCE OF CHAPERONES FOR HEPADNAVIRUS REPLICATION IN INTACT CELLS

Both the Hsp70 and the Hsp90 chaperoning activities are subject to regulation by a multitude of co-chaperones[117,129]. Hence which of the *in vitro* reconstitutable processes is the relevant one inside cells is not trivial to address, in particular because the chaperones are crucial for very many fundamental and regulatory cellular processes. For

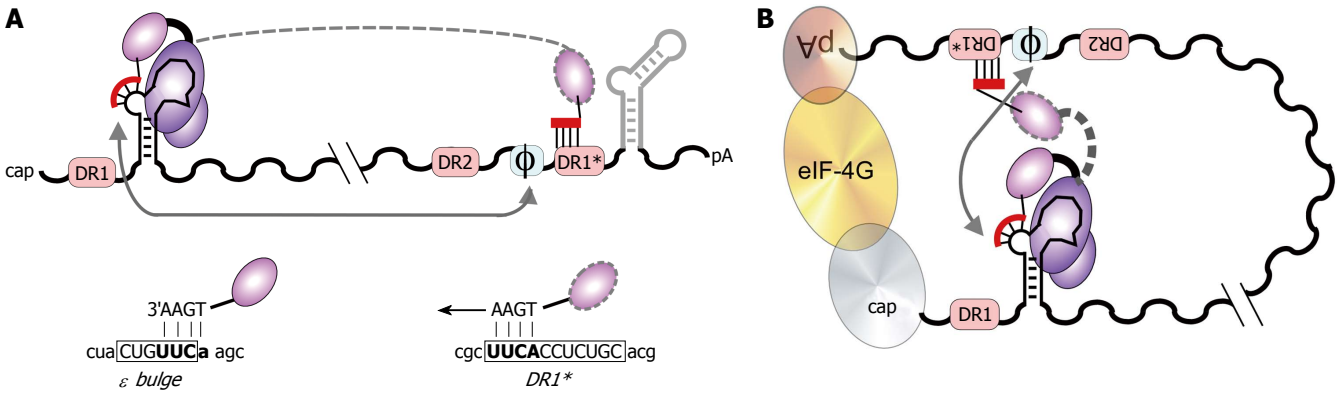

Figure 7 DNA primer translocation (first template switch). P copies 3 to 4 nt from the 5´ ε bulge, yielding the TP-linked DNA oligonucleotide which is translocated to the complementary motif in the 3´ proximal DR1*. **A**: Linear representation. DR1* is nearly 3 kb apart from 5´ ε in primary sequence, and numerous other UUCA motifs are not used as acceptors. ϕ denotes a newly identified cis-element with partial sequence complementarity to the 5´ half of ε. 3´ ε (light grey) is dispensable. The HBV specific sequences in the ε bulge, and in DR1* are shown below in capitals, flanking sequences in lower case; **B**: Models for juxtaposition of 5´ ε and DR1*. A general mechanism would be closed loop formation[80] of the pgRNA by cap-binding and poly-A binding factors (ovals), e.g. *via* elongation initiation factor 4G (eIF-4G; large oval). More specifically, ε might base-pair with ϕ[146,147], as indicated by the grey arrows. In such an arrangement, a small movement, rather than a big jump, of TP with the bound DNA primer (dashed outline) would suffice for specific translocation to DR1*.

instance, geldanamycin (GA), an inhibitor of Hsp90, strongly interfered with DHBV replication in transfected cells at a concentration of 10 ng/mL whereas significant reduction of the *in vitro* priming activity required much higher concentrations[113]. Therefore, the antiviral effect in cells might be indirect, perhaps *via* one of the cellular kinases that are regulated by Hsp90[130]. Such effects on the cell make it also difficult to imagine that chaperone inhibitors could therapeutically be used against HBV infection without causing severe adverse effects. That GA analogs, nonetheless, exert tumor-specific therapeutic value[131] is probably due to the selective presence, in cancer cells, of Hsp90 in a high GA affinity state[132]. Whether this also holds for HBV infected cells is not known.

Various additional chaperones have, in part indirectly, been implicated in affecting P protein, for instance Hsp60[133-135] and the Hsp90 family member GRP94[136]; however, GRP94 is an endoplasmic reticulum (ER) resident protein for which a role in P protein activation is difficult to imagine. Overexpression of p50/cdc37, a co-chaperone of Hsp90 involved in activation of several signal transduction kinases, stimulated DHBV replication in transfected cells and a dominant negative mutant of p50/cdc37 inhibited DHBV P protein priming *in vitro*[137]. Again, the physiological relevance of these observations remains to be confirmed.

One possible solution would be to map the contact sites of the various chaperones on P protein using biochemical and biophysical methods, and then to generate P mutants with specific interaction defects. Monitoring their replication phenotypes in transfected, or better in infected, cells should then allow to narrow down which of the various reported interactions are truly significant for virus propagation *in vivo*.

Though we have stressed the uniquely strict chaperone-dependence of hepadnaviral P protein activation, there is accumulating evidence that other polymerases are also affected by chaperones. Probably the closest analogy exists to telomerase, the cellular reverse transcriptase that maintains chromosome end integrity[138,139]. However, also the DNA polymerase of Herpes simplex virus[140], and the RNA polymerases of flock house virus[141] and influenza virus appear to require chaperone assistance[142,143]. Closely watching progress in those areas might also provide clues as to the mechanism of chaperone-assisted hepadnavirus replication; however, for polymerases without a similarly sophisticated protein-primed initiation mode the chaperones could act at rather different levels.

DNA PRIMER TRANSLOCATION AND (-)-DNA COMPLETION

In contrast to initiation, the subsequent steps for RC-DNA formation are currently not amenable to *in vitro* analysis, and they appear intimately related to the proper environment of intact nucleocapsids. Hence reverse genetics is still the most rewarding approach to address these equally puzzling and complex events.

The initial model of hepadnaviral (-)-DNA formation assumed that synthesis would start inside the 3´ copy of DR1 (DR1*), for HBV at the motif 5´ UUCA. As discussed above, the complementary sequence 3´ AAGT at the 5´ end of (-)-DNA is instead copied from the UUCA motif in the ε bulge (Figure 7A). Hence the oligonucleotide bound to TP must specifically be translocated to the 3´ DR1*, nearly 3 kb apart from 5´ ε. Given that there are about 20 further UUCA motifs on the pgRNA, and that even fewer than 4 nt of identity between the template region in ε and the target site in DR1* are sufficient for specific transfer[58], additional elements ensuring proper translocation must be operating. One model is that DR1* and 5´ ε are brought into close proximity (Figure 7B). A general mechanism would be closed-loop formation of the pgRNA *via* cellular proteins such as elongation initiation factor 4G (eIF-4G) which links 5´ cap and 3´ poly-A binding proteins[80]. A recent more specific model is a long-range RNA interaction between ε and a new cis-element ("ϕ" or "β5") slightly upstream of DR1* that is involved in proper (-)-DNA synthesis[144-146]; it contains a sequence that

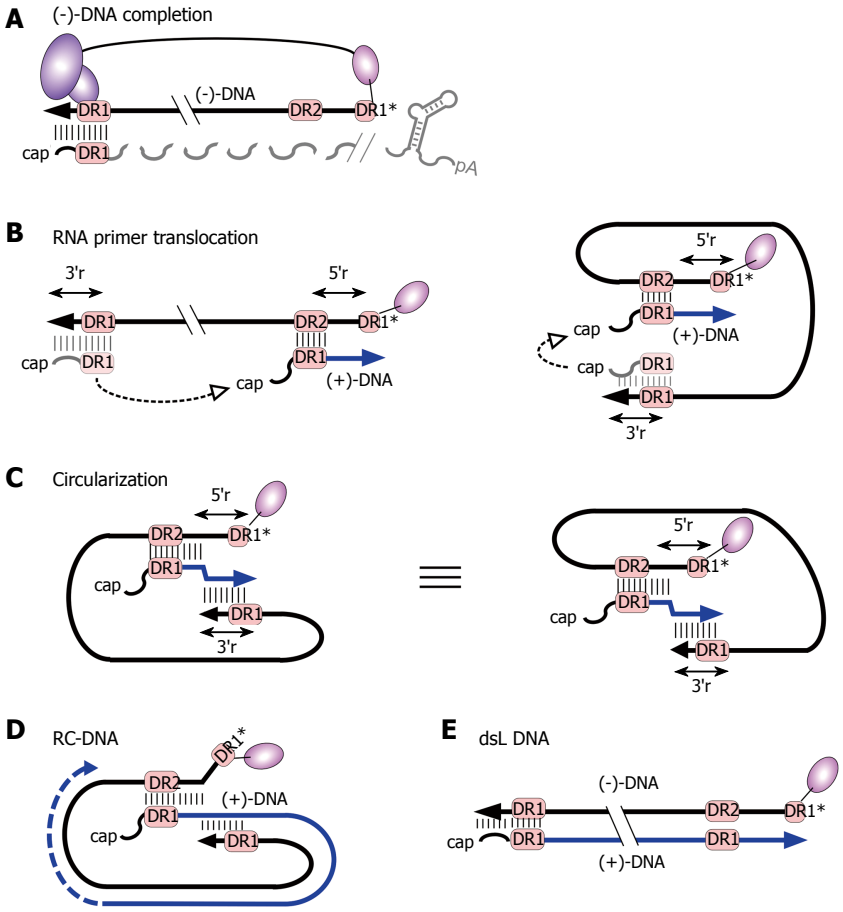

Figure 8 RC-DNA formation. **A**: (-)-DNA completion. The DNA primer, still linked to TP, is extended from DR1* to the 5′ end of pgRNA. The RNA is simultaneously degraded by the RH domain, except for its capped 5′ terminal region including 5′ DR1; the fate of the poly-adenylated 3′ end is unclear; **B**: RNA primer translocation (second template switch). The RNA primer translocates to DR2, and is extended to the 5′ end of (-)-DNA. 3′ r and 5′ r denote an about 10 nt redundancy on the (-)-DNA. As above, several cis-elements appear to promote close proximity of the DR1 donor and the DR2 acceptor, as schematically indicated in the right hand figure; **C**: Circularization (third template switch). Having copied 5′ r, the growing 3′ end of the (+)-DNA switches to 3′ r on the (-)-DNA, enabling further elongation. This reaction must involve juxtaposition of 5′ r and 3′ r. For easier comprehension, the switch is also depicted on the basis of the representation shown on the right of Figure 8B; both are topologically equivalent; **D**: RC-DNA. Extension on the (-)-DNA template creates a set of (+)-DNA strands of various length; **E**: Double-stranded linear (dsL) DNA. This minor DNA form originates when the RNA primer, having failed to translocate to DR2, is extended from its original position ("in *situ* priming").

is partially complementary to the 5′ half of ε. Mutations affecting the base-pairing potential between the two sequences can, indeed, influence the efficiency of (-)-DNA synthesis[147]. Exactly how an ε-ϕ interaction would aid in primer translocation is unclear because base-pairing would simultaneously affect the proper ε structure, implying some temporal regulation. Similar ϕ elements have been proposed to be present in the other hepadnaviruses[146]; for DHBV we noted, however, that Dε mutants with reduced potential base-pairing to the supposed ϕ element showed no obvious (-)-DNA synthesis defects ([71] and K. Dallmeier, B. Schmid and M. Nassal, unpublished data).

The primer translocation process must also involve remodeling of the P-ε complex; the priming Y residue of TP must give way to the growing DNA oligonucleotide, then ε must be replaced by DR1* as the template. Hence P, like other protein-priming polymerases, must have distinct initiation and elongation modes. The recently solved structure of the bacteriophage ϕ29 DNA-dependent DNA polymerase with its (separately expressed) terminal protein[101] gives an impression of the dynamic changes that have to occur. ϕ29 uses for protein priming an S residue in one of three distinct TP domains. In the initiation mode, the priming domain mimics the primer-template nucleic acid that occupies the same site during elongation. The initiation reaction continues until 6 to 10 nt have been attached; at this time the priming domain is pushed out of the polymerase´s active site, marking the transition to elongation mode. Similar events must happen in P protein. Notably, initiation of ϕ29 replication does not start at the very terminal template nt; rather the (identical) penultimate nt is copied. Next, the copied nt slips back to the terminal template position, and the penultimate nt is copied again; adenovirus uses a similar mechanism[100]. Interestingly, such slip-back and re-copying can also occur with HBV P protein on mutant ε templates[58]. A full understanding, however, will obligatorily require high resolution structural data on the hepadnaviral replication complex.

Notably, the single-stranded (-)-DNA is an intermediate that, as in retroviral replication, could be a target for cytidine deamination, and consequently inhibition of replication, by APOBEC enzymes[148]. APOBEC3G, one of several family members, has indeed been reported to interfere with HBV replication[149], however, the mechanism does not involve editing[150]. Also, expression of the corresponding APOBEC mRNA is low in hepatic cells, although it might be inducible[151]. Further research is needed to clarify whether any of the APOBEC enzymes is genuinely involved in the innate response against HBV infection. At any rate, sequestration into intact nucleocapsids of this potentially vulnerable single-stranded DNA replication intermediate may be an efficient counter-defense of HBV.

(+)-STRAND DNA SYNTHESIS AND CIRCULARIZATION

The end product of (-)-DNA synthesis is a unit length DNA copy of the pgRNA from its 5′ end to, in HBV, the UUCA motif in the 3′ DR1* (Figure 8A); hence it contains a small, about 10 nt, terminal redundancy ("r"). Most of

the pgRNA template is degraded concomitantly to (-)-DNA synthesis by the RNase H domain of P. The fate of the non-copied 3´ end of the pgRNA from DR1* to the polyA tail is not exactly known. It seems to be underrepresented in the packaged RNA, both in DHBV[152], and in HBV with mutations in the nucleic acid binding domain of the core protein[46]. Whether some of these 3´ ends are never completely encapsidated, or whether they dissociate out of the capsid, has not yet conclusively been shown.

Well established is, however, that the 5´ terminal about 15 to 18 nt of the pgRNA including the 5´ DR1 sequence are spared from degradation, probably because the active site of RH is halted at this distance when the RT domain reaches the template 5´ end[153]. This capped 5´ RNA oligo is essential as primer for (+)-DNA synthesis. Extension of the RNA from its original position ("in situ priming") gives rise to a double-stranded linear (dsL) DNA (Figure 8E) which occurs to a small percentage in all hepadnaviruses[154]. Lacking the core promoter/enhancer for pgRNA translation upstream of the pgRNA start site it is unsuited for virus propagation but may be of pathogenic potential[155]. For RC-DNA formation, the RNA primer must be transferred to the 3´ proximal DR2 (Figure 8B); pgRNAs with improper 5´ ends, not containing the DR1 sequence in the RNA primer, fail to undergo this essential second template switch. Why the RNA primer predominantly jumps to DR2 although its complementarity to the initial site is larger (more than 15 versus 11 or 12 nt) is not obvious. An interesting explanation has been proposed for DHBV, according to which burying part of the 5´ DR1 sequence in a competing intramolecular hairpin structure effectively shortens the sequence available for hybridization with the RNA primer[156].

From its new location on DR2 the RNA primer is extended towards the P bound 5´ end of the (-)-DNA, including the 5´ r redundancy. Further elongation requires a third template switch, i.e. circularization (Figure 8C). In effect, the growing (+)-DNA end is transferred from 5´ r to 3´ r on the (-)-DNA template from where it can further be extended to yield RC-DNA (Figure 8D).

Though sequence identity between 5´ r and 3´ r is important, additional cis-elements are again required to ensure efficient RNA primer translocation and circularization. Using quantitative genetic techniques, the Loeb laboratory has defined, mostly in DHBV, several such cis-elements on the (-)-DNA[144,156-161], e.g. 3E, M, and 5E, which are located at both termini and in the middle of pgRNA. Collectively, these data provide evidence that these cis-elements, *via* long range base-pairing, allow for a close juxtaposition of the corresponding donor and acceptor sites, and thus facilitate the proper template switches (Figure 8B, right panel). How this is achieved inside the replicating nucleocapsid is not easily envisaged. Also, we noted that chimeric heron-duck HBVs are less sensitive than DHBV to reduced base-pairing in some of these elements (K. Dallmeier and M. Nassal, unpublished). There is evidence, however, that similar potentially base-pairing cis-elements are present in human HBV[144, 162] and assist in circularization (E. Lewellyn and D.D. Loeb, pers. comm.).

Hence intramolecular base-pairing is probably an important mechanism that ensures proper shaping of the viral genome for the various template switches that eventually yield RC-DNA. More details than is possible to show here can be found in informative schematic representations in references[144,160].

CONCLUSIONS AND PERSPECTIVES

Since the original discovery that hepadnaviruses replicate through reverse transcription numerous novel and unique aspects of the replication process have been unraveled by using reverse genetics in transfected cells. Indisputably, this system will continue to be an important tool in future HBV research; in some cases because even a cancer-derived stable cell line mimics many aspects of the complex natural environment for viral replication, allowing to look into the interplay between virus replication and cellular networks by genomics and/or proteomics; in others because we have currently no alternative due to the complexity of the interactions between the viral components themselves. An example is the control of the various template switches during RC-DNA generation by long-range nucleic acid interactions which apparently can only occur, and be monitored, in the context of assembled nucleocapsids.

For a mechanistic understanding, however, cell-free systems are indispensable. Two striking examples for their power are the discovery of the replication origin function of ε and the chaperone-dependence of P protein activation; both would have gone unnoticed for a long time without the *in vitro* translation system. To disentangle these multifactorial processes on the molecular level, *in vitro* reconstitution from purified components is the approach of choice. Despite the bewildering complexity of the chaperoning systems, it will now be possible, starting from relatively simply composed systems, to systematically add-in additional factors and monitor their contributions to P protein activation; this should, *inter alia*, help answer the pressing question why human HBV P protein shows no enzymatic activity under conditions where its DHBV counterpart is active. An *in vitro* activity assay for HBV P protein would also be an important screening tool for better HBV antivirals. Similarly rewarding should be the development of *in vitro* systems that address other steps of the viral replication cycle, foremost perhaps inclusion of the core protein into the now available replication initiation systems.

Ultimately, structural biophysics needs to enter the field. At this time the HBV core protein, without its nucleic acid binding domain, is the only hepadnavirus component for which a high resolution structure is available[163]. Fortunately, 3D structural analyses of the ε element are well underway, and at least individual domains of the P protein may become amenable to direct structure investigations. The most exciting, though also most challenging aim given its multifactorial composition, will be to obtain high resolution data for the P protein-ε RNA complex, caught in the act of DNA synthesis. However, the relevance of any such biophysical and biochemical

data will have to be corroborated in the context of the complete virus replication cycle, whenever possible in an *in vivo* setting.

Finally, although the complex interactions between viral, and viral and cellular, components may seem of foremost interest for basic HBV biology, they also hold the keys for novel, and more efficacious therapies of chronic hepatitis B. The few currently approved anti-HBV drugs are all nucleos(t)idic inhibitors of the reverse transcriptase. Their long-term efficacy is limited by the virtually unavoidable emergence of resistant HBV variants[30]; any new compounds with the same mechanism of action will also face this problem, which will be aggravated by cross-resistance, particularly against different analogs of the same natural nucleotide. Each of the steps in the HBV replication cycle that is now being elucidated in molecular detail provides new, unconventional targets for interference. Replication initiation alone depends on many specific interactions, including the TP and RT/RH domains of P protein, ε RNA, and different chaperones. Blocking any of these interactions, e.g. by small molecules binding to, and altering the structure of ε or preventing chaperone binding to P, would abolish reverse transcription by mechanisms entirely different from that of nucleoside analogs. Knowledge of the 3D structure of P would allow to design better nucleosidic as well as non-nucleoside inhibitors, and additional opportunities will certainly arise once the process of cccDNA formation is better understood. Especially in combination with conventional antivirals, such potential new drugs should greatly increase the chances for curing, rather than just controlling, chronic hepatitis B.

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TOPIC HIGHLIGHT

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Hepatitis B virus morphogenesis

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Abstract

The hepatitis B virus (HBV) particle consists of an envelope containing three related surface proteins and probably lipid and an icosahedral nucleocapsid of approximately 30 nm diameter enclosing the viral DNA genome and DNA polymerase. The capsid is formed in the cytosol of the infected cell during packaging of an RNA pregenome replication complex by multiple copies of a 21-kDa C protein. The capsid gains the ability to bud during synthesis of the viral DNA genome by reverse transcription of the pregenome in the lumen of the particle. The three envelope proteins S, M, and L shape a complex transmembrane fold at the endoplasmic reticulum, and form disulfide-linked homo- and heterodimers. The transmembrane topology of a fraction of the large envelope protein L changes post-translationally, therefore, the N terminal domain of L (preS) finally appears on both sides of the membrane. During budding at an intracellular membrane, a short linear domain in the cytosolic preS region interacts with binding sites on the capsid surface. The virions are subsequently secreted into the blood. In addition, the surface proteins can bud in the absence of capsids and form subviral lipoprotein particles of 20 nm diameter which are also secreted.

Key words: Hepatitis B virus morphogenesis; HBsAg; Hepatitis B virus capsid; Virus envelopment

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INTRODUCTION

In vitro systems for efficient production of hepatitis B virus (HBV) capsids and subviral particles and for experimental examination of their morphogenesis are available. These systems allowed to draw a quite detailed, although still fragmentary, picture of these processes. However, *in vitro* production of virions by transfection of certain cell lines derived from hepatocellular carcinomas, such as HepG2 or Huh7, with cloned genomic HBV DNA[1,2] or by *in vitro* infection[3] is still quite inefficient, and this hampers many approaches to study the morphogenesis of the complete virus. In natural HBV infections one single hepatocyte in the liver releases 1 to 10 viruses per day[4]. *In vitro* the production rate seems to be similar. Therefore, the direct observation of HBV budding by electron microscopy or the characterization of the process by biochemical approaches is difficult to achieve[5]. It seems that the virus production rate in duck hepatitis B virus (DHBV) infection is higher at least *in vitro*, making these techniques suitable for studying DHBV morphogenesis[6].

THE HBV CAPSID FORMATION AND STRUCTURE

The C protein forming the shell of the HBV capsid consists of 183 or 185 amino acid (aa) residues depending on the genotype. The protein is relatively conserved among HBV isolates[7]. It can be expressed in a broad range of pro- and eukaryotic cell types and self-assembles into capsids. The first step is the formation of homodimers[8] linked by a disulfide bridge between the cystein residue 61[9,10]. Higher oligomers containing chaperons have been described[11] but the pathway leading from dimers to complete capsids has not been elucidated in more detail. In the final capsid, the inter-dimer interactions are rather weak[12]. Two different types of capsids are formed[13]: particles with an icosahedral T = 3 symmetry have a diameter of 30 nm and consist of 90 C dimers, whereas particles with an icosahedral T = 4 symmetry are larger (the diameter is 34 nm) and contain 120 C dimers. Both particle species can also be found in infected human liver[14]. In infectious virions, T = 4 capsids have been found[15].

The primary amino acid (aa) sequence of the C protein can be divided into two parts (Figure 1): the N-terminal 149 or 151 aa (depending on the genotype) form the so called assembly domain because this part of the protein is sufficient to direct the self-assembly of capsids. The C-terminal 34 aa are dispensable for capsid formation, rich in arginine residues, and involved in packaging of the pregenome/reverse transcriptase complex. Deletion of this domain abolishes the encapsidation of nucleic acid[16].

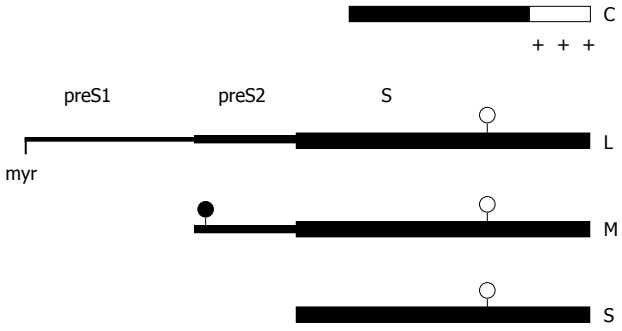

Figure 1 Linear map of the main structural HBV proteins. The C-terminal region (open box) of the capsid protein C is rich in arginine residues (+). The sequence of the small envelope S is also present at the C termini of the M and L protein. The two larger envelope proteins contain the additional N-terminal preS2 and preS2 + preS1 domain, respectively. The L protein is myristylated at glycin 2 (myr), the preS2 domain of M is N-glycosylated (filled circles), and the S domain of all 3 proteins is partially N-glycosylated (open circles).

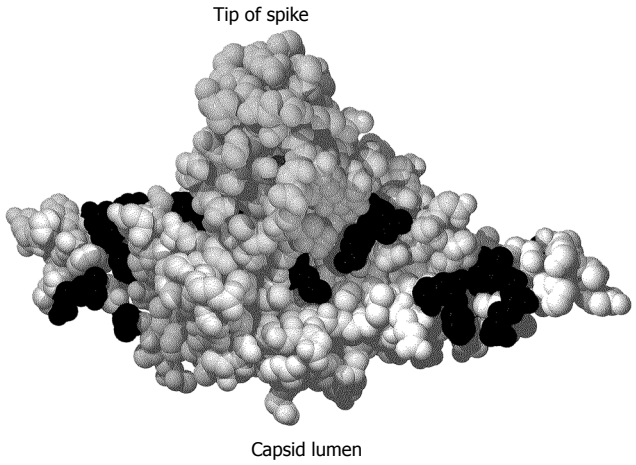

Figure 2 Crystal structure of a C-terminally truncated C protein dimer[21]. The spike protrudes upwards. The lumen of the capsid would be below the figure. Mutational analysis identified aa (shown in black) where the mutation was compatible with capsid formation and viral DNA synthesis in the lumen of the particle but blocked nucleocapsid envelopment[121].

Expression of the C-terminally truncated C protein in *E coli* produces high amounts of T = 4 capsids and relatively little T = 3 particles[17]. Using this material a model for the folding of the C protein in the capsid first at lower resolution by cryo-electron microscopy[18,19] and finally after crystallization at a resolution of 3.3 Å[20,21] has been proposed. The C protein dimer forms a structure like an upside down "T". The horizontal bar mediates the inter-dimer contacts with 5 and 6 dimers arranged around the 5-fold and quasi 6-fold symmetry axes, respectively, and the vertical bar forms a spike protruding outwards from the capsid surface (Figure 2). The tip of the spike forms the major epitope of the capsid antigen (HBcAg). The capsid shell contains pores with a diameter between 12 Å and 15 Å. These pores allow the diffusion of nucleotides into and out of the capsid lumen during the synthesis of the viral DNA genome.

The arginine-rich domain is not present in the capsid crystals but thought to interact with the viral genome in the lumen of the particle[16,22,23]. However, a monoclonal antibody directed against this region binds to intact HBV capsids[24], and trypsin can clip off this domain from approximately half of the C protein chains in recombinant HBV capsids (Daniela Lieder, PhD thesis, Goettingen, 2002). It therefore seems possible that the C-terminal region of one fraction of C proteins reach into the lumen of the particle, while the domains from the other fraction appear on the external surface of the same particle. The peptide at the boundary between the assembly and arginine-rich domains of C forms a mobile array[25] and may allow an extreme mobility of the C terminal domain.

Capsid formation during recombinant expression of the C protein requires a higher concentration of C protein dimers relative to nucleocapsid formation in the context of an infection[26]. During authentic capsid formation, not only the viral pregenomic RNA bound to the viral P protein[27-30] but also cellular factors such as chaperones[31-33] and a protein kinase phosphorylating serine residues in the arginine-rich domain of C[34-37] are encapsidated. Apparently, the threshold concentration of C dimers needed for the initiation of capsid formation is lowered by one or more of these factors. This mechanism assures the efficient encapsidation of replication complexes and prevents that large amounts of empty capsids are formed in the presence of free replication complexes.

C proteins from human and woodchuck HBV can form mixed capsids, while this is not possible between human and duck C proteins[38] which are less homologous. Foreign protein domains can be incorporated into capsids when fused to the N or C terminus or at the tip of the spike[39-44]. The assembly of HBV capsids can be blocked by low molecular weight compounds, possibly offering new options for antiviral treatments in the future[45-48].

THE HBV ENVELOPE PROTEINS

The HBV envelope contains three related viral surface proteins. They are expressed from one open reading frame (ORF) referred to as E containing 389 or 400 codons depending on the genotype and three start sites for translation[49] (Figure 1). Transcription is initiated at a promoter upstream of the ORF and, in addition, at an internal promoter upstream of the second translation initiation site[50]. Translation of the larger mRNA yields the large envelope protein (L) consisting of 389 or 400 aa. Translation of the shorter transcripts gives rise to the middle sized, 281 aa long M protein and, in addition, to the small S protein consisting of 226 aa[51] depending on which translation initiation site is used. The aa sequence present at the C termini of L and M is identical to the S protein and is referred to as the S domain. The 55 aa long additional N-terminal domain of M being central in L is called preS2, and the 108 or 119 aa long N-terminal domain unique to L is named preS1. The E ORF of avian hepadnaviruses contains only 2 start codons, therefore, these viruses possess only two envelope proteins (L and S).

Like typical membrane proteins, the HBV envelope proteins are synthesized at the endoplasmic reticulum (ER). They gain a relatively complex topology (Figure 3). Insertion of the S protein into the ER membrane is initiated by an N-terminal signal sequence (aa 8 to

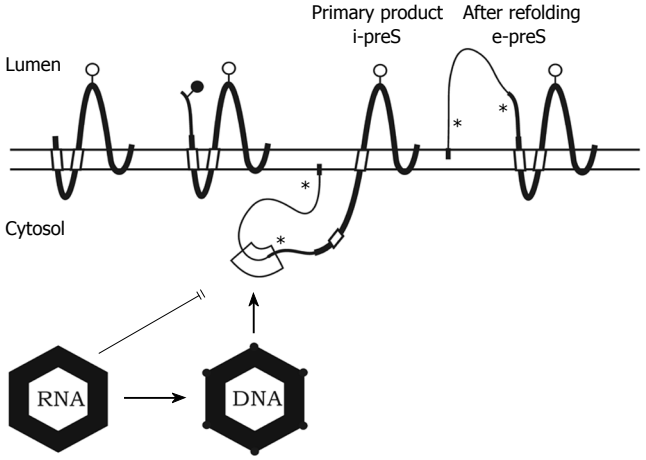

Figure 3 Transmembrane topology of the HBV envelope proteins and model for envelope-capsid interaction. The transmembrane folding of the S protein is determined by an N-terminal and an internal signal shown as open boxes. The C-terminal domain is hydrophobic and probably embedded in the lipid bilayer (horizontal open bar). The C terminus is oriented towards the ER lumen. The folding of the M protein is similar to S. The preS2 domain of M (thinner line) is located in the ER lumen. In the initial folding of the L protein, the preS domains are located in the cytosol (i-preS). Whether the N-terminal myristate group (filled box) is inserted into the membrane as shown here is unknown. After refolding approximately half of the L chains expose the preS domains at the luminal side of the membrane (e-preS). Open and filled circles: see Figure 1. Asterisks indicate potential but unused N-glycosylation sites in preS of L. A domain in i-preS (boxed area) and in the cytosolic loop of S may interact with the capsid during budding. Immature capsids containing pregenomic RNA are not capable to bud. During viral DNA synthesis, the capsid shell changes (indicated by filled circles at the edges) and becomes competent for envelopment.

22) which is, however, not cleaved by the host's signal peptidase. A second signal (aa 80 to 98) directs the translocation of the peptide chain downstream of this signal through the ER membrane into the ER lumen[52], whereas the region upstream of the signal remains in the cytosol. The signal itself anchors the protein as a transmembrane domain in the lipid bilayer. The C-terminal hydrophobic 57 aa of S are believed to be embedded in the ER membrane. Foreign domains fused to the C terminus of S are oriented towards the ER lumen, suggesting that the C terminus of S is also oriented toward this compartment[52]. This configuration causes the region between residues 23 and 79 to form a loop at the cytosolic side of the ER membrane, whereas the loop between aa 99 and approximately 169 is on the luminal side. The luminal loop carries the major conformational epitope of the HBV surface protein antigen (HBsAg) and is N-glycosylated in approximately half of the S molecules at asparagine (asn) residue 146[53]. After budding the HBsAg epitopes are located at the external surface of viral particles.

The transmembrane topology of the M protein is identical to S. The N-terminal preS2 domain (55 aa) is translocated into the ER lumen probably by the action of the first signal in the S domain[54]. The M protein is N-glycosylated at asn 4[55]. In addition, the preS2 domain is O-glycosylated in some but not all HBV genotypes[56].

Glycine residue 2 of the L protein is myristylated[57]. The preS1 and preS2 domains at the N terminus of L initially remain at the cytosolic side of the ER membrane during L translation (i-preS conformation). The central signal in the S domain of L anchors the protein in the ER membrane and causes the translocation of downstream sequences. Therefore, asn 146 in the S domain of L is partially N-glycosylated, while asn 4 of the preS2 as well as a further potential N-glycosylation site in preS1 remain unmodified (Figure 3). These sites are used when the N terminus of L is forced to cotranslationally translocate by the artificial fusion of a signal sequence to the N terminus of preS1[58].

In about half of the L chains, the transmembrane topology changes after translation[59-63]. The preS domains then appear on the luminal side of the ER membrane (e-preS conformation). Probably the N-terminal signal in the S domain crosses the membrane in this conformation similar to the M and S proteins. How the preS domains move post-translationally through the membrane is not known. Cytosolic chaperones like Hsc70 bind to preS1 and deletion of the binding site causes cotranslational translocation of preS[64-66]. Luminal chaperones binding to the e-preS domain[67] could support this process. It might be possible that oligomerized S domains form a channel in the membrane for the preS transport[68]. For HBV, the S and M proteins are not required for the translocation process[69], whereas the S protein is essential for DHBV[70,71]. In DHBV, a C-terminally truncated S protein has been described to fulfil a chaperone function during preS translocation[72]. These facts suggest that the preS translocation mechanism might be different between HBV and DHBV[73]. Both L isoforms have their own function: in the e-preS conformation, the preS1 and preS2 domains of L are exposed on the surface of virions and participate in virus receptor binding[74,75], while in the i-preS conformation, the preS1 and preS2 domains of L are internal in the virion and probably important for contacting the nucleocapsid (see below). In addition, the i-preS domain can activate a variety of promoter elements[76]; however, the significance of this function is not clear.

The S domain but not the preS domain contains multiple cystein residues. Cystein residues in the luminal loop crosslink the envelope proteins with each other by multiple disulfide bridges. Shortly after synthesis, disulfide-linked homo- and heterodimers between S, M, and L proteins can be found[77,78]. The cytosolic loop contains 4 cystein residues. Mutational analysis demonstrated that the exchange of 1 out of 3 of the 4 cysteine residues in this loop by a serine residue blocked subviral particle formation[79]. However, these cysteins are not involved in disulfide bridge formation in subviral particles[77], and a covalent modification of these sites, for example, by fatty acid acylation, has not been found[57]. The DHBV L protein is partially phosphorylated[80,81], mainly at serine 118[82]. However, mutational analyses could not demonstrate an essential role for this modification in the DHBV life cycle[83]. Phosphorylation of the HBV L protein could not be found.

SUBVIRAL PARTICLES

The HBV surface proteins are not only incorporated into virion envelopes. Rather, they also bud very efficiently

from intracellular, post-ER pre-Golgi membranes[84,85] without envelopment of capsids, appear as subviral quasi-spherical or filamentous lipoprotein particles in the lumen of the compartment, and are released from the cell by secretion. The quasi-spherical particles have a diameter of 20 nm and an octahedral symmetry[86], the filaments have variable lengths. Subviral particles are highly over-expressed relative to virions and reach a 10 000-fold higher concentration in serum. Subviral particles and virions carry identical surface antigens (HBsAg), although the protein composition is not identical. Spherical subviral particles contain only low amounts of L protein, whereas the relative amount of L is higher in filaments and even higher in the virion envelope[49]. It is assumed that the massive HBsAg overproduction influences the host's immune system in a way that is advantageous for the virus.

Recombinant expression of the S protein (e.g. in yeast) yields highly immunogenic intracellular 20-nm HBsAg particles which can be used as an active vaccine against hepatitis B[87]. S protein expressed in mammalian cells is efficiently secreted as 20-nm HBsAg particles. How the protein escapes the membrane and mobilizes lipid during subviral particle formation is unclear. Chaperons, such as calnexin[88] and BiP[67], bind to S and support the maturation of the protein. The relative amount of lipid is only 25% by weight in subviral particles[89], suggesting that the lipid is not organized as in a conventional membrane bilayer.

The M protein essentially behaves like the S protein with respect to subviral particle formation. However, the L protein can not be secreted from cells when expressed by itself. In fact, the L protein causes a dose-dependant inhibition of particle release when coexpressed with the S protein[90,91] and a storage of subviral particles in the ER lumen[88]. This can cause cell stress and even cell death or cancer[92,93]. The significance of the secretion inhibition function of L for the viral life cycle has remained unclear. This function can be abolished by blocking L myristylation (e.g. by a point mutation of the acceptor glycine residue) or by the deletion of the N-terminal 19 aa of preS1[94-96]. Also, the fusion of a secretion signal to the N terminus of L forcing the protein to exclusively generate the e-preS conformation abrogates secretion inhibition[58]. This, however, is different for the DHBV L protein[97].

Host proteins are efficiently excluded during the morphogenesis of subviral particles. Even the HBV and DHBV S proteins sharing 25% identical aa do not form mixed particles during coexpression[98]. However, this is possible with the more closely related S proteins from HBV and the woodchuck hepatitis B virus (WHV). Apparently, the S protein subunits interact tightly with each other during 20 nm particle formation. However, foreign protein domains can be incorporated into subviral HBsAg particles when they are fused to the S protein[99,100].

CAPSID MATURATION

During HBV nucleocapsid formation, the RNA pregenome is packaged into the particle's lumen and first converted into single stranded and than into partially double-stranded DNA. While nucleocapsids showing all stages of the viral DNA synthesis can be found within cells, secreted virions contain only rather mature circular, partially double-stranded DNA[101,102]. Therefore, it was proposed that early RNA-containing capsid can not be incorporated into virions and that the viral DNA synthesis is associated with a structural change in the capsid shell that allows only mature capsids to be enveloped[103]. This hypothesis was supported by several genetic experiments. C-terminal truncations of the DHBV core protein blocked viral DNA synthesis and also inhibited capsid envelopment[104]. Missense mutations inhibited the reverse transcriptase activity of HBV and DHBV P protein and locked nucleocapsids in an immature state. The incorporation of these capsids into virions was greatly reduced[105,106]. Using a synchronized DHBV replication system, it was shown that envelopment of capsids happened only late in the replication cycle[107].

The nature of the maturation signal has only been described insufficiently. A comparison of capsids containing RNA and DNA by cryo-electronmicroscopy revealed structural differences[15]. There is also evidence that the phosphorylation state of the arginine-rich domain of the C protein might be part of the signal[108-110]. A validation of this hypothesis by a genetic approach is not suitable because substitutions of phosphorylation sites with alanine or glutamic/aspartic acid also influence pregenome packaging and DNA synthesis[111]. Interestingly, the point mutation of isoleucine 97 to leucine in the C protein caused the envelopment of immature capsids[112]. The side chain of this residue is located in the inner space of the spike and close to a hydrophobic pocket showing structural differences in mature *versus* immature capsids[15]. Possibly, the I97L mutation induces a conformational change causing a constitutive or early expression of the envelopment signal. An additional point mutation (P130T) in a quite distant area of the core protein restored the wild-type phenotype[113], demonstrating the complex nature of the maturation signal for envelopment.

The exclusion of immature nucleocapsids from envelopment causes only replication-competent capsids to become part of virions. This may be one reason for the high specific infectivity of DHBV which is close to the optimum of 1 infectious particle per virion[114]. Also for HBV the specific infectivity seems to be very high[115].

The disassembly of capsids occurs in the basket of nuclear pores upon nuclear transport of the viral genome[116] either during infection or intracellular amplification of the viral genome copy number. Capsid destabilization can also be induced by a tumor necrosis factor alpha-mediated non-cytopathic pathway and may play a role as an antiviral mechanism in natural infections[117].

CAPSID ENVELOPMENT AND VIRION FORMATION

In contrast to retroviruses or togaviruses, it is difficult to directly observe the envelopment of HBV capsid by electron microscopy probably because budding events are less frequent in the available *in vitro* expression systems.

It has even not been clarified whether the HBV envelope contains lipid, although this seems to be likely due to the composition of subviral HBsAg particles. Nevertheless, based on the molecular characterization of HBV formation (see below), it is assumed that hepatitis B virions are formed by budding in analogy to other enveloped viruses.

Mature hepadnaviral nucleocapsids originate in the cytosol. How the capsids move to post-ER, pre-Golgi membranes where the envelopment by the surface proteins supposedly occurs[84,85] is unknown. For DHBV capsids, there is evidence that mature capsids lacking C protein hyperphosphorylation, like capsids in virions, attach to intracellular membranes independent of viral envelope proteins[118]. Immature capsids are hyperphosphorylated and do not bind. This observation suggests that the discrimination between immature and mature capsids happens during the transport of the particle to budding sites before the contact to envelope proteins is established.

Several enveloped viruses utilize a host cell machinery for budding of vesicles into the lumen of so-called multivesicular bodies for their own virus budding[119,120]. Viral capsid proteins interact with the host factor of this pathway via so-called late domains. The HBV C protein contains the sequence PPAY (aa 129-132) exposed on the capsid surface[21] resembling the late domain motif PPXY. However, mutations at this site either blocked capsid formation or reproduced the wild-type phenotype[121]. Therefore, future experiments have to decide whether this pathway is involved in HBV morphogenesis.

The envelopment of HBV capsids strictly depends on viral envelope proteins[122-124] in contrast to type C retroviruses or lentiviruses where mutants blocked in envelope protein expression still release capsids wrapped with a lipid layer. A natural HBV point mutant unable to express the M protein was isolated from a patient and demonstrated that this protein is not required[125], whereas suppression of L or S expression impeded virion formation[122,123]. An L construct with an N-terminally fused secretion signal generating only the e-preS conformation was secreted as a component of subviral particles[126] but failed to support virion formation[58]. Apparently, the i-preS conformation of L exposing the preS domain at the cytosolic side of the ER was essential for nucleocapsid envelopment. This finding is compatible to a model where this part of the L protein contains regions (matrix domains) mediating a contact to the capsid required for budding.

In DHBV, the L protein influences the fate of cytoplasmic capsids[124,127]. If the L protein is absent, capsids deliver the viral genome to the nucleus like in the initial infection of the cell and amplify the intracellular viral genome copy number, whereas capsids are mainly secreted as enveloped virions when the L protein is present. This function mapped to aa 116 to 137 of the 161 aa long DHBV preS domain[128]. A similarly short linear stretch between aa 103 and 124 (or aa 92 and 113 depending on the genotype) genetically mapping in HBV preS was found to be important for virion formation[129]. The exchange of two adjacent aa by alanine residues in this area also prevented nucleocapsid envelopment.

Therefore, it is hypothesized that this part of L interacts with the capsid during envelopment and serves the function of a matrix domain similar to the cytoplasmic tail of the alpha virus E2 protein[130]. This model is supported by an HBV double point mutant where the I97L C protein mutation causing the envelopment of relatively immature capsids is suppressed by the A119F mutation in the putative matrix domain of L[131]. Also *in vitro* binding assays, using HBV envelope-derived peptides and liver-derived as well as recombinant capsids favour the model[132]. These results also suggest that the discrimination between immature and mature capsids might not occur on the level of capsid-envelope protein interactions. As aforementioned, this selection may happen during surface protein-independent membrane association of capsids.

The loop between the first and second transmembrane region in the S protein is also located at the cytoplasmic side of intracellular membranes and may establish a contact between envelope and capsid. Indeed, short deletions in the C-terminal half of this loop inhibited virion but not 20-nm particle formation[133]. However, point mutations (substitutions of two adjacent aa by alanine residues) in this area were not sufficient to block envelopment (V. Bruss, umpublished).

Potential binding sites on the capsid for envelope protein domains have also been mapped by mutational analyses. A screening of random insertions and deletions in the C protein[134] identified a few mutations allowing nucleocapsid formation and genome synthesis but blocking nucleocapsid envelopment[135]. A similar phenotype was found for naturally occurring HBV mutants isolated from chronically infected virus carriers[136]. Eleven point mutations generated on the basis of the crystal structure of the HBV capsid also induced a loss of nucleocapsid envelopment[121] (Figure 2). They are clustered around the base of the spike and in the grove between spikes. The minimal distance from the matrix domain in the preS region of L to the transmembrane region in the S domain of L allowing virion formation as mapped by deletion mutagenesis[137] is sufficient to allow the matrix domain to reach these sites on the capsid surface. Mutations at the tip or stem of the spike had no impact on capsid envelopment. However, HBV budding from transfected cells can be suppressed by a peptide binding to the tip of the spike[138,139], possibly by steric hindrance.

As in the case of subviral 20-nm particles, the incorporation of foreign proteins into the virion envelope is strictly suppressed. Host membrane proteins could not be detected in virions and even envelope proteins from avian hepadnaviruses do not mix. However, the L protein from WHV can substitute with low efficiency for the HBV L protein in HBV morphogenesis[98]. The matrix domains of WHV and HBV L protein are highly conserved in contrast to the matrix domains of DHBV and HBV L. Foreign domains can be integrated into the HBV envelope by fusion to the N terminus of the S protein and addition of an N-terminal secretion signal[122]. This configuration results in a transmembrane topology similar to the M protein with the preS2 domain substituted by the foreign

sequence. When coexpressed with wild-type virus, the chimeric protein is phenotypically mixed into virions and the foreign domain is exposed on the virus surface.

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TOPIC HIGHLIGHT

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Hepatitis B virus-induced oncogenesis

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world with an annual incidence of more than 500 000 in the year 2000. Its incidence is rising in many countries. Recently, it has been estimated that about 53% of HCC cases in the world are related to hepatitis B virus (HBV). The epidemiological association of HBV with HCC is well established. In recent studies, it was revealed that HBsAg carriers have a 25-37 times increased risk of developing HCC as compared to non-infected people. At present, HBV-associated carcinogenesis can be seen as a multi-factorial process that includes both direct and indirect mechanisms that might act synergistically. The integration of HBV DNA into the host genome occurs at early steps of clonal tumor expansion. The integration has been shown in a number of cases to affect a variety of cancer-related genes and to exert insertional mutagenesis. The permanent liver inflammation, induced by the immune response, resulting in a degeneration and regeneration process confers to the accumulation of critical mutations in the host genome. In addition to this, the regulatory proteins HBx and the PreS2 activators that can be encoded by the integrate exert a tumor promoter-like function resulting in positive selection of cells producing a functional regulatory protein. Gene expression profiling and proteomic techniques may help to characterize the molecular mechanisms driving HBV-associated carcinogenesis, and thus potentially identify new strategies in diagnosis and therapy.

Key words: Hepatitis B virus; Hepatocellular carcinoma; Regulatory proteins; Signal transduction

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INTRODUCTION

With an estimate of more than 500 000 incidences in the year 2000 hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors worldwide and its incidence is rising in many countries[1-4]. Despite being the 5th most frequent cancer in the world, HCC is the third leading cause of cancer death behind lung and stomach cancer. The high mortality associated with HCC is due to its unresponsiveness to treatment in many cases and symptoms of HCC often are recognized lately[5]. When viewed as estimated age-adjusted incidence rates of liver cancer per 100 000 men, the figures ranged as follows: in Asia, from 35.5 in Eastern Asia, 18.3 in South-eastern Asia to 5.6 in Western Asia; in Africa, from 24.2 in Middle Africa, 14.4 in Eastern Africa, 13.5 in Western Africa, 6.2 in Southern Africa to 4.9 in Northern Africa; in Europe, from 9.8 in Southern Europe, 5.8 in Eastern and Western Europe to 2.6 in Northern Europe; and to values of 4.8 in South America; 4.1 in North America; 3.6 in Australia/New Zealand and, finally, 2.1 in central America. In all regions, the rates recorded were two to three times higher in men than in women.

These significant differences in the geographic distribution of HCC incidence have led to identify chronic HBV infection as a leading risk factor for HCC[6-9]. Recently, it has been estimated that about 53% of HCC cases in the world are related to HBV[3]. The lifetime risk to develop a HCC was found to be increased even in patients that have cleared hepatitis B virus surface antigen (HBsAg) or with an occult HBV infection. Further risk factors include chronic HCV infection, exposure to aflatoxin B_1, alcohol abuse, obesity and diabetes. Aflatoxin B_1 (AFB_1) is a fungal metabolite that contaminates the food supply in certain areas of the world. It is produced by *Aspergillus flavus* and related fungi that grow on improperly stored foods, such as corn, rice and peanuts. AFB_1 requires metabolic conversion to its *exo*-8,9-epoxide in order to damage DNA. Coexistence of these risk factors, such as HBV and HCV infection or HBV infection and aflatoxin B_1, increases the relative risk of HCC development[11-13]. While a variety of risk factors have been identified in the last years, here a short review describing the current state of knowledge of the molecular pathogenesis of HBV-associated HCC

is given. A focus of this review will be on the role of the HBV-regulatory proteins in this process.

EPIDEMIOLOGY OF HBV-ASSOCIATED HCC

The epidemiological association of HBV with HCC is well established. In recent studies, it was revealed that HBsAg carriers have 25-37 times increased risk of developing HCC as compared to non-infected people[14,15]. Moreover, it was analyzed in more detail whether the viral status of the patients are correlated with the risk of developing HCC. HBV has been designated eight genotypes (A-H) based on genetic divergence. Each genotype has a distinct geographical and ethnic distribution. While genotypes B and C are prevalent in Asia, genotypes A and D occur frequently in Africa, Europe and India. There are conflicting data about the influence of HBV genotypes on HCC development[16-18]. Recent studies from Taiwan provide profound evidence for hepatitis B virus e antigen (HBeAg)-positive patients that HBV genotype C causes a more aggressive disease course as compared to genotype B[19-21]. On the other hand, there are reports from Taiwan describing that more than 50% of the HBV-related HCC patients are infected with genotype B. A study on Taiwanese pediatric patients with chronic HBV infection, who were followed for 15 years, showed that genotype B was identified in 74% of the children with HBV-associated HCC[22]. A further interesting observation is the prevalence of the T1762/A1764 mutation in the basal core promoter region which increases with the progression of liver disease. Since this mutation seems to be associated with HCC development, it might represent a helpful prognostic biomarker[23,24].

The risk of HCC seems to be elevated with increasing HBV viral load[25]. Therefore, it is important to consider that most epidemiological analyses were based only on HBsAg positivity. A recent study revealed that the relative risk of HCC was increased by 6-fold among patients who were positive for both HBsAg and HBeAg, compared to those who were positive for HBsAg alone[15]. Based on this, it can be concluded that HBeAg could be an additional useful marker for risk of developing HCC, since HBeAg reflects productive HBV replication.

DIRECT EFFECTS TRIGGERED BY THE INTEGRATION OF HBV-DNA INTO THE HOST GENOME

Integration is not essential for the viral replication but it allows persistence of the viral genome. Almost all of the HBV-associated HCCs harbor chromosomally integrated HBV DNA[26-28]. In many cases, these integrated viral genomes are characterized by rearrangements and/or partial deletions. HBV integration can induce deletions in the host chromosome at the integration site[29]. Based on these observations, it was tempting to speculate that the integration event *per se* causes a deregulation of key regulators of cell cycle control. This cis-hypothesis (place of integration = place of function) seems to be supported by the woodchuck hepatitis B virus (WHV)-related HCC. Here, insertions of WHV-DNA into the c-myc or, preferentially the N-myc2 gene, have been frequently detected[30-34]. However, in case of the HBV-associated HCC, site-specific integration of the HBV genome or integration of the HBV genome into known oncogenes seems to be a rare event. Interesting examples are the integration of HBV DNA in a cyclin A gene[35], in the retinoic acid receptor beta gene, in the mevalonate kinase gene or in the sarco/endoplasmic reticulum calcium ATPase1 gene[28,36].

It was recently confirmed, using a PCR-based approach, that HBV insertion into cellular genes is a frequent event that occurs early during HBV infection even after acute self-limiting hepatitis[37] and that integration can occur in genes regulating cellular signal transduction cascades, proliferation control and cell viability. Recently, hTERT (human telomerase reverse transcriptase) that is part of the telomerase ribonuclear protein complex was found to be targeted in different HBV-associated HCCs[28,38,39].

In light of these recent data, it will be an important issue to reconsider the role of the integration process for HBV-associated carcinogenesis. A helpful tool will be combining the analysis of putative HBV-specific integration sites with functional genomics of HBV-associated HCCs[40].

INDIRECT EFFECTS OF INTEGRATED HBV-DNA: HBX AND THE PRES2 ACTIVATOR FAMILY

HBx

In most integrated subviral HBV genomes, the open reading frame for HBx or PreS2 regulatory protein is conserved and can be transcribed[41]. The HBx gene is conserved among all mammalian hepadnaviruses. HBx is a small polypeptide (17 kDa) that is produced at very low levels during chronic and acute hepatitis. Recently, a HBx-like regulatory protein was identified for duck hepatitis B virus (DHBV)[42]. Since the time when HBx initially was described to act as a transcriptional activator[43,44], a variety of functions have been ascribed to the still enigmatic HBx[45,46]. While the X protein is essential for viral replication in case of WHV[47], there are conflicting results about the relevance of HBx for the viral life cycle in case of HBV. There are reports describing that expression of the viral genome occurs independently from HBx functionality[48-50]; other papers describe a relevance of HBx for HBV replication[51]. In transgenic mouse models harboring an overgenomic HBV integrate, it could be observed that HBV replication does not depend on the presence of a functional HBx[52]. Comparable results were obtained in cell culture models based on huh-7 cells[48,50] while in case of HepG2 cells a reduction in HBx-deficient HBV genomes could be observed[53,54]. Moreover, infection experiments of primary *tupaia* hepatocytes revealed that HBx-deficient HBV particles are infectious (J. Köck, personal communication).

HBx activates a broad variety of different promoter elements. Based on the pleiotropic nature of the HBx-dependent transcriptional regulation, it was concluded that HBx interferes with signaling cascades upstream from the transcription complex. These signaling cascades trigger activation of transcription factors like AP-1 (activator protein-1), NF-κB (nuclear factor kappa B), SP1, and oct-1[46,55]. HBx affects the expression of a variety of genes that are involved in the control of the cell cycle, proliferation or apoptosis. From the beginning, HBx was considered as a crucial viral protein for the process of HBV-associated carcinogenesis[45,56-58] and this might have affected the focus of HBx research. In light of the putative role of HBx for viral carcinogenesis, the major focus of many research projects has been and is the interference of HBx with signal transduction cascades that affect the control of the cell cycle, proliferation or apoptosis. However, one should consider that selective over-expression of HBx reflects a situation that is different from the situation in an infected cell expressing the complete HBV genome. For example, it is well established that HBx is able to promote cellular proliferation[59]. On the other hand, it was shown that expression of the complete HBV genome that harbors the HBx and the PreS2 regulatory protein inhibits cell cycle progression[60] (Figure 1).

The analysis of HBx/protein kinase C (PKC) interaction is such an example for many reports analyzing the interference of HBx with signaling cascades and correlating this with a putative role of HBx for HBV-associated carcinogenesis. There are conflicting reports about the interference of HBx with PKC signaling. On the one hand, there are reports describing an HBx-dependent activation of PKC, mediated by an elevated DAG level in HBx-producing cells. In these studies, PKC is considered as an essential factor for the HBx-dependent activation of NF-kB or AP-1[61,62]. Other reports provide evidence that HBx neither affects activity of PKC nor that PKC is essential for HBx-dependent transcriptional activation[63-65]. The interesting aspect of an HBx-dependent activation of PKC is that a conclusive model for the role of HBx in the process of HBV-dependent carcinogenesis can be deduced. According to the two-step model of carcinogenesis[66] (initiation and promotion), the HBx-dependent activation of PKC could exert a tumor promoter-like function[61]. Independent from the point whether or not the HBx-dependent activation of PKC exerts a tumor promoter-like function, there is profound experimental evidence from experiments with transgenic mice that HBx indeed could exert a tumor promoter-like function. Irradiation of HBx transgenics or exposure of these transgenics to mutagens (diethylnitrosamine) caused a significant increase in the amount of pre-neoplastic lesions as compared to the wild-type control animals[67,68]. Apart from this, a variety of HBx transgenic mouse models were established, but only in one model system so far direct formation of liver cancer could be observed[69].

A tumor promoter-like function of HBx does not necessarily require an activation of PKC. Other pathways as the activation of c-Raf-1-MEK/MAP2 (mitogen-activated protein kinase 2) kinase cascade could fulfill this function as well. Starting with the finding that HBx increases the Ras/GTP complex formation and thereby activates the c-Raf-1 signal transduction cascade[70], more and more data were collected elucidating the interference of HBx with signaling cascades upstream of Ras. One of the next steps was the observation that Src is activated in HBx-producing cells[71,72], followed by the observation that HBx is able to activate the cytosolic Ca^{2+}-dependent praline-rich tyrosine kinase 2 (Pyk)[54]. Pyk is able to activate Src. A recent report describes an HBx-dependent activation of FAK (focal adhesion kinase), a well known regulator of Src kinases[53]. The activation of these signaling cascades requires the presence of HBx in an extranuclear compartment. On the other hand, there is evidence that a fraction of HBx is localized within the nucleus. The subcellular distribution of HBx is still a matter of debate. There are reports providing evidence that HBx is localized in the cytoplasm as well as in the nucleus[49,73]. The different localizations are associated with different functions. HBx localized in the cytoplasm is able to modulate intracellular signal transduction cascades as described above. Moreover, an association of HBx with the outer membrane of mitochondria that induces oxidative stress was described[74-76]. HBx localized in the nucleus is suggested to interfere directly with transcription factors or to exert a transcription factor-like function. A direct interaction with CREB (cAMP responsive element–binding protein) and ATF-2 (activating transcription factor 2) resulting in their increased DNA binding affinity[77] was reported as well as an interaction with RNA polymerase Ⅱ in the transcription complex[78,79].

In addition to the interaction of HBx with the transcription machinery, there is evidence that HBx interferes at multiple steps with DNA repair and so confers to an increase of critical mutations. HBx was found to bind to DDB1[80,81], a subunit of the damaged DNA binding protein that is bound to damaged DNA, the first step in nucleotide excision repair. In cell culture experiments indeed the expression of HBx significantly inhibited the ability of cells to repair damaged DNA.

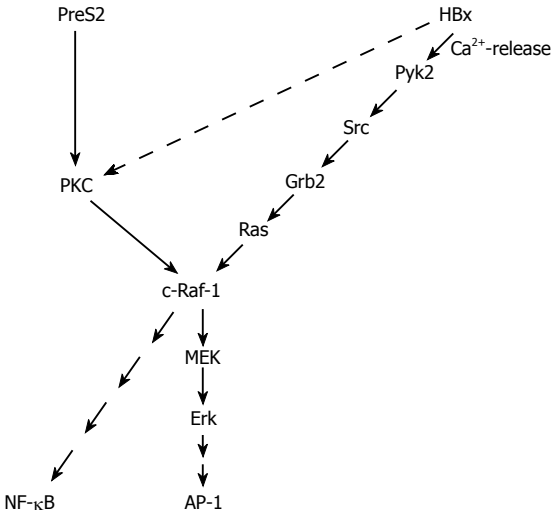

Figure 1 Major signaling pathways activated by the regulatory proteins HBx and PreS2 of HBV.

Therefore, it was tempting to speculate that HBx could confer by this to an increase in the amount of critical mutations in the host genome[80]. However, analysis of mutation frequency in HBx transgenic mice did not corroborate this hypothesis[82]. Other reports focus on the interaction of HBx with p53. On the one hand, it has been shown an indirect inhibition of p53 by HBx: HBx causes a transcriptional repression of the human p53 gene[83]. On the other hand, there is evidence for the capacity of HBx to bind to p53[84,85]. However, if the intracellular amounts of HBx and p53 are considered, there exists a tremendous excess of p53 as compared to HBx in the hepatocytes. The physiological significance of the direct p53/HBx interaction remains questionable.

The family of the PreS2 activators

Apart from the HBx-regulatory protein, the HBV genome encodes a second family of regulatory proteins: the PreS2 activators. Based on a subcloned HBV integrate of the human hepatoma cell line huH4[86] and of an integrate isolated from an HBV-associated HCC[87], preS/S genes that were truncated at the 3´ end were identified[41]. These preS/S^t genes encoded for C-terminally truncated surface proteins (MHBs^t) that display a regulatory protein function. Initial analysis revealed that generation of the regulatory protein function requires at least deletion of the last transmembrane region in the S-domain (transmembrane region 3)[88-91]. This results in C-terminally truncated MHBs molecules that are endoplasmic reticulum (ER)-membrane associated by the remaining transmembrane regions I and Ⅱ of the S domain[92,93]. A prototype of the ER-membrane-associated MHBs^t activator is encoded by the integrate isolated from the human hepatoma cell line huH4[86]. This integrate is truncated at ntHBV 221 of the HBV genome resulting in a C-terminally truncated MHBs protein at amino acid (aa) 76 (MHBs^{t76}). A detailed analysis revealed that a variety of differences exist between the structural protein MHBs and its C-terminally truncated variant MHBs^t. In contrast to the structural protein MHBs and the regulatory variants, MHBs^t are not secreted and lack the glycosylation at asparagine (asn) 4 of the PreS2 domain[92]. The intracellular retention of ER-membrane-associated MHBs^t proteins gave raise to the hypothesis that the observed activator function is due to ER stress, induced by intracellular retention and subsequent accumulation in the ER[93-96]. More detailed analysis revealed, however, that the structural protein MHBs and the regulatory protein MHBs^t differ in the topology of the PreS2 domain[97]. In case of the structural protein, the PreS2 domain faces the lumen of the endoplasmic reticulum and in accordance with this glycosylation at asn 4 can occur. In case of the activator protein MHBs^t, the PreS2 domain directs into the cytoplasm. This explains the lack of N-glycosylation at asn 4. The PreS2 domain facing the cytoplasm interacts with cytosolic binding partners, thereby triggering intracellular signal transduction cascades. In accordance with this, a minimal PreS2 activator was identified lacking any membrane insertion domain (MHBs^{t55})[88,67,98]. This minimal activator encompasses the complete PreS2 domain and is localized within the cytoplasm. Since the PreS2 domain is sufficient to exert the regulatory protein function, this class of regulatory proteins was designated PreS2 activator. The family of PreS2- regulatory proteins encompasses the membrane-associated regulatory proteins, such as MHBs^{t76} or MHBs^{t167}, and the non-membrane-associated short proteins, such as PreS2 domain (MHBs^{t55}). There is no functional difference between the ER and the cytoplasmatically localized Pres2 activators clearly arguing against the ER-overload hypothesis[97].

The PreS1-PreS2 domain of the large hepatitis B virus surface protein (LHBs) displays a dual membrane topology[99-101]. In one fraction of LHBs, the first transmembrane region that is located at the beginning of the S-domain (aa 8-21) is used: in this case, the PreS1-PreS2 domain of LHBs faces the lumen of the endoplasmic reticulum. In case of the other fraction, this transmembrane region is not used, resulting in a PreS1-PreS2 domain that directs into the cytoplasm. As described above, the cytoplasmic orientation of the PreS2 domain in case of the MHBs^t proteins is causative for their regulatory protein function. In accordance with this, LHBs displays a regulatory protein function[102] and belongs to the family of PreS2 activator proteins.

The PreS2 activators bind PKC-α in the cytoplasm. This interaction with PKC results in a DAG (1, 2, sn diacylglycerol)-independent activation of PKC and phosphorylation of the PreS2 domain. The activation of PKC is transduced by the c-Raf-1/MEK/ERK (extracellular signal-regulated kinase) signal transduction cascade[63]. This signal transduction cascade can exert a tumor promoter-like function according to the classical two-step model of carcinogenesis[66]. Indeed, transgenic mice expressing the PreS2 activator MHBs^{t76} develop liver tumors at an age above 10 mo. Although the MHBs^{t76} protein is produced in very small but clearly detectable amounts in the MHBs^{t76} transgenic mice, a permanent activation of the Raf-1/MEK/ERK signal transduction cascade can be observed, resulting in an increased proliferation rate of the hepatocytes. The fact that MHBs^{t76} is produced in very small amounts ensures that the observed effects are not due to any overload-associated effects. The tumor formation in these mice can be explained by the permanent activation signal transduction cascades that exert a tumor promoter-like function[63]. Since tumor formation is observed in older animals, it can be assumed that during the aging process critical mutations are accumulated (initiation) and then the tumor promoter function positively selects these cells.

In case of the LHBs-transgenics, tumor formation can be observed as well[103]. In these mice, a very strong overproduction of the LHBs protein occurs, resulting in an intracellular accumulation of the protein and subsequent formation of ground glass hepatocytes. This permanent accumulation results in a situation comparable to a storage disease. Tumor formation in these transgenics was explained by the resulting permanent inflammation[103-105]. In light of the observation that LHBs can act as a regulatory protein, however, the regulatory protein function that is immanent to LHBs should be considered as an additional factor conferring to tumor formation in these mice. The overload-associated stress

and inflammation results in the formation of critical mutations (initiation) and the permanent activation of the PKC/Raf/MEK/ERK signal transduction cascade which exerts a tumor promoter-like function.

Immune pathogenesis of HCC

A major factor in the process of HBV-associated HCC development is the immune system[104,106,107]. The relevance of a chronic, virus-specific immune response for development of HBV-associated carcinoma was shown in an elegant experiment from F. Chisari`s laboratory[108]. Transgenic mice that produce non-cytopathic amounts of HBsAg were used. In these mice, immunologic tolerance against the transgene product can be observed. In accordance with this, no evidence of the liver disease was observed. These mice were subjected to thymectomy and lethally irradiated. One group was reconstituted with the bone marrow and spleen cells derived from non-transgenic littermates that were vaccinated with a recombinant HBsAg encoding vaccinia virus resulting in HBsAg-specific cytotoxic T lymphocytes (CTLs) and antibodies. The other group was reconstituted with the bone marrow and spleen cells derived from transgenic donors that were immunologically tolerant.

In this animal model, the development of hepatitis and later of chronic hepatitis and finally HCC development could be exclusively observed in the mice that were reconstituted with the bone marrow and spleen cells derived from the vaccinated non-transgenic animals, but not in the control groups. Based on this, it was concluded that the immune system-mediated chronic inflammation of the liver, continuous cell death and subsequent cell proliferation might increase the frequency of genetic alterations and the risk of cancer[104,109-111]. This scenario is not exclusively restricted to HBV. Chronic inflammation, degeneration and regeneration are common to a variety of human liver diseases, such as glycogen storage disease or alcoholism or HCV infection, that can finally result in liver carcinoma development[5]. This means that an ineffective immune response can be the principal oncogenic factor during a chronic HBV infection in man. In other words, the same T-cell response can have complete different effects: if the T cell response is strong enough, HBV can be eliminated from the liver, if not, a pro-carcinogenic effect can be induced by permanently triggering necro-inflammatory disease without resulting in a final eradication of HBV from the liver. An interesting aspect is that the nucleoside analogue on lamivudine in patients with chronic hepatitis B can induce the recovery of antiviral T cell responses. However, restoration of HBV-specific T cell reactivity is only transient. The transient nature of the immune reconstitution may represent a favorable condition for virus reactivation once lamivudine therapy is withdrawn.

CONCLUSION

At present, HBV-associated carcinogenesis can be seen as a multi-factorial process that includes both direct and indirect mechanisms that might act synergistically. The integration of HBV DNA into the host genome occurs at early steps of clonal tumor expansion. The integration has been shown in a number of cases to affect a variety of cancer-related genes and to exert insertional mutagenesis. The permanent liver inflammation resulting in a degeneration and regeneration process confers to the accumulation of critical mutations in the host genome. In addition, the regulatory proteins HBx and the PreS2 activators that can be encoded by the integrate can exert a tumor promoter-like function, resulting in positive selection of cells producing a functional regulatory protein.

Based on new technologies, including gene expression profiling and proteomics, it should be possible to further reveal the molecular mechanisms underlying HBV-associated HCC development and to identify novel diagnostic markers as well as therapeutic and preventive targets.

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TOPIC HIGHLIGHT

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Pathogenesis of hepatitis B virus infection

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Abstract

Infection with hepatitis B virus (HBV) leads to a wide spectrum of clinical presentations ranging from an asymptomatic carrier state to self-limited acute or fulminant hepatitis to chronic hepatitis with progression to cirrhosis and hepatocellular carcinoma. Infection with HBV is one of the most common viral diseases affecting man. Both viral factors as well as the host immune response have been implicated in the pathogenesis and clinical outcome of HBV infection. In this review, we will discuss the impact of virus-host interactions for the pathogenesis of HBV infection and liver disease. These interactions include the relevance of naturally occurring viral variants for clinical disease, the role of virus-induced apoptosis for HBV-induced liver cell injury and the impact of antiviral immune responses for outcome of infection.

Key words: Host response; Viral hepatitis; Mutants; Pathogenesis; Resistance

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MOLECULAR VIROLOGY OF HEPATITIS B VIRUS INFECTION

Hepatitis B virus (HBV) is a small DNA virus and belongs to a group of hepatotropic DNA viruses (hepadnaviruses)[1,2]. The virus consists of a nucleocapsid and an outer envelope composed mainly of three hepatitis B surface antigens (HBsAgs) that play a central role in the diagnosis of HBV infection. The nucleocapsid contains hepatitis B core antigen (HBcAg), a DNA polymerase-reverse transcriptase, the viral genome as well as cellular proteins[1,2].

The genome consists of a partially double-stranded circular DNA molecule of about 3200 base pairs in length with known sequence as well as genetic organization. The pre-surface 1 [pre-S1]/pre-surface 2 [pre-S2]/and surface genes [S] code for the various HBsAgs. The protein encoded by the pre-core [pre-C]/core gene [C] undergoes post-translational modification to yield hepatitis B e antigen (HBeAg), which is a seromarker for high viral replication[2]. The core gene codes for HBcAg, the major structural protein of the nucleocapsid. Finally, the X gene codes for the hepatitis B x antigen (HBxAg). HBxAg has been shown to be a potent transactivator of cellular and viral genes. A variety of interactions with cellular proteins has been proposed as potential targets of HBxAg. The precise functions of HBxAg, however, in the viral life cycle and the natural course of HBV infection remain to be established. In addition to the known viral genes, several *cis*- and *trans*-acting genetic elements involved in the fine control of gene expression, RNA packaging and viral replication have been identified[1,2].

The viral DNA polymerase-reverse transcriptase is encoded by the polymerase gene [P] and is of central importance for viral replication. Different from all known mammalian DNA viruses, hepadnaviruses replicate *via* reverse transcription of a RNA intermediate[3,4], the pregenomic RNA, which is a strategy central to the life cycle of RNA retroviruses. Similarities and differences between retroviral and hepadnaviral replication have been defined[1]. Based on the unique replication cycle of HBV, antiviral therapeutic strategies aimed at the reverse transcription of HBV RNA or at HBV reverse transcriptase have been successfully used as antivirals to treat HBV infection[5-13].

VIRAL VARIANTS AND PATHOGENESIS OF INFECTION

Evidence has been accumulating that certain HBV mutants are associated with unique clinical manifestations, may affect the natural course of the infection and confer

resistance to antiviral agents (Table 1)[14-17]. Naturally occurring mutations in the context of various genotypes have been identified in the structural and non-structural genes as well as regulatory elements of the virus. The best characterized mutants are the pre-core (pre-C) stop codon mutations resulting in a loss of hepatitis B e antigen[18], defined clusters of mutations in the core promoter resulting in enhanced viral replication[19-21], and mutations in the reverse transcriptase/polymerase genes conferring resistance to antivirals[16,22]. Furthermore, several mutations in the HBV surface gene have been identified which alter the antigenicity of the viral surface proteins (HBsAg) and structure of the viral envelope[15,23].

Table 1 HBV variants and their potential impact for pathogenesis of HBV infection

HBV region	Mutation	Molecular phenotype	Clinical relevance
Pre-S/S	Pre-S1/ pre-S2/ S-promotor	Misassembly	Fibrosing cholestatic hepatitis
	S	Alteration of B- and T-cell epitopes	Vaccine escape
	S splicing		Immune escape
			Diagnostic escape
Pre-C	Pre-C-stop	Loss of HBeAg	Severe hepatitis
			HBeAg-deficiency
Core	Core	Alteration of T-cell epitopes	Viral persistence
			Severe hepatitis
RT/Pol	Pol	Replication deficiency	Viral latency
			Viral persistence
	Pol	Resistance to antivirals	Therapy escape
Regulatory Elements	Core promotor	Enhanced replication and core expression	Severe hepatitis
			Modulation of drug resistance
		Decreased HBeAg synthesis	HBeAg seronegativity
	Enhancer I	Decreased replication	Chronic hepatitis

HBV genomic region, mutation, molecular phenotype and clinical relevance are indicated.

HBeAg variants and HBeAg-seronegativity

One of the first HBV mutations clinically recognized and functionally characterized was the pre-C stop codon mutation, resulting in a loss of HBeAg. Not all patients with chronic hepatitis B become HBV DNA negative, despite seroconversion from HBeAg to anti-HBe. Numerous studies have shown that these patients are infected with a pre-C/C mutant[24,25]. This mutant has a translation stop codon at the end of the pre-C gene. Thus, the pre-C/C fusion protein, a precursor of HBeAg, cannot be synthesized. In these patients, therefore, viral replication may persist despite elimination of HBeAg and seroconversion to anti-HBe. While the loss of HBeAg appears irrelevant for the biology of the virus, it may play an important role in the interaction of the virus with the immune system. Secreted HBeAg has been proposed to have an immunoregulatory function *in utero* by establishing T-cell tolerance to HBeAg and HBcAg that may predispose neonates born to HBV-infected mothers to develop persistent HBV infection[26]. Recent studies have further demonstrated an immunomodulatory role of HBeAg in antigen presentation and recognition by $CD4^+$ T-cells[27]. The selection of HBeAg mutants in the host may be due in part to immunomodulatory properties of HBeAg resulting in a survival advantage for the virus[28]. Whether and how this mutation - either alone or in combination with other mutations - affects the clinical course of HBV infection is still unclear.

Of interest in this respect is the observation that pre-C stop codon mutants are found not only in patients with fulminant hepatitis[18,29-32] or chronic active hepatitis B[24,25,33-35], but also in asymptomatic HBV carriers[32] or acute, self-limited hepatitis [36]. In the woodchuck model, the pre-C stop codon mutation was found to exert no effect on viral replication or the severity of liver disease. Infections with the pre-C stop codon mutant, however, did not take a chronic course[37]. Interestingly, in the duck hepatitis B virus model the pre-C stop codon mutant replicates less well and is overgrown by wild-type virus during the natural course of coinfection[38].

Core promoter variants and enhanced viral replication

During the last couple of years mutations have been identified in regulatory genetic elements of the HBV genome. Several independent studies have identified and functionally characterized distinct mutations clustered in enhancer Ⅱ of the HBV core promotor. Core promoter mutations are predominantly found in patients with a more aggressive course of disease such as fulminant[19,39-41] or chronic hepatitis B[21,33,42-45]. Some of the patients have a decrease or loss of HBeAg[39,43].

A common hallmark of core promoter mutations is the biological phenotype of enhanced viral replication in transfected hepatoma cell lines[19,21,33,39-44] and primary hepatocytes[20]. The most prevalent mutant comprises a double mutation (A to T at nucleotide 1764 and G to A at nucleotide 1766, nucleotide numbering according to[46] located at the 3`end of enhancer Ⅱ of the basal core promotor being present in up to 80% of individuals chronically infected with HBV[47].

Several other core promotor mutations in immuncompromized patients and severe or fulminant liver disease have been identified[41,42,45]. A common phenotype of these mutations seems to be the enhanced viral encapsidation by altering the balance between pre-C and C RNA transcript levels[42]. Several of these mutations have been shown to create additional transcription factor (HNF-1, HNF-3, HNF-4 or C/EBP) binding sites[41,42,48]. The magnitude of mutant-induced enhancement of viral replication seems to be dependent on the HBV subtype/genotype[40,49].

The phenotype of enhanced viral replication may be the reason why core promoter mutants seem to be selected in immunosuppressed patients or patients with chronic hepatitis. Interestingly, one study suggested the presence of core promotor mutations was significantly associated with the development of hepatocellular carcinoma (HCC)[50]. Furthermore, the high-replication phenotype of viral strains containing core promotor mutations may play a role in the pathogenesis of more aggressive or severe disease associated with these mutations. Interestingly, the

transmission of a high-replication strain containing two core promotor mutations and the pre-C mutation to HBV-naive patients has resulted in an outbreak of fulminant fatal hepatitis[19]. Enhanced viral replication with the concomitant increase in viral protein expression may result in a differential immune response as well as a more rapid and widespread infection in naive patients[19,20,40]. Altered viral kinetics accompanied by a more vigorous cellular immune response may be important mechanisms resulting in more severe liver injury and potentially fulminant hepatitis. Another factor contributing to fulminant hepatic failure associated with defined core promoter mutations may be hepatocyte apoptosis induced by the HBV variant[20]. Since not all patients with fulminant hepatitis are infected with HBV strains exhibiting a high-replication phenotype[51] additional mechanisms for the pathogenesis of fulminant hepatitis likely exist[52].

Variants of HBsAg and immune escape

A variety of mutations have been identified in the HBV structural genes resulting in differential antigen recognition and immune response[53]. During the course of a passive-active HBV immunization program in southern Italy, several children were infected with HBV despite a primary response to the HBsAg vaccine. Molecular analyses showed that one of these children was infected with a HBV mutant[54,55]. This mutant exhibited a defect in the S region of the HBV genome (glycine (Gly) to arginine (Arg) at amino acid position 145) with loss of the group-specific antigenic determinant a, which is the main target of the vaccine response. Further biological characterization of this mutant in the context of replication-competent viral genomes revealed that this mutation also results in impaired S secretion and decreased virion stability[56]. This viral mutant was able to escape the immune surveillance and thereby resulted in an infection despite the presence of anti-HBs antibodies ('vaccine escape mutant'). Similar mutants have been detected in Japan[57] and the Gambia[58] and presumably occur world-wide[53]. Consistent with these initial observations, a recent study demonstrated the accumulation of HBsAg a determinant mutants during the implementation of universal vaccination programs in Taiwan[59]. In contrast to these epidemiological observations, a study in the chimpanzee model using currently available US-licensed HBV vaccines demonstrated protection against the 'classical' vaccine escape (Gly-145-Arg) mutant *in vivo*. This study provides strong evidence that immunization with recombinant HBV vaccines stimulates anti-HBs that is broadly reactive and protects the host efficiently from infection with HBV strains containing the Gly-145-Arg mutant[60]. Since this study only evaluated the protective properties of licensed HBV vaccines against the Gly-145-Arg mutant, it does not exclude the possibility that other genuine vaccine escape mutants result in immunization failure[60]. Further studies are needed to address this issue. In addition to immune escape on the B cell level, vaccine escape mediated by defined HBsAg epitopes can occur on the T cell level[61]. Taken together, these findings indicate, that careful epidemiologic monitoring of vaccine failure caused by infection with HBV mutants may be crucial for the success of global immunization strategies.

'Immune escape mutants' have also been reported in patients after liver transplantation for HBV-related chronic liver disease who had received monoclonal or polyclonal anti-HBs antibodies to prevent reinfection of the graft[62-65]. 'Immune escape mutants' have also been identified in anti-HBs positive individuals[57,66].

In addition to 'vaccine escape mutants' and 'immune escape mutants', 'diagnosis escape mutants' have been described, since most of the HBsAg detection assays are based on anti-HBs antibodies[57,67]. Diagnostic escape may also be the result of a posttranscriptional effect of viral mutations on HBsAg expression as described by Hass *et al* in an elegant study isolating a naturally occurring mutation targeting splicing of subgenomic RNA[68,69]. The emergence of these variants may potentially contribute to occult HBsAg-negative HBV infection[70].

Mutations have further been detected in the pre-S1 or pre-S2 regions of the HBV genome. Their clinical significance is as yet unknown, however. Since the preS1 region appears to be important for the very first steps of viral infection[71,72], it is conceivable that variants affecting this region may also affect the biological phenotype of the virus by altering its ability to bind or infect hepatocytes. A pre-S2 defective HBV variant has been associated with fulminant hepatitis B[73]. A cluster of mutations in the S promotor (two deletions and a point mutation in the regulatory element CCAAT) isolated from a patient with fibrosing cholestatic hepatitis after HBV reinfection of the transplanted liver has been shown to result in virus retention and misassembly[74,75]. Furthermore, a recent study demonstrates evidence that patients with progressive liver disease have a higher frequency of pre-S deletion[76]. HBsAg variants resulting in a defect or impairment in virion secretion have also been described in severe and fulminant hepatitis B[56,77].

Mutations conferring resistance to antiviral therapy

In recent years polymerase gene mutants have been identified in patients treated with antiviral drugs, resulting in drug resistance, respectively[78]. Lamivudine, a nucleoside analogue, is a potent inhibitor of HBV replication and is clinically used as an antiviral for the treatment of chronic hepatitis B and advanced HBV-induced liver disease[11,12,79,80]. Adefovir is an alternative nucleotide analogue previously licensed for the treatment of chronic hepatitis B[9,10]. Other nucleoside analogues currently under clinical investigations include entecavir, emtricitabine, clevudine and telbivudine[16,78]. Several of these drugs act as chain-terminators during the synthesis of the nascent DNA strand, thereby terminating viral replication[16]. Other nucleoside or nucleotide analogues such as adefovir and entecavir interfere with priming and minus-strand DNA elongation[16]. Adefovir and-to a lesser extent-lamivudine also target initial plus-strand DNA repair[81].

The selection of drug resistant mutants depends on several factors. As the viral polymerase is subjected to a spontaneous error rate, viral mutants accumulate during the natural course of the disease. When an antiviral pressure is applied, the mutations exhibiting the best replication capacity in the presence of the drug

are selected. The mutant spread depends on its level of intrinsic resistance and on its replicative fitness[16]. This may explain in part why the peaks of drug resistance observed with lamivudine (23% at year one and up to 65% at year five of treatment[82,83]) and with adefovir dipivoxil (2% at two years and 3.9% at three years of treatment[84,85]) are different.

These mutant viral genomes are characterized by selective amino acid changes in various domains of the HBV reverse transcriptase/polymerase. In particular, lamivudine resistance has been extensively studied. The reverse transcriptase polymerase of HBV and HIV share a common and highly conserved tyrosine, methionine, aspartate, aspartate (YMDD) nucleotide-binding motif in the catalytic domain of the enzyme. Similar to the development of lamivudine-resistant HIV mutants, lamivudine treatment of patients with chronic HBV infection results in drug-resistant strains, characterized by YMDD to YIDD (tyrosine, isoleucin, aspartate, aspartate; "M204I") or YVDD (tyrosine, valine, aspartate, aspartate; "M204V") mutations in the catalytic C-domain of the reverse transcriptase[16,86]. Interestingly, the mutant genomes exhibited lower levels of replication when compared with wild-type genomes[87]. Further studies identified additional mutations in the neighboring B domain (V173L and L180M[88]). Interestingly, these mutations can restore partially the replication capacity of the C-domain mutations[88].

In contrast to lamivudine, viral resistance to adefovir appears to be mediated pre-dominantly by a mutation in the D-domain[84]. The N236T mutation confers only a 5-10-fold resistance to adefovir[84], which may explain the delayed emergence of this mutant[16]. Another mutation A181V located in the B-domain has been described[16]. Three recently isolated cases of primary adefovir resistance were due to a mutation comprising I233V. Interestingly, the viral variants containing this mutation were sensitive to tenofovir[22]. Drug resistance due to viral mutations in the reverse transcriptase/polymerase represents an important clinical issue in the management of chronic hepatitis B and may ultimately require the development of novel treatment approaches including the combination of various antiviral strategies[16,78].

In summary, a large variety of HBV mutations associated with various pathological conditions as well as drug resistance have been isolated and described in detail[15]. Studies *in vitro* as well as *in vivo* have defined the functional relevance of several viral mutants. Furthermore, these studies provided important clues for the understanding of the impact of these mutants on the pathogenesis of disease and the molecular characterization of drug resistance. Functional studies of mutants in established and emerging HBV *in vivo* models[89] may ultimately allow confirmation of the relationship between defined mutations and their clinical relevance[15].

MECHANISMS OF HBV-INDUCED LIVER DISEASE: APOPTOSIS

The induction of apoptosis is a hallmark of many viruses infecting humans. Although HBV is considered as a non-cytopathic virus[14], hepadnavirus-induced apoptosis and cytopathic effects have been described in several experimental model systems: First, a duck hepatitis B variant containing a single amino acid change in the large surface antigen resulting in accumulation of cccDNA resulted in a strong cytopathic effect in hepatocytes in *vitro* and *in vivo*[90-92]. In this system, the level of viral replication and cccDNA formation correlated with cytopathic effects in infected hepatocytes[90]. Second, intracellular retention of the HBV large surface protein has been shown to induce apoptosis in cell lines[93,94]. In this model overexpression of the large surface antigen resulted in cellular vacuolization and apoptosis of transfected hepatoma cells[94]. Third, the HBX protein has been suggested to induce apoptosis in both a p53-dependent and p53-independent manner[95-97]. Exploring the mechanism of these previous observations, a recent study has elegantly demonstrated that HBX interacts with c-FLIP, a key regulator of the death-inducing signaling complex[96]. Recruitment of c-FLIP to the death-inducing signaling complex is inhibited by HBX resulting in hyperactivation of caspase-8 and caspase-3 by death signals[96]. Finally, a viral variant containing two core promoter mutations associated with fulminant hepatitis has been shown to induce apoptosis in primary Tupaia hepatocytes[20]. Interestingly, in the latter model induction of apoptosis was independent of viral replication suggesting that viral protein synthesis was sufficient for the virus-induced hepatocyte cell death. Since the two core promoter mutations resulted in two amino acid changes of the HBX protein, the HBX protein may be a potential candidate mediating this effect[20]. Further studies in animal model systems are needed to elucidate the impact of HBV-induced apoptosis for HBV-induced liver injury[20].

IMMUNOPATHOGENESIS OF HBV INFECTION

Apart from direct biological effects of viral variants (Table 1) there is growing consensus that the host immune response, especially the virus-specific T cell response[98], is the key determinant influencing the course of disease and the onset of liver disease.

Successful immune response.

A genomic array analysis using liver RNA obtained at multiple time points after HBV infection in chimpanzees demonstrated that HBV did not induce any genes during entry and expansion[99], suggesting it is a stealth virus early in the infection. In contrast, a large number of T cell-derived IFN-gamma-regulated genes were induced in the liver during viral clearance[99], reflecting the impact of an adaptive T cell response that inhibits viral replication and kills infected cells, thereby terminating the infection. These results are in agreement with several studies performed in acutely infected patients suggesting an important role of the adaptive T cell response[98,100-105]. Indeed, several studies have shown that the peripheral blood cytotoxic T lymphocyte (CTL) response to HBV is polyclonal and multispecific in patients with acute viral hepatitis and that

it persists indefinitely after recovery, when it is maintained by continued antigenic stimulation by residual virus that persists, apparently harmlessly, in healthy convalescent individuals[106]. In contrast, the CTL response to HBV is relatively weak in patients with chronic HBV infection, except during spontaneous disease flares or interferon (IFN) induced recovery, when it is readily detectable[101,104]. These earlier studies have been confirmed by using new techniques, such as the tetramer technology. By using this approach, Maini *et al*[100] could, for example, show that multispecific HBV-specific $CD8^+$ T cell responses are detectable during the acute phase of self-limited infection and decline thereafter. Of note, a recent study could also demonstrate that HBV-specific $CD8^+$ T cells accumulate in the infected organ, i.e., the liver, where they remain detectable at high frequencies even after HBsAg seroconversion[107].

Studies in the HBV transgenic mouse model revealed that, in addition to causing viral hepatitis, virus-specific T cells as well as NK and NKT cells can abolish HBV expression and replication without killing the hepatocytes and that this antiviral activity is mediated by interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα)[104,108,109] (Figure 1). Importantly, studies in the chimpanzee model indicated that the same events are operative during natural infection. Indeed, in these studies it was shown that the early phase of HBV clearance was temporally associated with the appearance of CD3, CD8 and IFNγ in the liver, which reflects the influx of T cells into the liver[110]. Of note, there was only limited liver disease during this time although almost 100% of hepatocytes were infected, clearly suggesting that noncytopathic mechanisms were active during this early phase of viral clearance. In contrast, the final elimination of the virus that occurred several weeks later occurred in the presence of significant liver disease, indicating the presence of cytopathic effector functions that are probably mediated by virus-specific T cells[110].

To determine which subset of T cells is responsible for viral clearance and liver disease, the course of HBV infection in chimpanzees that were depleted of its $CD4^+$ or $CD8^+$ cells was monitored[111]. Depletion of $CD4^+$ cells did not significantly alter the course of infection. In contrast to the minor effects observed during CD4-depletion, CD8-depletion greatly prolonged the infection and delayed the onset of viral clearance and liver disease until $CD8^+$ T cells reappeared in the circulation and virus-specific $CD8^+$ T cells entered the liver[111]. Indeed, in the absence of $CD8^+$ cells, the duration of peak infection was prolonged and the time of onset of the initial decrease in HBV DNA levels and increase in serum ALT activity was delayed. In addition, the time required for both the first phase of viral clearance and for the final elimination of the virus was markedly delayed and prolonged in the CD8-depleted animal. It is important to note that the reappearance of $CD8^+$ cells correlated with the appearance of IFNγ producing virus-specific $CD8^+$ T cells in the liver, the onset of a mild liver disease, the appearance of IFNγ mRNA in the liver and a 50 fold reduction in total liver HBV DNA[111]. These results suggest that HBV

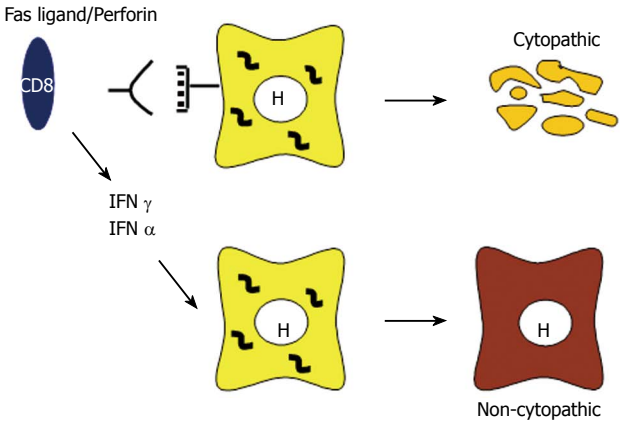

Figure 1 Cytopathic and non-cytopathic T cell responses against HBV infection.

replication is inhibited early and non-cytopathically in a CD8 dependent and probably IFNγ associated manner. The final elimination of the virus occurred several months later and was associated with a rebound of $CD8^+$ cells to baseline levels, a surge of the intrahepatic $CD8^+$ T cell response, a surge in intrahepatic IFNγ mRNA and a surge in sALT activity. Thus, these results demonstrate that intrahepatic HBV-specific $CD8^+$ T cells are required for rapid viral clearance during acute HBV infection. In addition, the data suggest the existence of dual antiviral functions that overlap temporally during natural infection but can be clearly separated by CD8 depletion: a primarily noncytolytic $CD8^+$ dependent mechanism that may be mediated by IFNγ and a primarily cytolytic mechanism that clears the remaining infected cells. It is also important to note that virus-specific $CD8^+$ T cells display oscillating effector functions during the course of infection that seem primarily influenced by the interaction of the T cells with the antigen-presenting cells[112,113].

T cell failure

In contrast to the strong and multispecific T cell responses observed during acute self-limited HBV infection, patients with chronic hepatitis B tend to have weak and narrowly focused immune responses[98]. The mechanisms that contribute to the failure of the virus-specific T cell response in chronically infected patients are only poorly understood and may include T cell deletion, anergy, exhaustion, ignorance and T cell dysfunction. HBV establishes chronic hepatitis mainly by vertical transmission from HBV infected mothers to neonates. The immunomodulatory effects of the HBeAg might play a role in this setting since it has been shown to be tolerable to T cells in transgenic mice. An important mechanism for the development of viral persistence in adults may be the development of viral escape mutations. Of note, mutational inactivation of B-cell and T cell epitopes has been demonstrated in chronic HBV infection but it seems to occur much less compared to the hepatitis C virus[101,104,114]. Little information is currently available about the intrahepatic T cell response during chronic HBV infection. Of note, one study showed functional, tetramer positive $CD8^+$ T cells in the blood and the liver of

chronically HBV infected patients. However, the number of intrahepatic HBV-specific, tetramer-positive T cells did not differ between HBeAg negative patients with normal ALT levels and HBeAg positive patients with increased ALT levels, even though the intrahepatic cellular infiltrate was greater in the latter group[115]. These results suggest a differential contribution of HBV-specific and HBV non-specific bystander lymphocytes in the pathogenesis of chronic hepatitis. T cell dysfunction might also contribute to viral persistence. For example, Reignat *et al*[116] have shown that HBsAg-specific $CD8^+$ T cells display abnormal HLA/ peptide tetramer binding properties in contrast to HBcAg-positive $CD8^+$ T cells. A high viral load may be one important factor that contributes to T cell failure. In this regard, it is important to note that antiviral treatment can overcome $CD8^+$ T cell hypo-responsiveness in subjects with chronic HBV infection, suggesting that the T cells are present but suppressed[117,118]. First evidence suggests that next to high viral loads and a lack of virus-specific $CD4^+$ T cell help, regulatory T cells may contribute to this T cell suppression[119,120]. Clearly, additional studies are required to better understand the complex host-virus interactions that determine the outcome of HBV infection.

CONCLUSION

Virus-host interactions, especially the virus-specific T cell response, are the key factors accounting for the pathogenesis of HBV infection. Viral variants may influence the course of disease and deserve special attention in the setting of antiviral therapy, immune escape and reactivation during immunosuppressive therapy.

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TOPIC HIGHLIGHT

Dieter Glebe, PhD, *Series Editor*

Avian hepatitis B viruses: Molecular and cellular biology, phylogenesis, and host tropism

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Abstract

The human hepatitis B virus (HBV) and the duck hepatitis B virus (DHBV) share several fundamental features. Both viruses have a partially double-stranded DNA genome that is replicated via a RNA intermediate and the coding open reading frames (ORFs) overlap extensively. In addition, the genomic and structural organization, as well as replication and biological characteristics, are very similar in both viruses. Most of the key features of hepadnaviral infection were first discovered in the DHBV model system and subsequently confirmed for HBV. There are, however, several differences between human HBV and DHBV. This review will focus on the molecular and cellular biology, evolution, and host adaptation of the avian hepatitis B viruses with particular emphasis on DHBV as a model system.

Key words: Hepadnavirus; Pararetroviruses; Evolution; Host range; Genome; Structure, Virions; Subviral particles; *In vitro* and *in vivo* infection; Transport; Fusion; Endocytosis; Hepatocellular differentiation; cccDNA; Gene expression; Morphogenesis and secretion

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INTRODUCTION

Full understanding of the molecular biology of the human hepatitis B virus (HBV) is hampered by a variety of experimental restrictions. There is no small animal model system available for infection studies and only few aspects of the viral life cycle are accessible to biochemical methods. A complete viral infection cycle mimicking natural HBV infection *in vitro* could only be achieved until recently with primary human hepatocytes. The disadvantages of this system are: (1) restricted accessibility to the cells, (2) infection inefficiency and (3) high variability in infection assays. The recent establishment of the HepaRG cell line is therefore a major breakthrough and allows HBV infection studies under defined conditions for the first time[1].

This review will focus on one of two established animal virus models; i.e., the DHBV model system. The human HBV and DHBV share several fundamental features. Both viruses have a partially double-stranded DNA genome that is replicated via an RNA intermediate and the coding open reading frames (ORFs) overlap extensively. In addition, the genomic and structural organization, as well as replication and biological characteristics, are very similar in both viruses. They both infect hepatocytes preferentially and have a very similar life cycle. Most of the key features of hepadnaviral infection were first discovered in the DHBV model system and subsequently confirmed for HBV. This includes replication of the viral genome by reverse transcription of a RNA intermediate[2], mechanisms of covalently closed circular (ccc) DNA formation and amplification[3], details of reverse transcription[4], and determinants of host tropism[5].

In light of the above mentioned austerities for HBV, the DHBV model of hepatitis B virus infection remains a convenient and reliable system that offers several unique advantages. Most importantly, steady availability and highly reproducible infection of primary duck hepatocytes (PDHs) provide the optimal basis for *in vitro* and *in vivo* studies of the molecular and cellular biology of hepatitis B virus infection under defined and controlled conditions. In addition, the chicken hepatoma cell line LMH produces progeny virus after transfection with cloned, mutant or wt DHBV genomes, which can be used to infect PDHs or ducks[6].

Thus, the DHBV model system is a unique system that allows elucidation of the hepadnaviral life cycle in considerable detail. However, there are several differences

Table 1 Comparison of HBV and DHBV

	HBV	DHBV
Natural host	Human	Pekin duck
Related viruses	WM-HBV, WHV	HHBV, SGHBV
Pathogenicity		
Chronic infection	yes	yes
Liver injury	yes	no
Experimental systems		
Cell transfection	yes	yes
In vitro infection	yes	yes
Transgenic mouse	yes	no
Small animal model	no	yes

WMHBV: woolly monkey HBV; WHV: woodchuck HBV; HHBV: heron HBV; SGHBV: snow goose HBV.

between human HBV and DHBV. First of all, DHBV infection normally results in chronicity since the virus is transmitted from the hen to the egg (see[7] and references therein). This infection usually does not lead to liver injury and the infected duck remains healthy throughout life. When an adult duck is infected, the infection is usually eliminated. When HBV is transmitted from mother to child, it also often results in chronic infection. However, in a large number of cases, this leads to liver injury and development of hepatocellular carcinoma or cirrhosis. When an adult is infected, this can either result in fulminant, acute or chronic hepatitis when the virus is not eliminated. Another difference between DHBV and HBV is expression of the X protein (for further differences see Table 1). This regulatory protein, with not fully understood function, is expressed by HBV from a conventional ORF, but in DHBV, an unusual cryptic ORF is used.

In the last two decades, parts of the hepadnaviral life cycle, especially the replication strategy, could be undeceived in considerable detail. In contrast, there is very little information available on the infectious entry or secretion pathway. The cellular partners involved in cell-virus interactions at these stages of infection remain unidentified and the molecular determinants of host specificity, hepatotropism and the nature of the receptor complex still await discovery.

For more information about human HBV and the other corresponding model systems please see the comprehensive reviews in this issue.

AVIAN HEPATITIS B VIRUSES

The duck hepatitis B virus was discovered in 1980 by William Mason and colleagues[8]. They found a virus very similar to HBV in about 10% of Pekin ducks from two different sources in the USA and they pointed out that no abnormal level of mortality or signs of hepatitis were found in the infected ducks. Naturally occurring DHBV infections have been reported in Pekin ducks and related species from China, USA, Canada, Europe, India, and South Africa[9-11].

Since then, avihepadnaviruses have been detected in various duck species[10] including exotic ducks and geese[10] (DHBV), in snow geese[12] (SGHBV), a Ross' goose

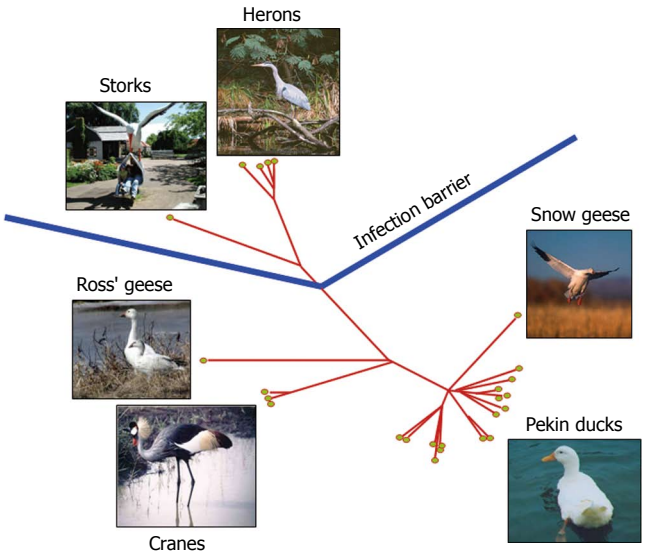

Figure 1 Phylogenetic tree of avian hepadnaviruses and related hosts. Phylogenetic relationship of all known avian hepadnaviruses based on preS/S gene sequence. The corresponding natural hosts are also indicated.

(RGHBV, GenBank Acc.No. M95589), white storks[13] (STHBV), demoiselle and grey crowned cranes[14] (CHBV) as well as grey herons[15] (HHBV). Like their mammalian counterparts, avihepadnaviruses have a rather narrow host range. For instance, DHBV infects only certain duck and goose species but neither infects Muscovy ducks nor chickens[16]. Little is known about the host range of HHBV or STHBV. Despite its substantial sequence homology with DHBV, HHBV does not infect ducks and only very inefficiently primary duck hepatocytes[15]. Recently, we reported that cranes are naturally infected with a hepatitis B virus, designated CHBV[14]. Cranes are phylogenetically very distant from ducks and are more closely related to herons and storks (Figure 1). Interestingly however, CHBV infects primary duck hepatocytes with similar efficiency as DHBV. Collectively this and related data suggest that the host range of hepadnaviruses cannot be simply predicted based on the evolutionary relatedness of their respective hosts. For a comprehensive sequence comparison and a phylogenetic tree of the host birds see[14].

Phylogenetic analysis of the various isolates demonstrated a rather high variability among DHBV strains, whereas genomes from other avihepadnaviruses, such as stork or crane hepatitis B virus, appear less variable. HHBV infection occurs not only in captive grey herons, but also with high prevalence in free-living birds. We have detected HHBV in another heron species (great blue heron) as well as in two of its sub-species (great white heron and Würdemann's heron). Thus, the virus persists in free-living birds and is an endogenous virus of several heron species.

DHBV will certainly remain the most important avihepadnavirus for research purposes since the infection system with PDHs and the important research tools are well established. It is possible to generate mutant viruses after transfection of cloned DHBV genomes into the chicken hepatoma cell line LMH[6]. Thus, different mutations can be studied not only concerning their effects

on replication but also on infection efficiency and events. In addition, *in vivo* infections can be performed in ducks without the need to establish expensive handling facilities and without risk. In these ducks not only the host range of hepadnaviruses can be studied in considerable detail (in addition to *in vitro* studies), but also the activity and toxicity of antiviral substances can be addressed[17].

THE AVIHEPADNAVIRAL LIFE CYCLE: AN OVERVIEW

Avian hepatitis B viruses belong to the family of DNA viruses that replicate their DNA genome by reverse transcription of an RNA intermediate. Thus, they belong, together with the cauliflower mosaic virus, to the group of pararetroviruses that do not integrate their genome into host cell chromosomes.

A schematic view of the DHBV genome and structure of virions is shown in Figure 2. The genome of DHBV has an unusual design. It consists of a ca. 3000 bp partially double-stranded DNA. The circularity of the genome is achieved by overlapping cohesive 5' ends[18]. The coding negative strand is complete and even has a short terminal redundancy but is not covalently closed. Its 5' end is covalently attached to the viral polymerase P[19]. The positive strand is not complete but encompasses between 40% and 85% of the genome[20]. However, the length of the gap varies among different hepadnaviruses and is smallest in DHBV[18]. Attached at the 5' end of the positive strand is a short ribooligonucleotide, which is a remnant of the pregenomic RNA (pgRNA)[21]. Both 5' terminal structures function as primers during viral replication[21]. As another particularity, the viral genome has two direct repeats (DR) with a length of 11 base pairs that have important functions in replication.

The viral genome organization is very compact and economic. All nucleotides have a coding function in at least one of the four open reading frames (ORFs). Regulatory sequences such as enhancers and promoters, as well as several cis-acting elements, overlap with coding regions. The first ORF encodes the surface proteins L and S, the second codes for the capsid protein as well as the e-antigen, the third for the polymerase and a cryptic fourth for the X protein[22].

Two different types of spherical viral particles can be detected in the serum of infected ducks: infectious virus particles (virions) with a diameter between 40 and 60 nm and subviral particles (SVPs) with a diameter between 30 and 60 nm, which lack the 27 nm nucleocapsid, including the viral genome[23]. After transfection of cell lines, a third particle entity can be detected in the cell culture supernatant. These cells secrete so called 'naked capsids' lacking the viral envelope for unknown reasons.

The virus is surrounded by a lipid envelope, which presumably originates from the host cell endoplasmic reticulum (ER) membrane, and contains both viral surface proteins. For HBV, it has been shown that the membrane is rich in phosphatidyl choline (60% of all lipids) as well as cholesterol (30% of all lipids)[24] and thus differs from the lipid composition of the cellular ER membrane,

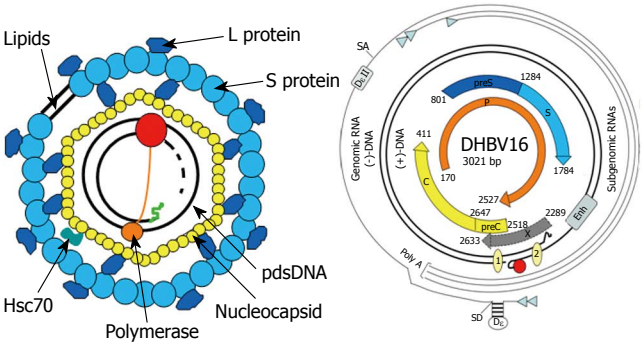

Figure 2 Virion structure and genome organization of avian hepadnaviruses. The viral envelope is derived from hepatocellular membranes and contains the viral surface proteins S and L. It covers the nucleocapsid harbouring the viral genome with the covalently linked terminal protein domain (TP, orange circle) of the polymerase (P, red circle) and cellular proteins like Hsc70. The genome is organized as depicted. The various transcripts are indicated by thin lines with the small arrowhead indicating the start sites. The partially double stranded, viral DNA with the covalently bound TP domain of P (red circle) is symbolized by the thicker lines. The numbered circles 1 and 2 on the viral DNA represent the direct repeats (DR). Enh represents the enhancer domain. The ORFs encoding core (C), polymerase (P), and the surface proteins (preS and S) are symbolized by thick arrows. Epsilon (Dε) is the stem loop structure on the pgRNA which acts as an encapsidation signal and replication origin. The second encapsidation element DεII is unique to avian hepatitis B viruses, since the mammalian counterparts lack this RNA structure. SD and SA represent the major splice donor and acceptor sites, respectively.

implicating that lipids are actively selected for the viral membrane. In total, the amount of lipids in comparison to the amount of protein is very small, in SVPs the weight ratio is about 1:4[25]. Thus, the lipids in the viral envelope are presumably not forming a lipid bilayer but protein aggregates seem to be separated through short lipid regions[26]. This strongly restricts the lateral mobility of the envelope components. The lipid composition of the DHBV envelope remains to be determined.

SVPs largely consist of the viral surface proteins S and L, which are incorporated into the envelope. They do not contain viral DNA and are thus not infectious. This entity is secreted from infected cells in excess compared to virions. It is assumed that per virion 1000 to 10 000 SVPs are secreted[27]. SVPs can compete with viral binding and thus infection[28]. In contrast, it has been shown that SVPs enhance infection when a very low multiplicity of infection (MOI) is used, which indicates an important role in the viral life cycle[29].

Complete virions contain an icosahedral capsid inside the envelope, which is about 35 nm in diameter as seen in cryo-electron microscopy or 27 nm in negative stain pictures and consists of 240 subunits of core protein[30]. The nucleocapsid holds the viral DNA genome with the covalently attached polymerase.

Cellular components can additionally be packed into viral particles. This is the case for Hsc70, which was detected in large amounts[31]. The identity and function of other proteins of cellular origin are still unknown.

A schematic view of the viral life cycle is shown in Figure 3. The life cycle of DHBV starts with attachment of the viral particles to their receptor/receptor complex on the surface of the target host cell. This is mediated by the preS domain of the viral L protein that binds to

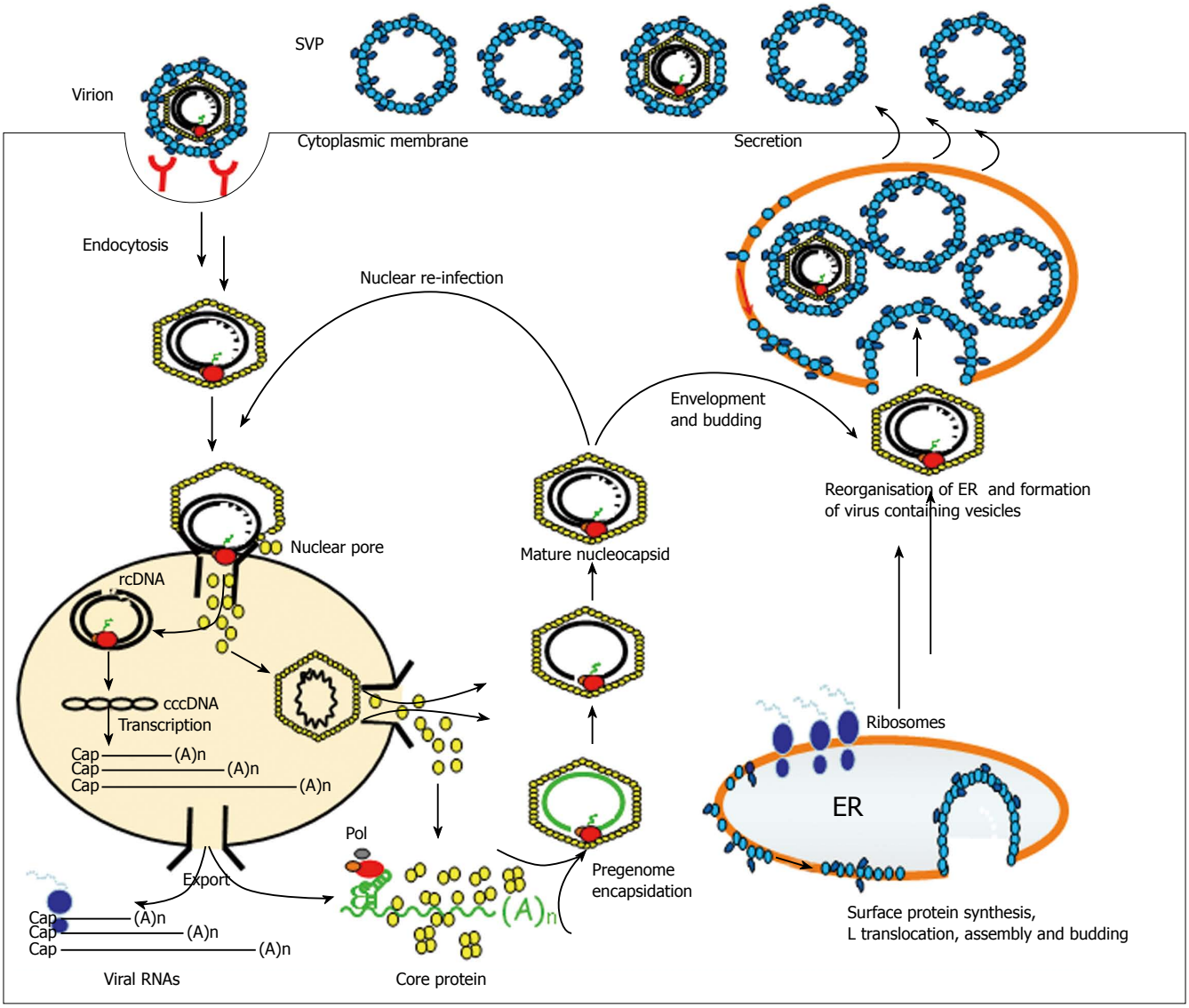

Figure 3 Model of the hepadnaviral life cycle. The hepadnaviral particle binds the hepatocyte via a specific receptor complex/molecule. It then enters the cell via endocytosis and is transported intracellularly in a MT-dependent fashion. After release out of the endosomal compartment, the nucleocapsid containing the viral DNA binds the nuclear pore complex and the viral genome is released into the nucleus. It is also possible that the whole nucleocapsid enters the nucleus and releases the viral DNA inside. Afterwards, the viral rcDNA is converted into cccDNA and viral transcripts are made. These are exported into the cytosol where the pgRNA is packaged together with the viral polymerase into the nucleocapsid. Inside the capsid, the RNA is reverse transcribed into the viral DNA genome. These matured nucleocapsids can then either be transported to the nucleus to add to the cellular cccDNA pool or they interact with viral surface proteins on the cellular ER membrane. There, the nucleocapsid buds into the lumen of the ER and is then transported in a yet undefined fashion to the cell surface where the viral particle is released into the cell exterior.

unknown cellular receptor compounds. After binding, viral particles are taken up into the cell by receptor-mediated endocytosis[32-34]. Then, the nucleocapsid is released from the endosomal compartment into the cytosol. It is currently under discussion whether a low endosomal pH is necessary for this release. The cytoplasmic nucleocapsids are then transported to the nucleus to initiate productive infection. This transport strongly depends on active microtubules (MT) but not on actin[27].

After reaching the nuclear membrane, the core protein is presumably phosphorylated and exposes a nuclear localization signal. This results in binding of nuclear factors; e.g., importins, to the capsid, which leads to uptake of the whole complex into the nucleus[35]. However, disassembly of the capsid near the nuclear membrane and subsequent transport of the viral DNA into the nucleus cannot be excluded.

In the nucleus, the relaxed form of the viral genome, the rcDNA (relaxed circular), is converted into the covalently closed, circular form (cccDNA). This is only possible after removal of the 5'-terminal structures (protein and RNA), repair of the gap and covalent ligation of the strands by cellular proteins. The cccDNA is usually not incorporated into the host genome but is organized in nucleosomes in the form of an episome[36].

Transcription of the viral genome is mediated by the cellular RNA polymerase Ⅱ[37]. *In vivo*, three different viral RNA classes could be identified, which are all polyadenylated and posses a cap structure[21]. They all have the same 3' end since only one polyadenylation site is present in the viral genome but have different 5' ends due to different transcription initiation sites. The different viral

RNAs are transported into the cytoplasm and translated. The two longest RNAs (3.5 kb), which stretch the whole genome, have two different functions. They code for the viral proteins core, the polymerase and e-antigen but one also serves as the pregenome[21]. Part of the 3.5 kb mRNA is spliced and serves as mRNA for L protein synthesis. A second class of viral RNAs (2.1 kb) encodes for the large surface protein, and the third class (1.8 kb) codes for the small surface protein, which is the most abundant one. The identity of the X-encoding RNA is unknown.

After export into the cytosol, the viral RNAs are translated and the viral surface proteins are directly inserted into the ER membrane. Once inserted, they can autonomously form subviral particles or interact with capsids to form virions.

In the cytosol, core protein dimers interact with each other and self assemble with the viral polymerase and the pgRNA into ribonucleoprotein complexes[38]. The pgRNA is packaged upon a chaperone-mediated interaction of the polymerase with the stem loop structure epsilon (ε). This structure also serves as the replication origin for the reverse transcription. Prior to packaging, the core protein is phosphorylated[39]. It is currently unclear whether reverse transcription initiates during ribonucleoprotein complex formation or after assembly of the capsid. However, when the pgRNA is inside the capsid, the particles mature, e.g. the RNA is reverse transcribed into the viral DNA genome and the core proteins are dephosphorylated[2,40]. The mature capsids interact with the viral surface proteins at the membrane of the ER and bud into the lumen, thus forming complete virions. The enveloped virions are then presumably transported through the constitutive secretion pathway to the cell surface and are released there. Alternatively, mature capsids can be transported to the nucleus and add to the cccDNA pool. After a successful infection, between one and 20 cccDNA molecules can be detected inside the nucleus[41]. This re-infection cycle preferentially occurs during establishment of an infection when the levels of large surface protein are low[42]. After a successful infection, the levels of L inside the cell rise and capsids preferentially form virions and are secreted.

In the absence of mature capsids and due to the autonomous budding activity of the viral surface proteins, subviral particles are formed. S as well as L proteins seem to accumulate in membrane domains of the ER where they reach quite high densities. When they reach a critical density, they presumably bud into the ER lumen and form subviral particles.

DHBV PROTEINS AND THEIR BASIC FUNCTIONS IN VIRAL ENTRY, REPLICATION, AND MORPHOGENESIS

Core protein and e-antigen

The viral core protein fulfils several opposing functions during the different stages of the viral life cycle; e.g., nucleic acid binding and assembly opposed to disassembly and nucleic acid release. These diverse functions are partly regulated by: (1) subcellular localization, (2) quaternary structure and (3) posttranslational modification by phosphorylation and dephosphorylation of its C-terminus.

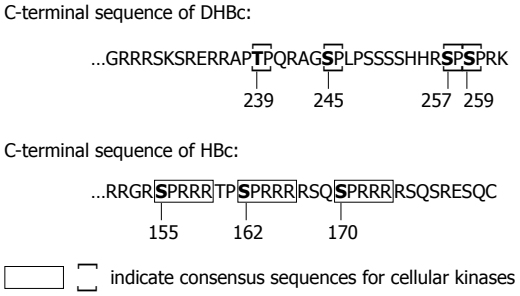

Figure 4 Phosphorylation sites of the viral core protein. The C-terminal sequence of the core proteins from DHBV and HBV are shown. Red brackets indicate the phosphorylation motifs, bold letters indicate the phosphorylated amino acid residue.

The viral nucleocapsid is composed of dimeric subunits of the viral core protein (DHBc) with a molecular weight of 32 kDa. The N-terminal region of the core protein (up to about 144 aa) is acetylated[43] and sufficient for assembly of the capsid shell[44,45]. The carboxyterminal end of the protein, the so called C-terminal domain (CTD), is extremely basic and binds nucleic acids. This is essential for packaging of the pgRNA into the nucleocapsid as well as progression of reverse transcription within[44,46]. In addition, a nuclear localization signal is present between aa 184 and 226 along with a nuclear export signal[47].

The major phosphorylation sites in the core protein are mapped to the arginine-rich C-terminus. This domain contains consensus sequences for different cellular kinases, such as PKC, SRPK1 and SRPK2 (Figure 4). Furthermore, Thr174 is highly conserved and is a cdc2 kinase phosphorylation site. Compared to the extracellular core protein, intracellular core is highly phosphorylated. Mutational analysis of the major phosphorylation sites revealed that individual or combined substitution had no overt effect on pgRNA packaging. However, the S245A mutant was deficient in genome maturation[48]. It has been shown that the CTD contains several phosphosites, which are heterogeneously phosphorylated intracellularly and hypophosphorylated or non-phosphorylated in the secreted virion[40,43]. This dephosphorylation, which occurs as nucleocapsids mature (meaning that the pgRNA is reverse transcribed into the rcDNA genome), is thought to be a maturation signal that results in secretion of only fully matured virions containing the DNA genome[43]. In addition, it has been shown that binding of hepadnaviral capsids to the nuclear pore complex depends on the phosphorylation status of the core protein[35]. The kinases or phosphatases implicated in these steps are not known. This is also true for the fate of the nucleocapsids after nuclear binding; it is not known whether they disassemble at the nuclear pore and thereafter release the viral DNA or if they are transported through the nuclear pore and then disassemble.

DHBc has the autonomous ability to assemble into particulate structures, which is dependent on the DHBc concentration[49]. The nucleocapsid is a dynamic structure and subject to regulated conformational transitions. Formation of progeny virions requires stable

nucleocapsids, whereas during viral entry, the incoming viral structure must disassemble and release the viral genome. Furthermore, reverse transcription of the viral genome occurs within the capsid and it is thought that this is linked with structural rearrangements in the capsid.

The ultrastructural analysis of the HBV core protein revealed that the monomeric form is dominated by a long alpha-helical hairpin structure[45]. The first step of capsid formation is the homodimerization of two core protein monomers that arrange in a way that leads to an antiparallel order, which results in an almost exact twofold symmetry. As a result, the characteristic spike on the capsid surface is formed by a compact four helix bundle. These spikes are the regions that interact in the virion with the viral envelope structures. Preliminary 3D reconstruction of the DHBV capsid suggests that the protein forms T-shaped dimers similar to the human core protein (M. Nassal, personal communication).

The open reading frame that encodes DHBc also codes for a nonstructural viral protein, which is the DHBe or precore. This e-antigen is, compared to the DHBc, truncated at the C-terminus and extended on the N-terminus. In addition it contains a type I signal recognition sequence. It is translocated into the ER during synthesis where the signal recognition sequence is cleaved and the C-terminus removed. After this processing, e-antigen is transported through the Golgi complex and secreted from the infected cell[50,51]. It has been shown that the e-antigen is glycosylated[50]. The glycosylation pattern seems to differ among the different avian hepadnaviruses and even among different isolates of DHBV due to the presence of different numbers of N-glycosylation sites. This is, for instance, obvious in a recent study of HHBV e-antigen that has one glycosylation site. This resulted in two bands in immunoblot analysis (one for glycosylated and for non-glycosylated e-antigen), while CHBV e-antigen with two N-glycosylation sites showed three bands on the immunoblot[14].

DHBe can be detected in the serum of infected ducks[52] and serves as a marker for efficient viral replication. Its exact function is unknown, but it has been shown that it plays no essential role in viral replication, morphogenesis or infectivity[53]. However, it seems to play a role in the establishment of chronic infections[54] and its absence may confer a growth advantage of precore-minus mutants over wildtype virus[55].

Polymerase

The viral polymerase is a multifunctional protein of about 90 kDa in size. It has a DNA-dependent DNA polymerase activity/domain to fill the gap in the viral DNA during replication[56] and an RNaseH activity/domain to selectively digest RNA from an RNA-DNA-hybrid molecule[57], as it has been shown for HBV. It also has reverse transcriptase (RT) activity to transcribe the RNA pregenome into the DNA genome during replication[2]. The polymerase homology domain is centrally located, whereas the RNaseH homology domain is located near the C-terminus of the protein. Viruses with point mutations in these regions are either defective for viral DNA synthesis or only allow negative-strand but not positive-strand DNA synthesis[56]. Most N-terminal sections of the polymerase domain are spacer regions without any other apparent function[58]. Most N-terminal sections are the region implicated in the covalent linkage of the P protein to the viral DNA often referred to as terminal protein.

In the process of viral genome replication, during which the pregenomic RNA is transcribed into the genomic DNA, the different domains can assert their different functions. The pgRNA is transcribed from the viral cccDNA by cellular polymerase Ⅱ. This RNA is then transported into the cytosol and binds the viral polymerase and the core proteins. The polymerase recognizes the epsilon and another downstream region on the viral RNA and binds there with the help of cellular proteins like Hsp90[59]. Inside the particle, the RNA is transcribed into the negative strand DNA by the RT domain of the polymerase. This process is primed by the protein itself and tyrosine 96 of the P protein serves as a primer[60]. This results in covalent attachment of the nascent DNA strand onto the terminal protein part of the polymerase. After attachment of about 4 nucleotides, this DNA product is transferred to the DR1 sequence on the viral pgRNA. This is possible since the epsilon signal and the DR1 share a 4 nucleotide identity. From this position, the DNA negative strand is elongated by the conventional mechanism[61,62]. As this elongation proceeds, the template RNA is degraded by the RNaseH activity of the viral polymerase[2,63]. The end product of this reaction is a negative-stranded DNA, which is terminally redundant by about 8 nucleotides.

Positive strand synthesis is initiated at DR2 and primed by a short oligoribonucleotide, which is a remnant from the pgRNA[64]. This primer is transferred to a complementary region at the 5' end of the negative strand DNA for positive strand synthesis. From there, synthesis of the positive strand proceeds.

Sometimes (in about 1% to 5% of cases) the primer fails to translocate. This results in a process called *in situ* priming, where a fully duplex linear DNA is the end product[65]. This is a dead end for viral replication but may be the cause for integration of some viral genomes into the chromosomal DNA of the host cell.

Elongation of the positive strand proceeds until the 5' end of the negative strand is reached. Then, an intramolecular strand transfer is needed to complete positive strand synthesis. This happens after circularization of the genome, which is possible because of the short redundancies at each strand end. After the circularization, positive strand synthesis can proceed. Usually elongation does not proceed until the end of the template is reached. In the case of DHBV, normally about 80% of the positive strands complete elongation[20].

Envelope proteins

The multiple functions of the viral envelope proteins are reflected by the domain organization and unique biochemical features. The DHBV envelope proteins are encoded by a single ORF consisting of the preS and S domain. The viral envelope proteins are inserted into the membrane of the virus that originated from intracellular membranes (presumably the ER) of the infected cell. In the case of DHBV, these envelope proteins are the small

protein S, which constitutes about 80% of total envelope proteins, and the large protein L, which constitutes about 20%[66,67]. S protein is thus the most abundant protein in the viral envelope. It determines the envelope curvature and is indispensable for both budding and secretion of viral particles. Both viral surface proteins are unique compared to other viral surface proteins in their relatively complex structure and topology[68]. They have a molecular weight of 18 and 36 kDa, respectively, and are anchored in the membrane by several transmembrane domains. The C-terminus of both proteins are identical, while L is N-terminally extended by 161 aa compared to S. The length of this extension varies with the isolate. This unusual organisation results from differential transcription of a single ORF[67] (Figure 2).

The L protein is modified with myristic acid at its N-terminal glycine after removal of the first amino acid methionine, which presumably anchors the protein in the membrane in addition to the transmembrane domains[69]. This myristoylation is required for infectivity of the virus but not for assembly[69]. Unlike the envelope protein of HBV, DHBV L and S are not modified by N-glycosylation although they contain consensus glycosylation sites. In contrast, it has been shown that the DHBV L protein (p36) is the only surface protein that is phosphorylated[70,71]. This phosphorylation occurs at serine 118 by an ERK-type MAP kinase and is not essential for viral replication, particle formation or infectivity[71].

As transmembrane proteins, L and S are incorporated cotranslationally into the ER membrane. The proteins have four transmembrane domains (TM1 to TM4) that anchor them in the membrane and consist of membrane-spanning hydrophobic alpha-helices (Figure 5). In addition, the preS domain of the L protein contains two translocation motifs (TLM), which are 12 amino acid encompassing domains that form an amphipathic alpha helix[32]. TLMs mediate an energy- and receptor-independent transfer of peptides, nucleic acids and proteins when fused to them across membranes without affecting their integrity[72]. The DHBV-TLMs are located between amino acids 20-31 (TLM1) and 42-53 (TLM2) and required for viral infectivity.

Worth mentioning is the dual topology of the L protein[73]. After cotranslational insertion of the protein into the cellular membrane, part of the proteins changes the topology (Figure 5). This leads to exposure of the N-terminus to the cytosolic side of the membrane in about half of the L proteins while the other half has the N-terminus directed to the luminal side[74]. In this way, the protein can fulfil two different functions: it can interact with the cellular receptor on the outside of the virus and it can interact with the nucleocapsid on the inside. Whether S also adopts a dual topology remains to be determined.

The viral surface proteins are able to autonomously form subviral particles without the help of an interacting nucleocapsid. The exact mechanism of this phenomenon is not known, yet it is assumed that the proteins interact with each other to form microdomains in the ER membrane from which they bud when they reach a critical density. A prerequisite for this budding activity would be a tight interaction of the viral surface proteins with the membrane lipids and a membrane bending activity. When

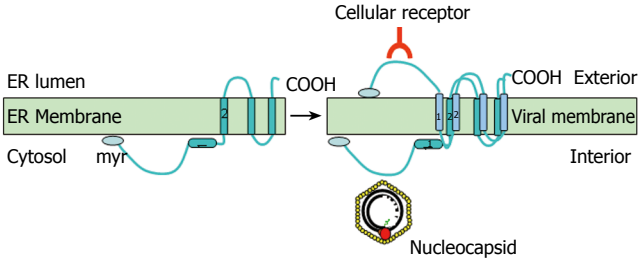

Figure 5 Dual topology of the viral surface protein L. The L protein is inserted into the ER membrane during synthesis with transmembrane domain 2 being inserted into the membrane. Half of the proteins then change their topology and insert the transmembrane domain 1 into the ER membrane. After this change and formation of the virus, L can exert its two basic functions; interaction with the nucleocapsid on the cytosolic preS domain and interaction with the host cell receptor on the surface of the viral particle.

L protein is expressed without the S component, particles are formed inside the cells, but are not secreted. This retention and secretion defect can be overcome by co-expression of the S protein[75]. This implies an important function for the S protein in secretion of viral particles.

Another function of the L protein is regulation of cccDNA amplification. As mentioned above, cccDNA is the replication template for all hepadnaviruses. It is a prerequisite for maintaining chronic infection of hepatocytes and is the main obstruction during antiviral therapy since it is mostly not eliminated from the cells. The amount of cccDNA inside the host cell nucleus is thus of great importance for the virus. To maintain its replication template in the nucleus, it re-infects and this process is regulated in an elaborate manner by the large viral surface protein[76]. During the early stage of infection when the amount of L protein is low inside the cell, mature core particles preferentially infect the nucleus to add to the cccDNA pool. Concomitant with the increase in cccDNA, the levels of L increase. This leads to redirection of the mature capsids into the secretory pathway and reduces cccDNA amplification.

The L protein is also responsible for superinfection exclusion, which prevents hepadnaviral infection of already infected hepatocytes[77]. This phenomenon was first ascribed to downregulation of the putative viral receptor protein gp180. This has not been confirmed and it has been suggested that superinfection exclusion may result from a role of L as a regulator of intracellular trafficking[77].

The L protein not only plays a role in viral morphogenesis or cccDNA amplification, but it is also implicated in additional regulatory functions such as signalling[71]. The cytosolically exposed preS domain of the L protein has the potential to activate gene expression from cellular promoter elements in *trans*. It is also phosphorylated by ERK2 at serine 118, which is induced by various stimuli and may play a role in intracellular virus-host crosstalk[71].

X protein

One major difference between the avian and mammalian hepatitis B viruses is the presence of an ORF called X in the latter. The function of this regulatory protein is still far from being understood. Recently it has been reported

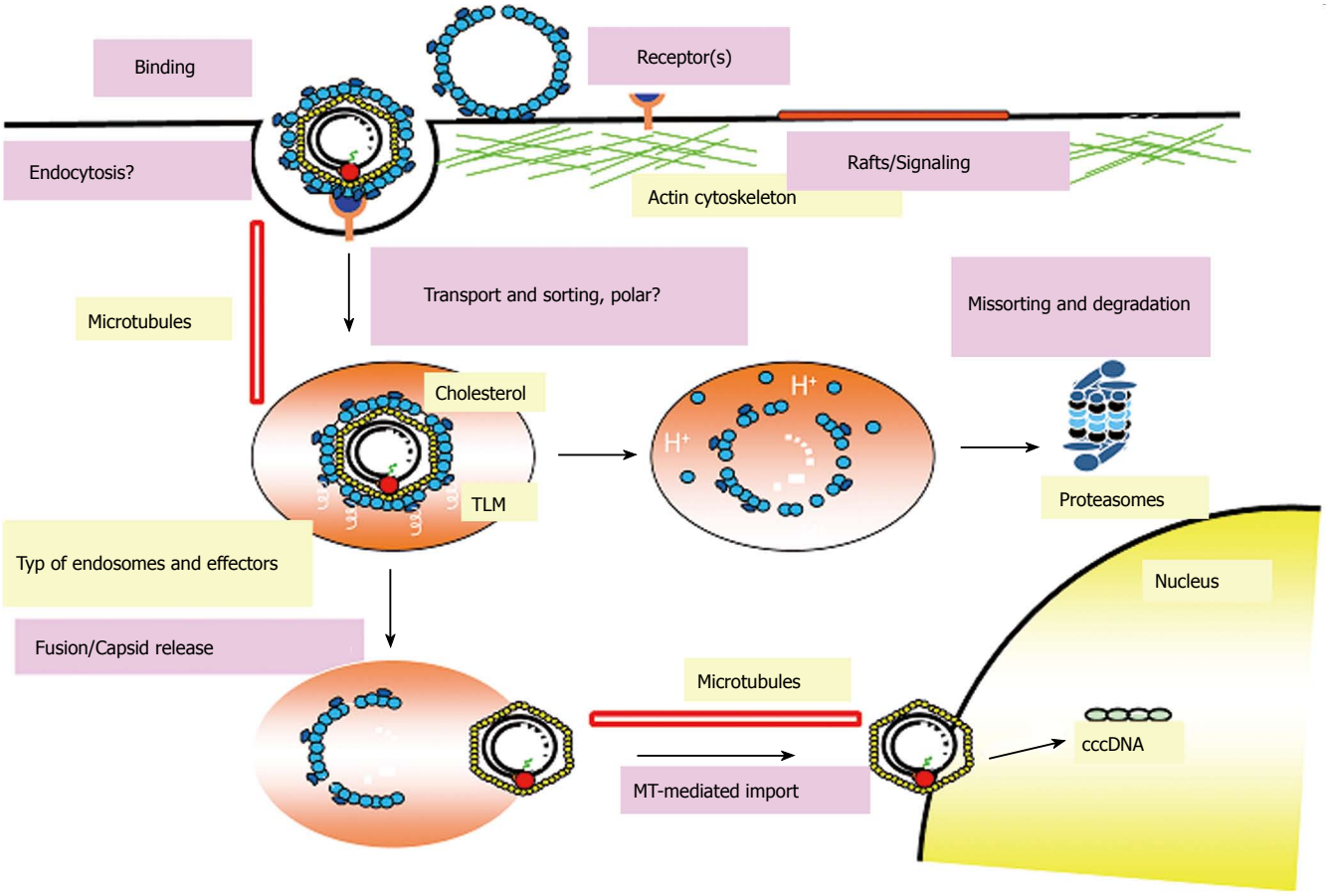

Figure 6 Current model for entry and intracellular trafficking of DHBV. The hepadnaviral life cycle starts with the attachment of virions to specific binding sites on the surface of hepatocytes mediated by the preS region of the large viral envelope protein L. Afterwards the virus enters the cell via endocytosis and resides in an endocytic vesicle which is transported in a MT-dependent and probably polar fashion. The endosomal release of the incoming particle requires the activity of the vacuolar H^+-ATPase and presumably the cholesterol of the viral envelope. After release, the particle is transported in a MT-dependent fashion mediated by dynein/dynactin to the nucleus and initiates infection after release of the viral genome at the nuclear membrane or within the nucleus. However, the majority of viral particles is missorted and degraded by the proteasome and other cellular proteases.

that X protein enhances the replication of transfected HBV genomes[78]. DHBV lacks an apparent X-ORF. But a cryptic X-like ORF has recently been reported[22]. *In vivo* experiments have suggested no functional role for this gene product in DHBV short term infection[79].

DHBV INFECTION

In vitro infection

Hepadnaviruses can only efficiently infect primary hepatocytes with the exception of the recently published HepaRG cell line, which can be infected with the human HBV[1]. Thus, primary hepatocyte cultures have to be established for use with hepadnaviruses. In the case of DHBV, either primary fetal or adult hepatocytes can be used. Fetal hepatocytes can be obtained easily from the livers of non-hatched duck embryos. These are digested with collagenase, washed and plated. This results in a mixed culture containing hepatocytes as well as other cells of the liver, which are the non-parenchymal cells, such as macrophages, sinusoidal endothelial cells, and fibroblasts. Alternatively, primary duck hepatocyte cultures can be obtained from adult ducks by liver perfusion. This results in a rather homogenous suspension of cells containing high amounts of hepatocytes (up to 90%) compared to non-parenchymal cells. Compared to primary human hepatocytes, PDHs are about 20 times more permissive to hepadnaviral infection.

The cells are cultured in a standard medium containing hydrocortisone, insulin as well as DMSO. The DMSO is important for maintaining differentiation and thus infectability of the cells[80]. Under these culture conditions, the cells remain infectable for up to 2 weeks in culture and viral spread occurs. The DMSO as well as omitting serum from the cell culture medium are essential conditions for maintaining cellular infectability since addition of serum to the cultures decreases the amount of cellular receptor proteins on the cell surface[81].

Research with the *in vitro* model of DHBV infection has lead to the discovery of many different features of the hepadnaviral life cycle. However, while factors involved in the early and most vulnerable steps of the viral life cycle (Figure 6) have been identified for a variety of viruses, little is known for hepadnaviruses (for a review see[7] and references therein).

Hepatoma cell lines, which can replicate the viral DNA and produce progeny virus after transfection of the viral genome into the nucleus, are not permissive for infection with the virus itself[82]. This phenomenon led to the assumption that the absence of a viral receptor or

receptor complex on the cell surface of these cells is the major determinant for infection. Great effort was invested to identify these molecules. But to date, the molecules responsible for the viral uptake that leads to productive infection are still unknown, although carboxypeptidase D (gp180) has been shown to specifically interact with the viral L protein and leads to internalization of viral particles after heterologous expression[83-85]. However, this did not lead to productive infection of these cells. In addition, the protein has been shown to not only bind DHBV, but also the viral surface protein from heron HBV, which does not infect PDHs, and this protein is not liver specific. It thus cannot be the determinant for viral host range or tissue specificity. This shows that either additional factors are required for the infection or that gp180 plays no role in the productive uptake and binding of the virus.

Initial binding of hepadnaviral particles seems to involve a component with low affinity without saturation and a component with high affinity and saturation[28]. This indicates that the binding involves at least two determinants and thus components. Several competition experiments with recombinant preS peptides, neutralizing antibodies and SVPs showed that the preS region of the viral L protein is essential for viral binding and the establishment of an infection[26,28,86,87]. In addition, it has been shown that preS peptides that were myristoylated were much more efficient in preventing DHBV infection than non-myristoylated peptides, which indicates that the myristoylation of L plays an important role during infection[86].

To gain more insight into the enigmatical early steps of hepadnaviral infection, we recently characterized the early attachment and entry events of DHBV infection in PDHs[27]. To do so, we established a sensitive, PCR-based assay that allowed us to investigate viral binding and entry. This binding and entry assay showed that only a small proportion of the inoculum binds to the cell surface of hepatocytes. Also the overall number of particles that bind to the cell surface is quiet low, after 2 h at 4℃, only up to 10 virions and 10 000 SVPs per cell were detectable. This indicates that the number of hepatocellular surface binding sites is about 10 000 per cell. Binding was prevented by the use of neutralizing antibodies as well as suramin, which also prevented viral infection showing that the detected binding sites are relevant for productive infection.

The steps after viral binding also remain quite elusive. It was shown years ago that the kinetics of DHBV uptake is unusual since binding and entry seem to be very slow. For a maximal infection efficiency, cells have to be incubated with the virus up to 16 h[80]. We have recently shown that viral uptake indeed needs an unusually long time period[27]. 1 h after attachment only about 70% of bound DHBV was taken up and internalization was complete within 3 h. This shows that virus uptake itself takes a relatively long time period, but since after 3 h all bound virions were internalized, uptake is very efficient. In contrast, viral trafficking inside the cell does not seem to be efficient since a high proportion of viral particles are degraded after viral entry into the cell and thus cannot establish infection[34].

It has been shown previously that DHBV entry into PDHs requires energy, which indicates that cellular and/or viral processes actively take place and that DHBV is presumably entering the cell via endocytosis[33,34]. Studies addressing the pH-dependency of DHBV infection by the use of chemical substances that increase endosomal pH led to contradicting results[33,88-90]. However, the weight of data favours pH-independent entry as well as an endocytic mechanism and shows that the virus does not require passage through a highly acidic compartment. The effect of the vATPase inhibitors seem to be due more to effects on viral trafficking inside the cell than on the endosomal pH alone[34].

After the virus is taken up by the cell via endocytosis, it has to be transported to the nucleus to establish infection. In the nucleus, the viral rcDNA is converted into the cccDNA. This conversion is detectable within the first 24 h after virus inoculation[27,91]. Thus, after efficient viral uptake there is an unusually long gap of 13 to 17 h before the appearance of nuclear viral cccDNA. This gap suggests that there is a rate-limiting post entry step that preceeds cccDNA formation, which involves viral uncoating and nuclear genome transport, or is required for rc- to cccDNA conversion. The intracellular transport has been shown to be independent of the actin skeleton, which in contrast seems to restrict entry, and is strictly dependent on the microtubule (MT) network of the cell[27]. Overexpression of dynamitin, a cofactor subunit of the motor protein complex dynactin-dynein, which mediates transport along microtubules, also reduced DHBV infection (our unpublished data). To date it remains unknown whether the MT-dependent transport of the virus (or the nucleocapsid alone) occurs only at the stage of the endosome or if the virus alone also interacts with microtubules.

To deliver the viral DNA into the nucleus, it has to be released from the viral particle and, prior to that, the viral particle has to be released from the endosomal compartment it resides in. It has been shown that this involves an unusual mechanism that depends on the integrity of a so called TLM[32]. The TLM thus mediates release of the viral particle out of the endosome into which it initially entered.

The infectious entry pathway of hepadnaviruses appears to involve a series of highly coordinated and directional steps leading to the nuclear delivery of viral genomes essential for the establishment of a productive infection. These steps may, alone or in combination, determine the species and host cell tropism common to all hepadnaviruses.

In vivo infection

DHBV-infected ducks exhibit age-related outcomes of infection, which is similar to HBV-infected humans. In principle, hepadnaviruses have the ability to cause either a transient or chronic infection. When infected with DHBV, young ducks develop persistent infection whereas adult ducks become transiently infected and eliminate the virus[92]. These different outcomes are viral dose dependent; persistent infection in young ducks develops more frequently with higher doses of virus[92]. In some cases, dependent on age and infection with a given mutant,

experimental infections can cause symptoms of a liver inflammation[93]. It has been shown that one genome equivalent is sufficient to infect a duckling and that spread of the virus within the liver is very efficient: 14 d after inoculation, virtually all hepatocytes were infected[94]. In addition, it has been shown that the difference between the infection outcome in older and neonatal ducks depends on the production of neutralizing antibodies against the virus[95]. The rapid production of neutralizing antibodies in older ducks led to an efficient inhibition of viral spread in the liver.

The duck hepatitis B virus infects Chinese domestic and American pekin ducks as well as geese. Normally, infection takes place through vertical transmission from the infected hen to the eggs and results in a chronic infection that is without symptoms and is tolerated by the immune system[23]. The virus then replicates in the egg yolk sack and is transferred to the embryonic hepatocytes by day 6 of development[96]. Thus, all offspring from an infected hen are DHBV-positive.

When DHBV infection is persistent in the duck, viral replication mainly occurs in the hepatocytes of the liver. Usually, the level of viral replication is then very high, with most hepatocytes infected and expressing the viral antigens. This is also reflected in the amount of viral antigens circulating in the blood stream. Up to 10^{10} virions and 10^{13} SVPs can be detected per ml of serum[97].

In vivo infections with DHBV are often used to study the growth kinetics of viral mutants[79]. This allows elucidation of specific mutations in the viral genome on the behaviour of this respective mutant in their natural host. In contrast to the *in vitro* situation, the role of the immune system and the spread of the virus within the infected liver can be assessed.

It has been shown that the addition of lipopolysaccharides (LPS, endotoxin) to PDH cultures inhibited DHBV replication efficiently[98]. This was due to the release of interferon alpha and gamma from non-parenchymal cells (i.e. Kupffer cells, resident macrophages of the liver). The exact mechanism behind this phenomenon is not known.

HOST SPECIFICITY

All known hepadnaviruses are strongly, but not exclusively, cell type specific and have a narrow host range restricting them to their natural host and a few closely related species (Figure 1). DHBV, for example, infects only certain duck and goose species, but either does not or very inefficiently infects chicken or Muscovy ducks, respectively. Despite its substantial sequence homology with DHBV, the heron HBV (HHBV) does not infect PDHs. Although ducks and duck-derived primary hepatocytes are virtually non-permissive for HHBV, substitution of a region of the HHBV-specific preS domain of the L protein by the corresponding sequence from DHBV overcomes this species barrier. As a consequence, the pseudotyped HHBV virions can efficiently infect primary duck hepatocytes[5]. The same is true for mammalian hepadnaviruses, as shown for woolly monkey hepatitis B virus pseudotyped with a small stretch of preS1 sequence from HBV, which was

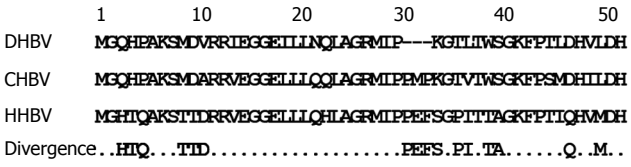

Figure 7 Sequence comparison of the viral L protein from different hepadnaviruses. The first 50 amino acids of the preS domain of hepadnaviral L from duck, crane, and heron HBV are shown. The divergent amino acids are shown in the lowest lane.

then infectious for human hepatocytes. Thus, although the sequence of this region is very divergent among the different hepadnaviruses, the biological functions seem to be conserved. The so called host-determining region (HDR) in the preS part of the avian L protein was mapped to amino acids 22 to 90, and an exchange of this small region also changed the species specificity[5]. These studies clearly indicate that the block to cross-species infection by hepadnaviruses is destined at the level of infectious viral entry. A small domain within the preS region of the L protein plays a pivotal role in host discrimination.

We showed that cranes are naturally infected with a novel hepadnavirus, designated crane HBV (CHBV)[14]. Phylogenetically, cranes are very distant from ducks and are closely related to herons and storks. However, we found that CHBV infects PDHs with similar efficiency as DHBV, indicating a rather broad host range of this virus at least *in vitro*. Whether CHBV can establish chronic infection in ducks *in vivo* and is as non-pathogenic as DHBV remains to be elucidated. Interestingly, comparison between the HDR of DHBV and the HDR of CHBV reveals a short insert of 3 amino acids (PMP) in the CHBV L protein, a sequence similar but not identical to the analogous region of HHBV and STHBV, whereas all other known duck and goose hepadnaviruses have no such insert (Figure 7). It remains to be shown which sequence features of the L protein are responsible for the unusual broad host range of CHBV and at which level of infection the block in cross-species infection is determined.

Accordingly, comparative genomic and subgenomic sequence alignment from different avihepadnaviruses facilitates the prediction of specific properties of each virus and helps to gain insight into the mechanisms controlling species specificity and host adaptation.

CHEMOTHERAPY AND VACCINATION

Antiviral drugs currently in use for therapy of chronic hepatitis B are nucleoside analogues and interferon. These therapies are unsatisfactory since the virus is usually not eliminated from the infected patient and resistant viruses frequently appear after treatment with nucleoside analogues. These data show the need for additional therapies and therapeutic strategies. The therapeutic effect of new vaccination strategies as well as chemotherapeutic agents can be assessed with the DHBV model system.

A long-term study showed that treatment of persistently infected ducks with 0.1 mg/kg per day of entecavir resulted in a rapid 4-log drop in serum DHBV

surface antigen[99]. However, a rapid rebound of levels of DHBV DNA and antigens in serum and liver was observed when entecavir was discontinued. When entecavir was administered at the time of DHBV inoculation, it was not effective to prevent infection but it led to a significant suppression of viral spread even after withdrawal of the drug[100]. Thus, short term suppression of DHBV infection shortly after infection provides the opportunity for the immune response to successfully control the infection.

Immunotherapy using DNA vaccines has been proposed as a way to improve viral clearance via the induction of an effective immune response. It has been shown that a DNA vaccine expressing DHBV surface antigens induces high levels of antibodies directed against these antigens, which protected or partially protected the animals against a challenge with DHBV[101]. This suggests that DNA vaccines may be an alternative to conventional vaccines for inducing immune response and protection against infection.

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TOPIC HIGHLIGHT

Dieter Glebe, PhD, *Series Editor*

The woodchuck as an animal model for pathogenesis and therapy of chronic hepatitis B virus infection

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Abstract

This review describes the woodchuck and the woodchuck hepatitis virus (WHV) as an animal model for pathogenesis and therapy of chronic hepatitis B virus (HBV) infection and disease in humans. The establishment of woodchuck breeding colonies, and use of laboratory-reared woodchucks infected with defined WHV inocula, have enhanced our understanding of the virology and immunology of HBV infection and disease pathogenesis, including major sequelae like chronic hepatitis and hepatocellular carcinoma. The role of persistent WHV infection and of viral load on the natural history of infection and disease progression has been firmly established along the way. More recently, the model has shed new light on the role of host immune responses in these natural processes, and on how the immune system of the chronic carrier can be manipulated therapeutically to reduce or delay serious disease sequelae through induction of the recovery phenotype. The woodchuck is an outbred species and is not well defined immunologically due to a limitation of available host markers. However, the recent development of several key host response assays for woodchucks provides experimental opportunities for further mechanistic studies of outcome predictors in neonatal- and adult-acquired infections. Understanding the virological and immunological mechanisms responsible for resolution of self-limited infection, and for the onset and maintenance of chronic infection, will greatly facilitate the development of successful strategies for the therapeutic eradication of established chronic HBV infection. Likewise, the results of drug efficacy and toxicity studies in the chronic carrier woodchucks are predictive for responses of patients chronically infected with HBV. Therefore, chronic WHV carrier woodchucks provide a well-characterized mammalian model for preclinical evaluation of the safety and efficacy of drug candidates, experimental therapeutic vaccines, and immunomodulators for the treatment and prevention of HBV disease sequelae.

Key words: Woodchuck; Woodchuck hepatitis virus; Hepatitis B virus; Neonatal-acquired infection; Adult-acquired infection; Resolution; Chronicity; Humoral immune response; Cellular immune response; Antiviral therapy; Immunotherapy; Combination therapy; Hepatocellular carcinoma

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INTRODUCTION

Infection of adult humans with the hepatitis B virus (HBV) results characteristically in self-limited hepatic disease with recovery based on serological and clinical parameters. Progression to chronic HBV infection occurs infrequently in infected adults, but HBV infections often persist in unvaccinated infants born to HBV-carrier mothers. Chronic HBV infection can lead to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) later in life. Estimates indicate that more than 2 billion people worldwide have serological evidence of previous or current HBV infection, with at least 350 million chronic carriers, and an overall mortality rate from HBV-induced liver disease of 1.2 million deaths per year[1]. Although highly effective vaccines are licensed and have been in use since the early 1980's to prevent HBV infection in neonates and adults, the large reservoir of chronic HBV

carriers currently remaining could benefit immensely from the timely development of effective antiviral and/or immunotherapies that cure the infection or reduce the risk of disease progression.

Evidence from HBV-infected humans, and from animal models of HBV (i.e., HBV-transgenic mice, chimpanzees, pekin ducks, and woodchucks), indicate that the success or failure of humoral and cellular immune responses to the virus determine the initial outcome of acute HBV infection (i.e., as self-limited versus chronic), and that defective responses appear to play a role in the progression of chronic HBV infection (i.e., to chronic hepatitis, cirrhosis, and possibly HCC)[2-10]. Self-limited infections by HBV involving successful immune responses represent by far the more favorable outcome. Chronic HBV infections, where immune responses have failed or are sub-optimal for virus clearance, represent a daunting challenge to successful therapy against a background of continuing disease progression. Current treatment strategies for chronic HBV infection are suboptimal when compared to the curative process observed in self-limited HBV infection. Understanding the prevention and pathogenesis of HBV infection has advanced greatly through clinical studies in humans, and through experimental studies in the chimpanzee model of HBV infection; however, neither of these models is well-suited for the routine testing of therapeutic strategies for treatment of chronic HBV infection.

Woodchuck hepatitis virus (WHV) is a naturally occurring hepadnavirus of the Eastern woodchuck (*Marmota monax*) (Figure 1). WHV was described initially in 1977 at the Penrose Zoo in Philadelphia in a colony of woodchucks where high rates of chronic hepatitis and HCC had been observed[11]. Several strains of WHV have been identified since then, which are all very closely related genetically[12-16], but which may induce differing proportions of chronic infections in neonatal woodchucks[17]. WHV, and another HBV-like virus, the duck hepatitis B virus (DHBV)[18-20] have been used most extensively in the modeling of HBV infection and antiviral therapy (for previous reviews see[21-24]).

Research using the woodchuck began in 1978 and it was developed further into a laboratory model by 1980 when a woodchuck breeding colony was established at Cornell University. Early progress in model development at the Georgetown and Cornell Universities involved: (1) the production and validation of reagents and assays for WHV and for disease markers, (2) the characterization of infectious WHV inocula that induced predictably high rates of chronic infection when inoculated in neonatal woodchucks, and (3) basic studies of the natural history of virologic responses and tumor development associated with experimental infection of neonatal and adult woodchucks. Since 1988, the neonatal chronic WHV infection model has been applied primarily in the testing of antiviral nucleoside analogues for chronic HBV infection (for previous reviews see[10,25-30]).

Early studies in woodchucks also involved the testing of conventional vaccines for the prevention of acute, self-limited WHV infection in neonatal and adult woodchucks[28], and also for prevention of the chronic outcome and HCC in the vaccinated neonates challenged with higher doses of inoculum to enable breakthrough infections[31,32]. Immunomodulation of acute and chronic WHV infections using immunosuppressive drugs, such as cyclosporine A[33,34], was performed to gain an initial understanding of the role of the woodchuck immune response in the outcome and maintenance of WHV infection. The focus of investigations using the woodchuck has ranged widely since 1980, with flexible emphasis on both model development and model application in many areas of HBV research. These included viral and disease pathogenesis, and the prevention and treatment of HBV infection and disease sequelae (including HCC) using vaccines, antiviral drug candidates, and immunomodulators alone and in combination. The purpose of this review is to highlight the woodchuck as an animal model for pathogenesis and therapy of chronic HBV infection.

Figure 1 Eastern woodchuck (Marmota monax).

NATURAL HISTORY OF WHV INFECTION AND DISEASE

Experimental infection of woodchucks with WHV is a well-accepted model for many aspects of the pathogenesis of human HBV infection[7,10,26-29,35-38]. Recent studies of the host response of woodchucks to WHV infection and therapy have revealed numerous parallels to the immunopathogenesis of HBV infection. Certain immune markers in woodchucks cannot be analyzed currently to the same extent as those in mice and in humans. However, the patterns and profiles of those immune responses measured thus far in the woodchuck model are highly consistent with the underlying immunologic mechanisms defined in humans.

Experimental infection of neonatal or adult woodchucks with WHV7P1[17], a well characterized inoculum of WHV, produces predictable proportions of acute, self-limited (i.e., resolved) infections versus chronic infections. This mimics the effects of age on outcome of HBV infection in humans[3,4]. In adult woodchucks, WHV7P1 infections result mainly in resolution, with less than 5% of woodchucks progressing to chronicity[17] (Figure 2).

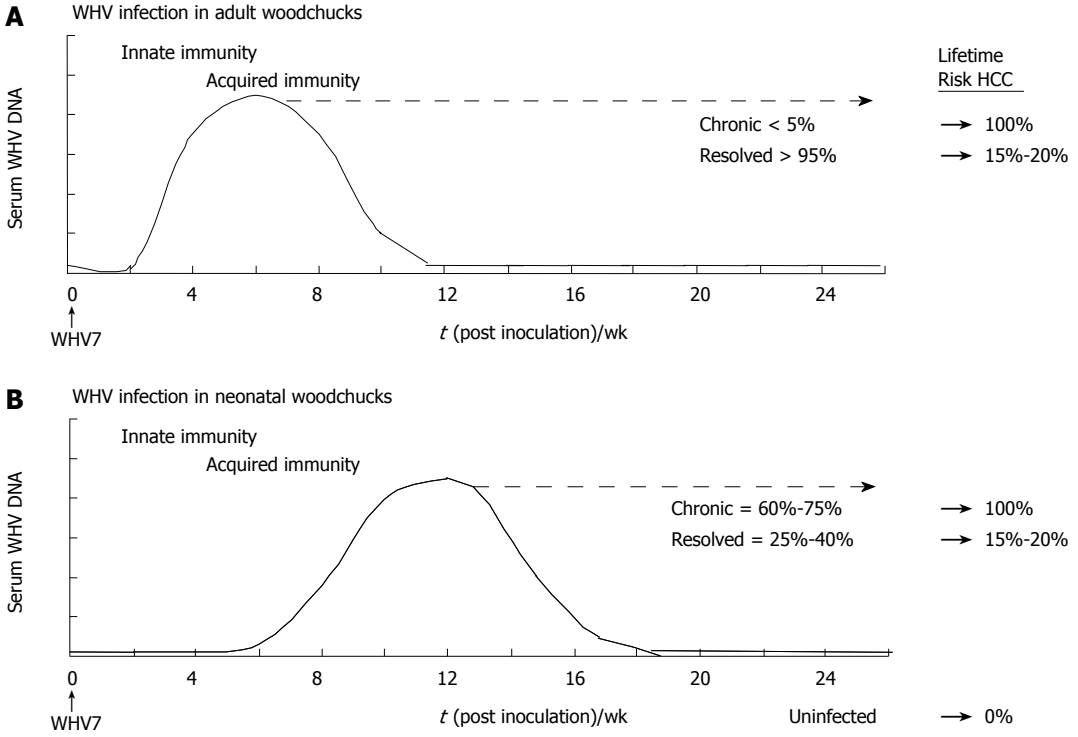

Figure 2 Schematic profiles for serum viremia in adult and neonatal models of experimental WHV infection. **A**: Adult woodchucks. Adult woodchucks born to WHV-negative dams are infected with 1 x 10^7 woodchuck infectious doses 50% of a defined WHV inoculum by the intravenous route. The proportions of chronic and resolved outcomes of adult woodchucks usually are less than 5% and more than 95%, respectively. The lifetime risk for the development of HCC in established chronic and resolved WHV infections is 100% and 15%-20%, respectively; **B**: Neonatal woodchucks. Neonatal woodchucks born to WHV-negative dams are infected with 5 x 10^6 woodchuck infectious doses 50% of a defined WHV inoculum by the subcutaneous route. The proportions of chronic and resolved outcomes range between 60%-75% and 25%-40%, respectively. The lifetime risk for the development of HCC in established chronic and resolved WHV infections is 100% and 15%-20%, respectively. HCC in uninfected, WHV-negative woodchucks is not observed. Approximate time intervals for the development of innate and acquired immunity are shown.

However, transient suppression of cellular immune responses with cyclosporine A (CsA) during the incubation and acute phase of adult WHV infections results in 92% of these infections progressing to the chronic outcome; with CsA given only during the incubation period and very early acute stage (0 to 4 wk post infection), the result is up to 50% chronic outcomes in adult WHV infections[33,34]. This shows the importance and timing of early immune responses in the resolution of acute WHV infection. Experimental immunosuppression, however, does not necessarily mimic natural processes associated with the progression to chronic infection.

Most chronic HBV infections occur as a result of neonatally-acquired infection[39-41]. Experimental infection of neonatal woodchucks with WHV7P1 usually results in a 60%-75% frequency of chronic carriers and a 25%-40% frequency of naturally recovered infections[17] (Figure 2). Viral and host response kinetics are relatively uniform when neonatal woodchucks are inoculated with WHV7P1 in the spring of the year, thus enabling statistical modeling of serologic and hepatic responses using samples collected in successive years. Such features also enable co-temporal comparisons of early acute phase immune responses before the self-limited and chronic outcomes become evident serologically, which can help to differentiate and identify the underlying mechanisms involved in the onset versus maintenance of chronic WHV infection[42-46] (Figure 3).

Chronic WHV infection involves life-long active viral replication and inevitable disease progression to chronic hepatitis and HCC[35,47-50]. In chronically infected woodchucks, there is no naturally occurring e-antigen to anti-e seroconversion and associated step-down of viral replication (i.e., as is commonly seen in chronic HBV infection; e.g.,[51-54]). In general, the high viral replication and high surface antigen and e-antigen loads present in the chronic WHV carrier appear to play a role in the maintenance of immunologic tolerance, and are associated with disease progression to HCC[43-45,55-57].

Self-limited WHV infection involves a relatively complete shut down of viral replication and a nearly complete clearance of virus from the system with full recovery. It has been suggested that trace amounts of residual WHV genomes often detected in long-term recovered woodchucks in liver, serum, and in peripheral blood mononuclear cells (PBMC), could actually represent an alternate form of persistent viral infection[58-65]. Residual HBV DNA has been documented also for humans recovered from self-limited HBV infection (e.g.,[66-71]). In woodchucks, even recovery from acute WHV infection incurs a discernable lifetime risk of HCC (5%-20%) when compared to control seronegative woodchucks (i.e., uninfected with WHV); however, this risk is significantly lower compared to the lifetime risk of HCC in chronic

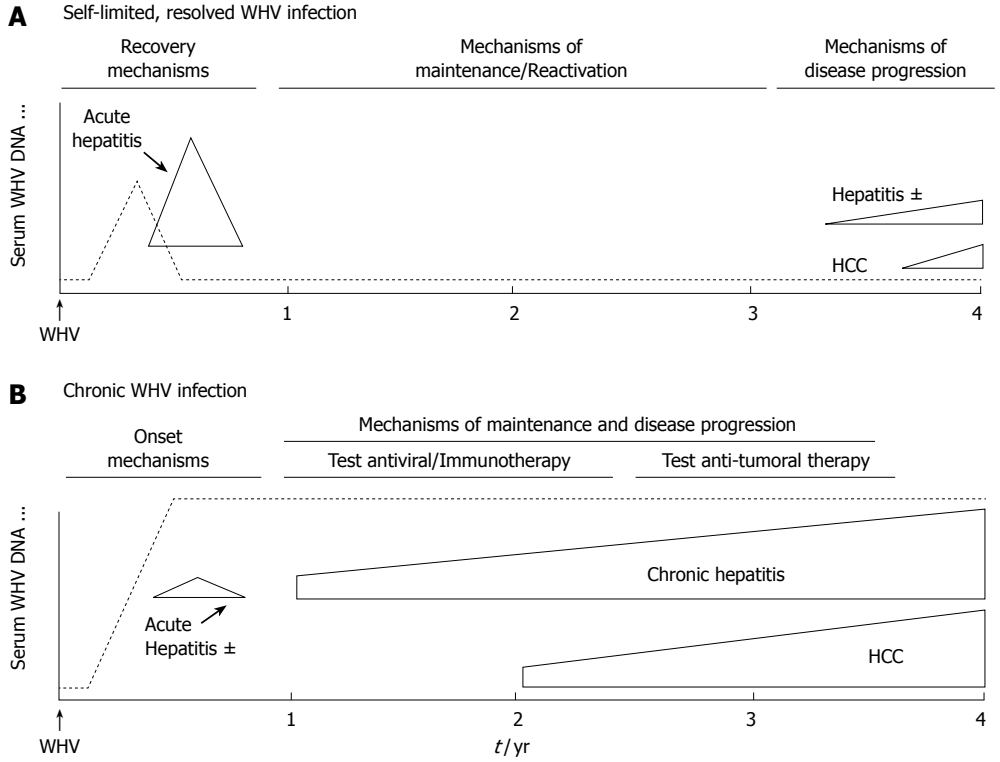

Figure 3 Schematic profiles for viremia, acute hepatitis, and disease progression in the neonatal model of experimental WHV infection. **A**: Self-limited, resolved WHV infection; **B**: Chronic WHV infection. Neonatal woodchucks born to WHV-negative dams are infected experimentally at 3 d of age with 5 x 10^6 woodchuck infectious doses 50% of a defined WHV inoculum by the subcutaneous route. Approximate time intervals for self-limited acute hepatitis and progressive chronic hepatitis are shown. Use of the neonatal WHV infection model enables co-temporal comparisons of acute self-limiting and chronic outcome of WHV infections as they develop. Comparison of acute phase events at early time points before or near the times when self-limited, resolved and chronic outcomes begin to segregate based on serologic criteria allows the definition of immune mechanisms involved in progression toward recovery versus chronicity. Comparison at later time points enables the investigation of mechanisms that are important in the maintenance of the established WHV infection state and that lead to disease progression and tumor development. During this time the established chronic WHV carrier woodchucks are used mainly for the testing of antiviral nucleosides, immunotherapeutic strategies, and for the prevention of onset and development of HCC.

WHV carrier woodchucks, which is essentially 100%[58,72] (Figure 2). Such results provide direct experimental evidence for the carcinogenicity of WHV and, by analogy, for HBV where chronic infection also is associated with HCC.

In a recent study, we examined the reactivation of WHV replication and the generation of infectious WHV in long-term resolved adult woodchucks during experimental immunosuppression with CsA (Menne *et al*, unpublished data). Administration of CsA to serologically recovered woodchucks with evidence of residual WHV DNA in liver and PBMC, and with durable recall cellular immune responses of PBMC to WHV antigens, resulted in a transient reactivation of WHV replication during CsA treatment. This supports the idea that replication-competent WHV (and by analogy, HBV) can persist for many years after recovery from acute viral hepatitis, possibly as part of a continuing process. That is, the virus may be controlled by virus-specific immune responses that are primed continuously by trace amounts of virus and viral antigens. In any case, the presence of long-term recall cellular immune responses with mutual persistence of residual WHV covalently closed circular DNA (WHV cccDNA) is significant to the durability of recovery responses over the long term for the stable control of replication and shut down of the infectious process. The apparent lack (or need) of such immune responses with the apparent loss of WHV cccDNA is significant to the extent of viral immune clearance possible in recovery. One implication from the above studies is how much a relatively successful antiviral and/or immunotherapy for chronic HBV or WHV infection will improve the prospects for disease outcome beyond that observed in natural recovery from infection.

MOLECULAR VIROLOGY STUDIES

WHV is classified as a member of the genus *Orthohepadnavirus*, family Hepadnaviridae[73]. The genetic organization of WHV is similar to that of HBV and other mammalian hepadnaviruses, and their biological properties and replicative strategies are essentially the same[74]. Filaments and spherical particles are found in the serum of WHV-infected woodchucks which are composed of the envelope protein of the virus. Complete virions are 42 to 45 nm in diameter and are composed of an exterior envelope protein (WHV surface antigen; WHsAg), an inner nucleocapsid or core protein (WHcAg), and, within the nucleocapsid, the DNA genome[75,76]. The replicative cycle of WHV seems to be identical to that of HBV[75-78]. The role of cccDNA as the template for viral transcription, the mechanism of replenishment of the cccDNA pool,

and the control of this pathway by surface antigen, have been investigated mainly using DHBV. For some studies, full-length clones of the WHV genome, cut and ligated to form a supercoiled cccDNA, have been used for *in vivo* molecular studies since direct injection into the hepatic parenchyma of woodchucks results in productive WHV infection[79]. Only a brief overview is provided below for background purposes.

During infection, HBV enters the hepatocyte, but the mechanism is poorly understood. No hepatocyte receptor has yet been defined for HBV, although studies suggest that the virus-cell recognition may be mediated all or in part by specific sequences located in the pre-S1 region of the large envelope protein. However, with numerous other potential envelope recognition sites for the cell suggested from *in vivo* neutralization studies with monoclonal antibodies, and the fact that antibodies elicited by vaccines to only the small envelope protein provide protective immunity, we are a long way from understanding the mechanisms of antibody-mediated neutralization of HBV attachment, entry, and uncoating during infection. It is known that the circular, partially double-stranded DNA genome makes its way to the nucleus where the partial DNA strand (i.e., positive strand) is completed via the endogenously linked virion reverse transcriptase-DNA polymerase, and the now fully circularized double strand is then ligated into a cccDNA. The cccDNA serves as the key template for viral mRNA transcription via the cellular RNA polymerase Ⅱ. One of the viral mRNAs (slightly larger than the genome length transcript) becomes encapsidated into maturing core particles along with the virion polymerase, where it is then reverse transcribed into the viral negative strand DNA *via* the RNA-dependent DNA polymerase activity of the encapsidated enzyme. The viral polymerase then uses its DNA-dependent DNA polymerase activity to partially complete the positive strand DNA to about 50%-75%, and this non-covalently closed circularized DNA is found in mature virions of HBV and WHV. Envelope acquisition occurs at the endoplasmic reticulum (ER) and mature virions are secreted from hepatocytes. Hepadnaviruses are not directly cytotoxic to infected cells.

Amplification and replenishment of cccDNA in the nucleus of the infected hepatocyte occurs when a portion of the maturing core particles complete positive strand DNA synthesis and are cycled back to the nucleus (i.e., instead of through the ER) where the new double strand DNA is processed into cccDNA. In HBV, most immunostaining of core is found in the nucleus, whereas in WHV, the core staining is primarily cytoplasmic, and not detected in the nucleus. This suggests a process of newly synthesized cytoplasmic core particles carrying out reverse transcription, partial or complete positive strand synthesis, and occasional re-entry into the nucleus for amplification of cccDNA (alternatively, cytoplasmic core staining may reflect incoming virus, but this seems far less likely). For HBV, cytoplasmic cores may go undetectable by immunostaining, and the denser staining of core particles within the nucleus may reflect maturation of HBV core particles there, with exit to the ER for envelope acquisition via a different cellular pathway. In established carrier woodchucks, WHV virions often circulate in 10- to 100-fold greater concentrations than do HBV virions in human chronic carriers. This may relate to the differential immuno-localization of core particles in the two models.

Transition of viral DNA to RNA during the life cycle of WHV has similarities to that of retroviruses[75,78], but integration of viral DNA into the host genome is not, however, essential for replication of hepadnaviruses, as is the case with retroviruses. Persistence of episomal cccDNA in infected hepatocytes is considered stable and this is problematic for its removal from the system, which appears to require elimination of the infected hepatocyte. It therefore represents the main target for attaining complete eradication of hepadnavirus from the system. When hepadnaviral DNA does integrate into host cell DNA, it is usually truncated and rearranged, and can target any number of sites in cellular DNA[80,81], some or all of which may be important in hepatocarcinogenesis. Morphological and molecular virological studies of the liver have shown that virtually 100% of hepatocytes become infected after experimental WHV infection[82]. Although replicative forms are cleared rapidly during recovery, WHV cccDNA persisted in a certain proportion of woodchucks long after evidence of WHV replication had ceased. That said, recovery is indeed durable and protective against disease progression in the vast majority of cases. On the other hand, persistence of the episomal cccDNA in chronic HBV (and WHV) infections remains a major conundrum in attempts to clear the virus via various therapeutic approaches (see below).

HBV generally is considered a hepatotropic virus, but hepadnavirus DNA can also be detected in extrahepatic tissues. For example, DHBV is often found replicating in the pancreas of ducks. HBV and WHV appear to infect the lymphatic system, although the exact significance of this observation is not well understood[58,60-63,65,83-86]. Some studies suggest that WHV replication and spread in the lymphatic compartment can proceed independently, even before infection of the liver[60,86]. Quiescent (non-replicating) WHV DNA molecules in PBMC from chronic WHV carriers can be activated to form replicative intermediates by stimulation of PBMC with lipopolysaccharide (LPS)[62]. The cell-free supernatants from LPS-stimulated WHV carrier PBMC (but not those from the unstimulated carrier PBMC) contain newly replicated infectious WHV that induce-acute hepatitis in WHV-susceptible adult woodchucks[84].

WHV quiescence versus replication in the lymphatic compartment may vary depending on the state of the host lymphatic target cell (i.e., resting, dividing, circulating in blood, within lymphatic tissue, *etc.*). WHV DNA can be detected in bone marrow cells as early as one month post neonatal WHV infection, but the first signs of WHV replication in PBMC, lymph nodes, and spleens occur during the acute stage of hepatic infection[83]. During recovery lymphatic WHV replication subsides to a quiescent state (or approaches complete elimination). In chronic infections, WHV replication also becomes quiescent in circulating PBMC, but often continues in the

spleen[83], and the quiescent WHV in PBMC often can be activated upon *ex vivo* stimulation using LPS, as indicated above[62,84]. More recent published studies indicate that long-term recovered woodchucks can also harbor infectious DNA in PBMC[58].

From the above, woodchucks recovering from acute WHV infection and those progressing to chronicity seem to have similar PBMC infection profiles, and in both cases the PBMC respond robustly in proliferation assays to polyclonal mitogens such as ConA, PHA, and LPS[44,55,87-89]. Even with similar PBMC WHV DNA profiles, the PBMC proliferative responses to viral antigens are generally more robust in the recovery outcome compared to the chronic outcome[44,55,56,87-90]. Thus, immune response function in viral infection does not appear to be affected adversely by the ongoing lymphatic infection. In fact, lymphatic infection by WHV, either acutely or in chronic WHV carriers, does not result in any lymphadenopathy, lymphopenia, lymphoma, or generalized immunodeficiency enabling opportunistic infections. As with natural recovery, therapy of chronic WHV infection presumes to target all reservoirs and molecular forms of the virus in both the lymphatic system and liver.

IMMUNOLOGICAL STUDIES

Resolution of experimental WHV infection in both neonatal and adult woodchucks involves a self-curative process with appropriate virus-specific immune responses in the periphery and liver (Figure 3). Natural recovery perhaps represents a benchmark for the possible induction of antiviral and/or immunotherapeutic effects in chronic WHV carriers. Specific activation of humoral and cellular immune responses is a prerequisite for viral clearance during acute HBV infection in adult patients, as reported in numerous studies[2-4,9,91]. However, the kinetic development of these responses during the early incubation and acute stages of adult HBV infection, and their influence on the course and outcome of infection, are less well characterized in humans, since patients usually do not present with clinical symptoms immediately after HBV transmission (except for a few rare cases involving known exposure times; e.g.,[92]).

Studies of self-limited WHV infection reveal numerous virus and host response patterns analogous to self-limited HBV infection[61,79,82,83,85,88,89,93-105]. In general, resolution of WHV infection in both the neonatal and adult settings is characterized by: (1) a transient peak of WHV DNA and antigen detection in serum and liver during the acute phase of infection, (2) timely and appropriate cell-mediated immunity (CMI) to viral antigens, (3) acute viral hepatitis with limited liver injury, (4) a transient peak and subsequent normalization of serum aminotransferases, and (5) seroconversion to virus-neutralizing antibodies, all leading to a substantial clearance of virus and viral antigens from the blood and liver. The humoral immune response to viral antigens (i.e., WHcAg and WHsAg) during resolution is associated with the development of robust titers of anti-core (anti-WHc), and of virus-neutralizing, protective, anti-surface antibodies (anti-WHs) with the onset and waning of the acute phase, all usually within several weeks after experimental infection[17,42].

In adult woodchucks, the CMI associated with recovery is characterized by activation of PBMC detected by *in vitro* stimulation of PBMC with WHsAg, WHcAg, and synthetic peptides of both antigens[87-90,93,94,106]. The successful PBMC response to WHcAg is associated co-temporally with viral clearance from serum, and this has been mapped extensively to several key epitopes of the WHcAg[87,93]. In fact, immunization with the dominant WHcAg epitope sequence between amino acids 97 to 110 significantly dampens acute WHV infections in adult woodchucks following experimental challenge with WHV, when compared to infections in unvaccinated control woodchucks[87]. Mapping of the PBMC responses to WHsAg and WHV x antigen (WHxAg) during the acute phase of resolution have been in progress.

The CMI to viral antigens during the acute phase in neonatal woodchucks experimentally infected with cWHV8P1 (from which neonates resolve more frequently) is similar to that in resolving adult woodchucks, which is independent of the WHV inoculum used[44]. Robust PBMC responses to WHcAg, WHsAg, and WHxAg, and to several non-overlapping core peptides, are associated temporally with the clearance of WHV DNA and WHsAg from serum. Detailed analysis of the WHcAg-specific PBMC responses revealed a broad recognition of several WHcAg epitopes representing apparently distinct regions of this antigen. Similar to adult WHV infections, neonatal woodchucks develop PBMC responses to important WHcAg peptides (residues 97 to 110, residues 100-113)[44].

In the liver, the CMI during resolution of adult WHV infection is characterized by moderate to marked hepatic inflammation and liver injury involving increased CD3- (cluster of differentiation 3) positive T lymphocyte accumulation, and apoptosis and regeneration of hepatocytes[96,97]. These events are accompanied by marked elevations of CD3, CD4, and CD8 mRNA expression and increased expression of the T-helper lymphocyte (Th)-type 1 cytokine mRNAs interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), and of the IFN-γ -inducible oligoadenylate synthetase (2'-5'-OAS) mRNA[96,97]. *In vitro* testing of cell-mediated killing of hepatocytes revealed activation of both FasL- (ligand for the apoptosis-inducing factor Fas) and perforin-dependent pathways during resolution, and a comparative analysis demonstrated that acute hepatitis, but not established chronic WHV infection, is associated with elevated hepatocyte killing as a consequence of increased activation of the perforin-dependent pathway[95]. Further acute phase studies of adult self-limiting WHV infections are in progress to better define the kinetic interrelationships between mRNA expression in liver and PBMC mRNA expression *ex vivo* or following *in vitro* stimulation with antigens.

Neonatal WHV infections can be studied prospectively, in proportionate and adequate numbers of woodchucks, as the dichotomy in outcome proceeds dynamically in real time toward recovery versus chronicity. As with recovering WHV infections in adult woodchucks, resolution of

neonatal WHV infection is associated with moderate hepatic inflammation and liver injury and accumulation of CD3-positive T-lymphocytes[42,43]. Hepatic inflammation is characterized further by significant accumulation of CD3, CD4, and CD8 mRNAs, with elevated expression of the Th-type 1 cytokine mRNAs IFN-γ and TNF-α, and of the intracellular transcription factor STAT4 (signal transactivator of transcription) and T-bet (T box expressed in T lymphocytes) mRNAs, and also of Fas ligand and perforin mRNAs. When taken together, the results indicate that both non-cytolytic and cytolytic clearance of WHV-infected hepatocytes is occurring[43,45,46]. The results from the above studies suggest further that early virus-specific CMI in both the periphery and liver play a pivotal role in resolution of acute WHV infection in neonatal and adult woodchucks, and that the responses to WHcAg and to selected core peptides are instrumental in controlling viral infection.

Although immune responsiveness has been well-characterized in the periphery and liver of established HBV chronic carriers during chronic hepatitis, the actual acute phase responses associated with the early onset of chronic HBV infection are less well understood. Lack of immune responsiveness to HBV antigens in some HBV carriers may ensue with the establishment of the carrier state and have little to do with the early onset at a time when other individuals may recover normally. Moreover, chronic hepatitis is defective by definition, when compared with the acute hepatitis that results in recovery, because the chronic inflammation is incapable of clearing the hepatic infection and lends itself only to progressive liver disease. Adult patients presenting with acute hepatitis B are often well into the infection and only rarely progress to chronic infection. Studies of the early onset of chronic HBV infection in humans after neonatal transmission have obvious limitations. While studies in established chronic HBV carriers show defective immune responses associated with tolerance and chronic hepatitis and a failure to clear the infection[2-4,9,91], it is unclear whether such deficient responses are representative of the primary acute phase responses that predispose to the chronic outcome. Understanding how the chronic infection first becomes established kinetically near the time of the acute stage of infection could lead to the identification of important cause-effect relationships that will facilitate the rational development of therapies for successfully treating established chronic infections.

For testing the hypothesis that chronic WHV infection develops due to a diminished host response to acute infection, co-temporal comparisons were performed in the neonatal WHV infection model[7,17,42-46,107-109]. Using a bank of control and WHV-infected liver specimens that were obtained surgically at two acute phase time points of neonatal WHV infection (wk 8 and 14) and that were assigned to recovered or carrier woodchucks once outcome was known based on later serological profiles, the early onset of the chronic WHV carrier state (compared to co-temporal resolving infections) was characterized by: (1) higher acute phase viral loads in liver (at wk 8 and 14 post infection), (2) diminished acute hepatitis (at wk 14), (3) detectable but significantly diminished hepatic inflammation (at wk 14), and (4) reduced liver injury (at wk 14)[42,43,45,46]. This was associated further with: (1) absent or suboptimal intrahepatic accumulation of CD3, CD4, and CD8 mRNAs, and (2) reduced expression of Th-type 1 cytokine mRNAs, especially IFN-γ and TNF-α, along with the key Th-type 1 transcription factor T-bet[43,45,46]. This represented an early primary deficiency in the Th-type 1 response in liver to acute WHV infection, and was not associated with any local antagonistic Th-type 2 immunoregulation[45].

Studies in the peripheral blood using serial measurements of PBMC responses in neonatal woodchucks experimentally infected with WHV7P1 or cWHV8P1 have shown thus far that all neonates with resolving infections had robust acute phase PBMC responses to WHcAg and to the key epitope of this antigen (core residues 97-110), with the majority of woodchucks also responding to WHsAg and WHxAg[44]. In contrast, prospective carriers responded less frequently or not at all, with only about one-third responding to WHcAg, and among these, only about half responded to the key core epitope and to other WHV antigens. Detailed mapping of the PBMC responses to WHcAg revealed that the epitopes recognized were localized to distinct regions of this antigen and were different from those recognized during resolving WHV infections[44]. In the prospective carriers with minimal acute phase PBMC responses to WHcAg, viremia and antigenemia developed later, and viral and antigen loads were lower compared to those seen in prospective carriers without any evidence of virus-specific PBMC responses[44]. In any case the levels of viremia and antigenemia in these prospective carriers were much higher than in neonates with resolving WHV infections[44]. Interestingly, the fact that virus-specific PBMC responses were undetectable in the majority of prospective carriers indicates an early genesis for the CMI defect commonly observed later in established chronic WHV infection[55,56,87-90,94]. Further studies to correlate the molecular immunologic responses of PBMC based on leukocyte surface marker and cytokine mRNA expression with outcome of neonatal WHV infection are in progress.

Established chronic HBV infection is associated with increased viral load and risk of severe liver disease sequelae[2-4,9,91]. Chronic HBV infections resulting from neonatal transmissions are characterized by T cell immunotolerance to viral antigens throughout most of life until end-stage disease, but may exhibit occasional exacerbations of liver disease before this time[51,110-114]. T cell proliferative responses to viral antigens in adult-acquired chronic HBV infections can be variable during disease progression, but are usually less responsive, except during periodic transient flare reactions and with seroconversion to anti-e antibodies (e.g.,[52-54,115,116]).

Studies in woodchucks indicate that viral antigen-specific CMI during established chronic WHV infection is also defective, similar to that observed in HBV infection[45,55,56,87-89,94]. In the neonatal woodchuck, following an occasional, early and transient, but suboptimal acute hepatitis, WHV chronic carriage is characterized for some

time with minimal chronic persistent hepatitis, and little or no liver injury based on serum enzyme markers up through at least 15 mo post infection[46]. This progresses subsequently to more active hepatitis and liver injury just before or at the time of HCC onset and tumor growth[46,47,49,50,55,57]. PBMC remain essentially immunotolerant to WHV antigens throughout all of the chronic phase of neonatal WHV infection, including end stage disease[55,56,87,89,90,94]. The baseline expression of Th-type 1 cytokines in liver that is usually observed during the chronic phase can sometimes increase above normal with progressive chronic hepatitis (usually with increased TNF-α and less IFN-γ)[45,97], but without affecting clearance of the infection. Less is known of the CMI in documented adult-acquired chronic WHV infections, but the apparent greater degree of chronic hepatitis in this setting may suggest some exception to the fully tolerant state as leading to and maintaining the chronic infection[96]. Indeed, some leukocyte surface and cytokine markers become elevated in liver during acute hepatitis in adult woodchucks that eventually become chronic carriers, although to a lesser extent than seen during the acute phase of woodchucks that resolve.

THERAPEUTIC STUDIES

Antiviral drugs

Woodchucks with experimentally induced chronic WHV infection have been used successfully in the empiric screening and preclinical assessment of antiviral drugs being developed for treatment of chronic HBV infection (for previous reviews see[21,24,25,30,35,117]). Current strategies aim to suppress viral replication in liver and the concentration of viral DNA in serum during chronic HBV infection (i.e., reduce viral load) by treatment with nucleoside and nucleotide analogues. As indicated above, it has been difficult to target the viral cccDNA directly in this process, and so potent inhibition of viral replication is the main means to reduce viral load in blood and tissues, and perhaps diminish replenishment of cccDNA indirectly, until cells harboring this intermediate can turnover or be eliminated by immune responses. Accordingly, lifelong therapy with antiviral drugs is currently the accepted procedure, even though this often results in the selection of drug-resistant mutants (i.e., mutations of the polymerase gene), which has been observed and modeled in the woodchuck (e.g.,[118-122]).

Before testing in woodchucks, potential drug candidates are screened for antiviral activity against HBV in the 2.2.15 cell system, a HepG2 cell line that is engineered to produce HBV constitutively[123]. Drugs with significant antiviral activity *in vitro* also have been tested in a HBV-transgenic mouse model designed and validated for this purpose[124]. However, drug efficacy in an *in vivo* infection model is most usually assessed in the woodchuck model. Most nucleoside analogues with intermediate antiviral activity *in vitro* against HBV had comparable antiviral activity against WHV in woodchucks, but some exceptions exist. For example, fialuridine (D-FIAU) had modest activity *in vitro*, and potent antiviral activity in woodchucks; however, this was associated with a marked and delayed hepatotoxicity characterized by microvesicular steatosis and mitochondrial injury[125], similar to the unfortunate hepatotoxic effects this drug had in humans, where it was first tested[126].

In recent years, numerous nucleoside and nucleotide analogues designed to inhibit HBV replication were tested in the woodchuck model (e.g.,[125,127-140]. Some of these nucleoside analogues had demonstrated antiviral efficacy in chronic WHV carrier woodchucks, such as lamivudine (3TC, Epivir)[21,120,141-143], adefovir dipivoxil (ADV, Hepsera)[144,145], and entecavir (ETV, Baraclude)[139,146], and are now approved by the FDA for treatment of chronic HBV infection. Other nucleoside analogues, also having activity in woodchucks against WHV, are used for the treatment of human immunodeficiency virus (HIV), such as tenofovir disoproxil fumarate (TDF, Viread)[147] and emtricitabine (FTC, Coviracil)[148-150], and still others are in advanced clinical testing, such as telbivudine (LdT)[151-153], valtorcitabine (val-LdC)[151-153], and clevudine (L-FMAU)[55,56,122,150,154-156]. FDA approval of these drugs for treatment of chronic HBV infection is expected in the near future (in fact, telbivudine was approved most recently). Table 1 summarizes the antiviral activities of these second and third-generation nucleosides in chronic WHV carrier woodchucks that were reported in selected studies. We note here in these experiments that viral recrudescence following cessation of drug is often a function of being unable to completely suppress viral replication sufficiently during a given treatment, or significantly enough over time in order to allow cells containing cccDNA to turnover or be eliminated by the immune response.

Lamivudine is a moderately potent antiviral drug in woodchucks and is without toxicity during daily, oral administration for up to 24 wk[21,141,157], and even longer[142]. The average reduction in serum WHV DNA after 4 or 12 wk of treatment with different doses (1, 5, or 15 mg/kg bodyweight) was approximately 2.5 and 1.5 logs, respectively. The average time to recrudescence of viral replication after drug withdrawal was within 1 to 2 wk. In woodchucks, lamivudine also has been shown to act synergistically both with alpha-interferon and with famciclovir[141,157]. An antiviral activity comparable to lamivudine has been reported for adefovir[144]. Daily oral administration of adefovir for 12 wk with doses of 5 and 15 mg/kg resulted in a reduction in serum viremia of 1.7 or 2.5 logs, respectively. Viral recrudescence after drug withdrawal occurred within 6 wk. No toxicity associated with administration of adefovir was observed. The antiviral activity of tenofovir in woodchucks[147] is comparable to those of lamivudine and adefovir. The reduction in serum WHV DNA observed after 4 wk of daily, oral treatment with tenofovir doses of 5 and 15 mg/kg was 1.5 or 1.2 logs, respectively. After drug withdrawal viral recrudescence occurred within 1 to 4 wk and treatment was without any evidence of drug-associated toxicity.

A higher antiviral activity on chronic WHV infection was reported for emtricitabine[149]. Daily oral treatment for 4 wk with doses of 10 or 30 mg/kg reduced serum viremia by 3.2 and 4.9 logs, respectively. Recrudescence of viral replication occurred within 1 to 2 wk after drug withdrawal.

Table 1 Antiviral activities of second and third-generation nucleosides and nucleotides in the woodchuck model of chronic HBV infection

Antiviral drug	Oral dose (mg/kg per day)	Treatment duration (wk)	Follow up duration (wk)	Serum WHV DNA reduction (log)	Time to viral recrudescence (wk)	Drug-associated toxicity	Other viral markers	Ref.
Lamivudine	1	24	24	1.5	within 1-2	none	WHV RI red. (3-fold) no WHV RNA red. no serum WHsAg red.	[141]
	5	4	12	3.4	within 1	none	WHV RI red. (4-fold) no WHV RNA red. no serum WHsAg red.	[21]
	5	12	12	1.9	within 1-2	none	WHV RI red. (3-fold) no WHV RNA red. no serum WHsAg red.	[157]
	15	4	12	5.4	within 1	none	WHV RI red. (12-fold) no WHV RNA red. no serum WHsAg red.	[21]
Adefovir	5	12	6	1.7	within 6	none		[144]
	15	12	6	2.5	within 6	none		[144]
Entecavir	0.02	12	12	7-8[1]	within 2-10	none	WHV RI red. in individual animals to undetectable levels	[139]
	0.1	12	12	7-8	within 6-10	none	WHV RI red. in most animals to undetectable levels	[139]
Tenofovir	5	4	12	1.5	within 1-4	none	no WHV RI red. no WHV RNA red. no serum WHsAg red.	[147]
	15	4	12	1.2	within 1-4	none	no WHV RI red. no WHV RNA red. no serum WHsAg red.	[147]
Emtricitabine	3	4	12	1.4	within 1-2	none	WHV RI red. (3-fold) no WHV RNA red. no serum WHsAg red.	[149]
	10	4	12	3.2	within 1-2	none	WHV RI red. (13-fold) no WHV RNA red. no serum WHsAg red.	[149]
	30	4	12	4.9	within 1-2		WHV RI red. (80-fold) no WHV RNA red. no serum WHsAg red.	[149]
	20[2]	4	4	1.4	within 1-2	none		[148]
	30[2]	4	4	1.8	within 1-2	none	WHV RI red. (2-fold)	[148]
Telbivudine	10	4	8	8	within 4-8	none	serum WHsAg red.	[151-153]
Valtorcitabine	10	4	8	4-6	within 1-8	none		[151-153]
Clevudine	3	4	12	9.2	within 2-10	none	WHV RI red. (28-fold) no WHV RNA red. serum WHsAg red. (2-fold)	[154]
	10	4	12	8.2	within 8-12[3]	none	WHV RI red. (68-fold) WHV RNA red. (2.7-fold) serum WHsAg red. (4-fold) WHV cccDNA red. (2-6-fold or to undetectable levels)	[154]

[1]Two of 6 woodchucks with modest reduction in serum WHV DNA of approximately 2.0 logs were not included. [2]Dosage was given twice daily by intraperitoneal administration. [3]One woodchuck had suppressed serum WHV DNA at the end of the study. WHV RI, hepatic WHV DNA replicative intermediates; WHV RNA, intrahepatic WHV RNA; red., reduction.

A dose of emtricitabine of 3 mg/kg in this study was less efficacious, but was comparable to those observed with 20 and 30 mg/kg, administered twice daily, intraperitoneally, for 4 wk[148]. There was no toxicity associated with this drug treatment. A more remarkable antiviral activity was obtained with valtorcitabine in chronic WHV carrier woodchucks after daily oral administration for 4 wk with a dose of 10 mg/kg[151-153]. In this case, serum WHV DNA became reduced by 4 to 6 logs with no evidence of drug-associated toxicity at the dose used. The time to recrudescence of viral replication after drug withdrawal was as little as 1 wk, but extended to 8 wk in many of the animals. Entecavir had an even higher antiviral activity in woodchucks[139]. Daily oral administration of entecavir for 12 wk at a dose of 0.02 mg/kg resulted in a reduction in serum viremia of 7 to 8 logs in 4 of 6 treated woodchucks (2 of the 6 treated woodchucks had only a modest antiviral effect). Recrudescence of viral replication after drug

withdrawal occurred in as little as 2 wk, but was extended to 10 wk in several of the animals. Administration of entecavir at a dose of 0.1 mg/kg reduced serum viremia by 7 to 8 logs and viral recrudescence was observed within 6 to 10 wk. No toxicity associated with drug treatment was reported.

The most potent antiviral drugs that have been tested so far in woodchucks are telbivudine and clevudine. Daily oral administration of telbivudine for 4 wk at a dose of 10 mg/kg resulted in an 8 log reduction in serum viremia and viral recrudescence was observed within 4 to 8 wk after drug withdrawal[151,152]. Daily oral administration of clevudine for 4 wk at doses of 3 or 10 mg/kg reduced serum viremia by 9.2 and 8.2 logs, respectively[154]. With the lower dose of clevudine viral recrudescence after drug withdrawal was observed within 2 to 10 wk. The higher dose delayed viral recrudescence and serum WHV DNA concentrations reached pretreatment levels within 8 to 12 wk, and in one woodchuck, serum viremia was still suppressed at the end of the study. No toxicity was associated with the above short-term treatments using either telbivudine or clevudine.

The above studies in the woodchuck model demonstrate that a significant antiviral effect on chronic WHV infection could be achieved with all drugs. The relative antiviral efficacy against WHV, at the doses administered and for the duration of treatment used, was clevudine $\geqslant$ telbivudine $\geqslant$ entecavir > valtorcitabine $\geqslant$ emtricitabine $\geqslant$ tenofovir = adefovir = lamivudine. A prolonged suppression of WHV replication after drug withdrawal was achieved with clevudine, telbivudine, entecavir, and valtorcitabine, and the magnitude of these responses was often associated indirectly with transient or sustained reductions in WHV cccDNA potentially enabling some turnover of residually infected cells. The favorable safety and efficacy profile obtained thus far in the woodchuck model using relatively short-term treatments with clevudine, telbivudine, and valtorcitabine suggest that these drugs should be of value in the long-term control of chronic HBV infection in humans and support their continued clinical development.

The preclinical evaluation of antiviral drugs for treatment of lamivudine-resistant HBV infection has been modeled in woodchucks by the experimental induction of lamivudine-resistant WHV with nine or more months of lamivudine treatment, followed by continued therapy with lamivudine along with the new drug candidate of interest[122,145]. Prolonged treatment with lamivudine led to the establishment of drug-resistant WHV mutants, characterized mainly by mutations in the B domain of the WHV polymerase gene (i.e., HBV mutations occur in B and C domains). Supplemental daily oral treatment of these circulating B domain mutants with adefovir or clevudine (10 mg/kg per day, 12 wk and 7 wk, respectively) demonstrated that both drugs could suppress replication of these lamivudine-resistant WHV mutants. In a different study, lamivudine-resistant mutants of WHV were found to be cross-resistant to treatment with clevudine[122]. Studies are in progress using engineered lamivudine-resistant mutants of WHV that mimic the additional polymerase C domain mutants observed in human HBV patients treated with lamivudine.

In addition to the testing of drugs in woodchucks for antiviral effects, applications also have been extended to the testing of entecavir, clevudine, or lamivudine for efficacy against disease progression[30,142,146,155]. Extended lamivudine treatment of woodchucks with chronic WHV infection delayed the development of HCC and significantly extended survival of woodchucks in one study[142]. In that study, twenty 8-mo-old chronic WHV carrier woodchucks were treated throughout the rest of their lifetime with lamivudine (5 mg and then 15 mg/kg, orally, daily). Twenty placebo control WHV carrier woodchucks were included for comparison. Serum WHV DNA decreased by 4 to 5 logs in lamivudine-treated carrier woodchucks, with an antiviral effect that was sustained for more than one year with continued treatment. Importantly, there was a significant delay in time to onset of HCC and death due to HCC among lamivudine-treated woodchucks compared to placebo controls. In another study of lamivudine in WHV carrier woodchucks, no delay in hepatocarcinogenesis was observed with treatment, most likely because drug treatment began when woodchucks were at an older age, was of shorter duration, and less of an antiviral effect on serum WHV DNA was observed[120]. In both studies, lamivudine resistance developed that was associated with a high frequency of mutation in the WHV polymerase gene B domain[118,121].

In another study, long-term oral treatment with entecavir[146] in 8-mo-old woodchucks at 0.5 mg/kg per day for 8 wk, and then with a weekly dose of 0.5 mg/kg for 14 or 36 mo, produced sustained antiviral responses in half of the woodchucks treated for 14 mo, and in 80% of the woodchucks treated for 36 mo (i.e., reduced serum viremia of 5 to 8 logs). Here, the drug-treated woodchucks had marked reduction in viral load and did not develop HCC during the next 2 years follow-up. Compared in this case with historical controls, entecavir treatment significantly delayed the development of HCC and prolonged survival.

In another study, clevudine was administered orally to chronic WHV carrier woodchucks at 10 mg/kg per day for 32 wk[30,55,56,155] starting at 1 to 2 years of age. Half of the clevudine-treated woodchucks and half of the placebo recipients then received 4 doses of a conventional WHsAg vaccine (alum-adsorbed, formalin-inactivated WHsAg) during the next 16 wk. Combination treatment with clevudine and vaccine resulted in a sustained antiviral effect with reductions in serum viremia of more than 8 logs in many cases (Figure 4), and prevented the development of HCC altogether in up to 38% of treated woodchucks. In a subset of the woodchucks studied, where clevudine or placebo treatment was initiated at 1 year of age (and the data analyzed independent of combination with WHsAg vaccine), the development of HCC in clevudine-treated woodchucks was delayed significantly and long-term survival after 4 years likewise was increased significantly compared to woodchucks that did not receive clevudine. These studies show that chemotherapy with antiviral drugs can delay and reduce disease progression in chronic carrier woodchucks, and also show the correlation between

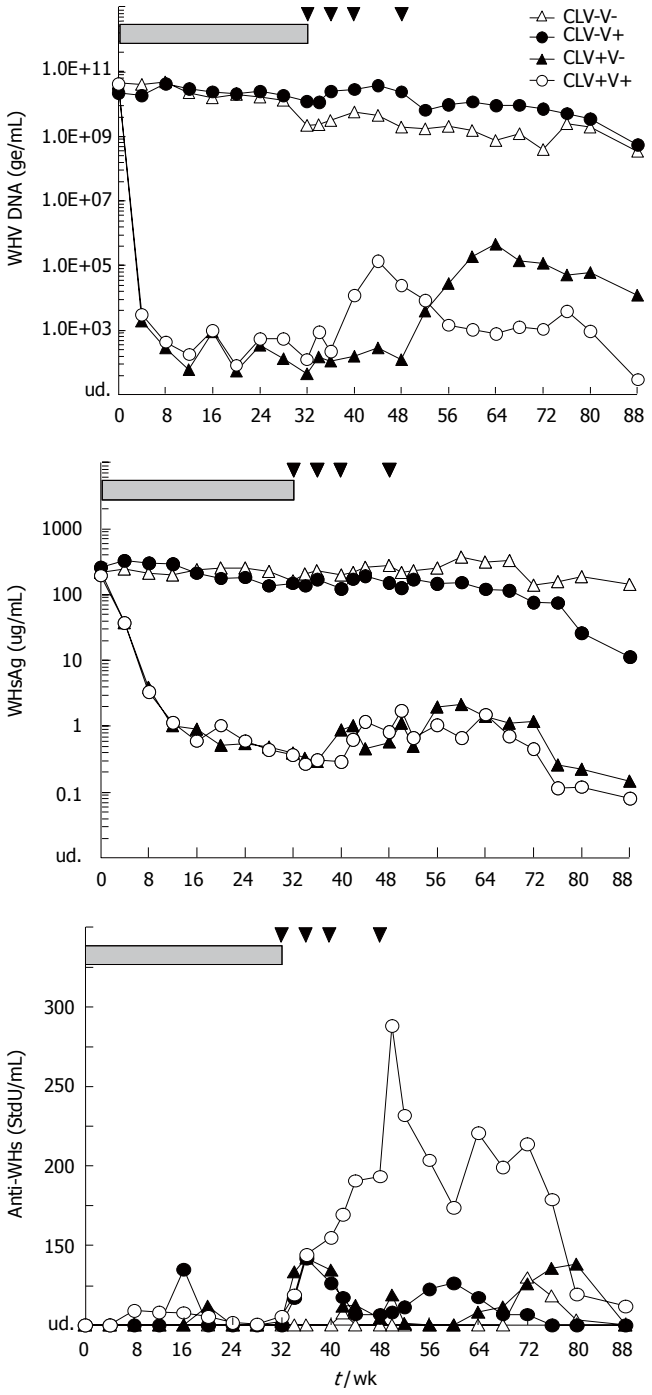

Figure 4 Combination treatment with clevudine and WHsAg vaccine suppresses serum viremia and antigenemia and induces a humoral response in chronic WHV carrier woodchucks. Changes in serum WHV DNA, serum WHsAg, and anti-WHs antibodies of chronic carriers in response to treatment with placebo (CLV-V-), vaccine (CLV-V+), drug (CLV+V-), and combination of drug and vaccine (CLV+V+) are shown. Horizontal bars denote the period of clevudine (CLV) administration for 32 wk. Arrowheads represent the 4 immunizations (V) using 50 μg doses of an alum-adsorbed, formalin-inactivated WHsAg vaccine at wk 32, 36, 40, and 48. WHVge, WHV genomic equivalents (virion or WHV DNA-containing particles).

reduced viral load and reduced disease progression, with noteworthy implications for HBV therapy in humans.

In addition to nucleoside or nucleotide analogues, various other compounds of organic and plant origin have been tested in woodchucks for their antiviral activity (e.g.,[141,158-161]), but these will not be discussed in detail in this review. Direct testing of anti-tumor agents against established HCC in woodchucks is also possible[162-164], but has not been fully developed to date.

Immunotherapy

The main goal of basic immunological studies described above in neonatal and adult WHV-infected woodchucks is to identify and differentiate factors that cause and maintain chronic infection from those that result from chronic carriage. By better defining cause-effect relationships, it should be possible to develop and test rational immunotherapies that can induce immune responses in the established chronic carrier that mimic those in recovery from WHV infection. In this way, it should be possible to enhance the immune elimination of cells harboring viral cccDNA (and/or control its level and expression), as occurs with successful immune responses leading to recovery.

As indicated in the sections above, chronicity as an outcome of neonatal WHV infection appears to result from a failed or suboptimal primary immune response relatively early during the acute phase of infection in the periphery and in the liver. The onset of chronic infection (compared to resolution) is characterized by deficiencies in the CD8-positive cytolytic T lymphocyte (CTL) response, and reduced expression of Th-type 1 cytokines and intracellular transcription factors, minimal acute hepatitis, and humoral and cellular immunologic tolerance to viral antigens[42-46]. Negative immunoregulation of the intrahepatic Th-type 1 response by excessive intrahepatic Th-type 2 immune responses is not a defining factor in this outcome[45,46]. Chronicity then appears to develop due to reduced immune-mediated clearance of infected hepatocytes by both non-cytolytic and cytolytic processes[45,46]. The above studies indicate further that early induction of immune tolerance may be a factor in the onset of chronic neonatal WHV infection, and a similar mechanism may be involved in the onset of chronic HBV infection in unvaccinated infants born to HBV-carrier mothers. This may include the deletion of higher affinity virus-specific T cells by negative selection of precursor T cells in the thymus (central tolerance), or clonal anergy or exhaustion of virus-specific T cells that escaped early negative selection in the thymus, which are then rendered unresponsive due to higher viral and antigen loads (peripheral tolerance).

Several studies have used WHV-naïve woodchucks for testing experimental vaccines, including conventional and DNA vaccines, and adjuvants, for later therapeutic vaccination of chronic WHV carrier woodchucks. In these studies antibody responses against WHsAg or WHcAg were induced[31,55,87,165-172], and partial or full protection against viral infection and disease by challenge with WHV was observed[31,87,165,166,168-172]. A few studies also determined that cellular immune responses were induced in addition to the humoral responses[87,168-171].

Unlike when WHV-naive woodchucks are immunized, the detection of free anti-WHs in serum of WHV carriers vaccinated with WHsAg is more problematic due to an excess of WHsAg in the serum samples. However, positive

Table 2 Immunotherapeutic approaches in the woodchuck model of chronic HBV infection

Treatment	Outcome	Additional results	Ref.
Vaccination			
WHsAg vaccine/adjuvant	Anti-WHs response	CMI to WHsAg	[55,56,155]
WHsAg vaccine/adjuvant	Anti-WHs response (antibodies mainly directed against preS region)		[173]
WHsAg vaccine/Th peptide epitope	Anti-WHs response Transient serum WHV DNA red. in a few animals (1 log)	Two woodchucks died	[174]
Cytokines			
IFN-α (adenoviral vector)	Transient serum WHV DNA red. (1 log)	Transient WHV RI red. (1 log)	[181]
IFN-α (adeno-associated viral vector)	Transient serum WHV DNA red. (2 logs) Sustained serum WHV DNA red. in 2 animals		[182]
IFN-γ (adenoviral vector)	No antiviral effect		[181]
Adoptive immunotransfer			
Liver transplantation	Serum WHV DNA red.	WHV RI red., WHV RNA red.	[188]
Combination treatment			
Lamivudine + WHsAg vaccine/ Th peptide epitope	No additional benefit beyond lamivudine-induced antiviral effect	CMI to WHsAg/WHcAg	
Lamivudine + β-galactosidase (adenoviral vector)	Transient but sustained serum WHV DNA red. (> 1 log) in addition to lamivudine-induced antiviral effect	WHV RI red., WHV cccDNA red., WHV RNA red.	[191]
Clevudine + β-galactosidase/+ IFN-γ/+ IFN-α (adenoviral vector)	Transient but sustained serum WHV DNA red. in addition to clevudine-induced antiviral effect	WHV RI red.	[156]
Clevudine + emtricitabine + IFN-γ (adenoviral vector)	No additional benefit beyond clevudine + emtricitabine-induced antiviral effect	Increased liver inflammation with IFN-γ	[150]
Clevudine + WHsAg vaccine	Anti-WHs response Sustained serum WHV DNA red. (> 6 to 8 log)	WHV cccDNA red., CMI to WHsAg/WHcAg Delay in onset of disease progression	[55,56,155]

WHV RI, hepatic WHV DNA replicative intermediates; WHV cccDNA, covalently closed circular WHV DNA, WHV RNA, intrahepatic WHV RNA; red., reduction.

signals for anti-WHs can often be detected by enzyme immunoassay under these conditions, even though it may be complexed in native serum. This is because of the exchange of bound anti-WHs between WHsAg in the sample solution, and the WHsAg adsorbed to the solid phase assay matrix. Unvaccinated WHV carriers rarely if ever show detectable anti-WHs of this nature, even though they may have some complexed anti-WHs in serum. Thus, the vaccination of WHV carriers with WHsAg most likely increases the levels of anti-WHs in complex, which enables its subsequent detection (at generally low levels) in the various enzyme immunoassay formats. Assays able to detect anti-WHs in complex with WHsAg are being developed to better study such responses.

One approach to immunotherapy is to modulate the deficient humoral and cellular immune responses of chronic HBV carriers by conventional vaccination (Table 2). In one study chronic WHV carrier woodchucks received up to 6 immunizations with a serum-derived WHsAg vaccine that was adsorbed to aluminum salt and contained monophosphoryl lipid A[173]. Following immunization, all of the carrier woodchucks developed an antibody response against WHsAg that was directed mainly against the WHV preS region, but there was little in the way of positive CMI to the antigen used in the vaccine. Despite the induction of anti-WHs antibodies, serum levels of WHV DNA and WHsAg in vaccinated carriers remained unchanged. This was consistent with another study in which four doses of an alum-adsorbed, formalin-inactivated WHsAg vaccine were administered[55,56,155]. In the latter study, CMI to WHsAg and WHsAg peptides was detected in the majority of vaccinated carriers, but, again, there was little effect on serum viral load (Figures 4 and 5). Therapeutic vaccination of chronic WHV carrier woodchucks with a serum-derived WHsAg in combination with an experimental adjuvant (i.e., a peptide carrying a Th epitope from sperm whale myoglobin) induced anti-WHs antibody responses and minor transient reductions in serum WHV DNA in a few of the vaccinated carriers[174]. Caution in the use of this therapeutic WHsAg vaccine was recommended, however, since some of the carriers died during the vaccinations. Such adverse effects could have been related to the experimental adjuvant and/or to liver disease present in the woodchucks at entry into the study. The results from these studies indicate that immunization of chronic WHV carrier woodchucks with WHsAg can partially induce (or boost) B cell responses to WHsAg. Additional modulation, however, seems necessary for inducing a response profile that resembles that observed during resolution of WHV infection.

Another approach to immunotherapy of chronic HBV infection involves direct reconstitution of the deficient Th-type 1 immune responses in the liver to mimic natural recovery from infection. Cytokines such as IFN-γ and TNF-α have been reported to have direct, non-cytolytic antiviral effects in HBV transgenic mice[8,175-177]. However,

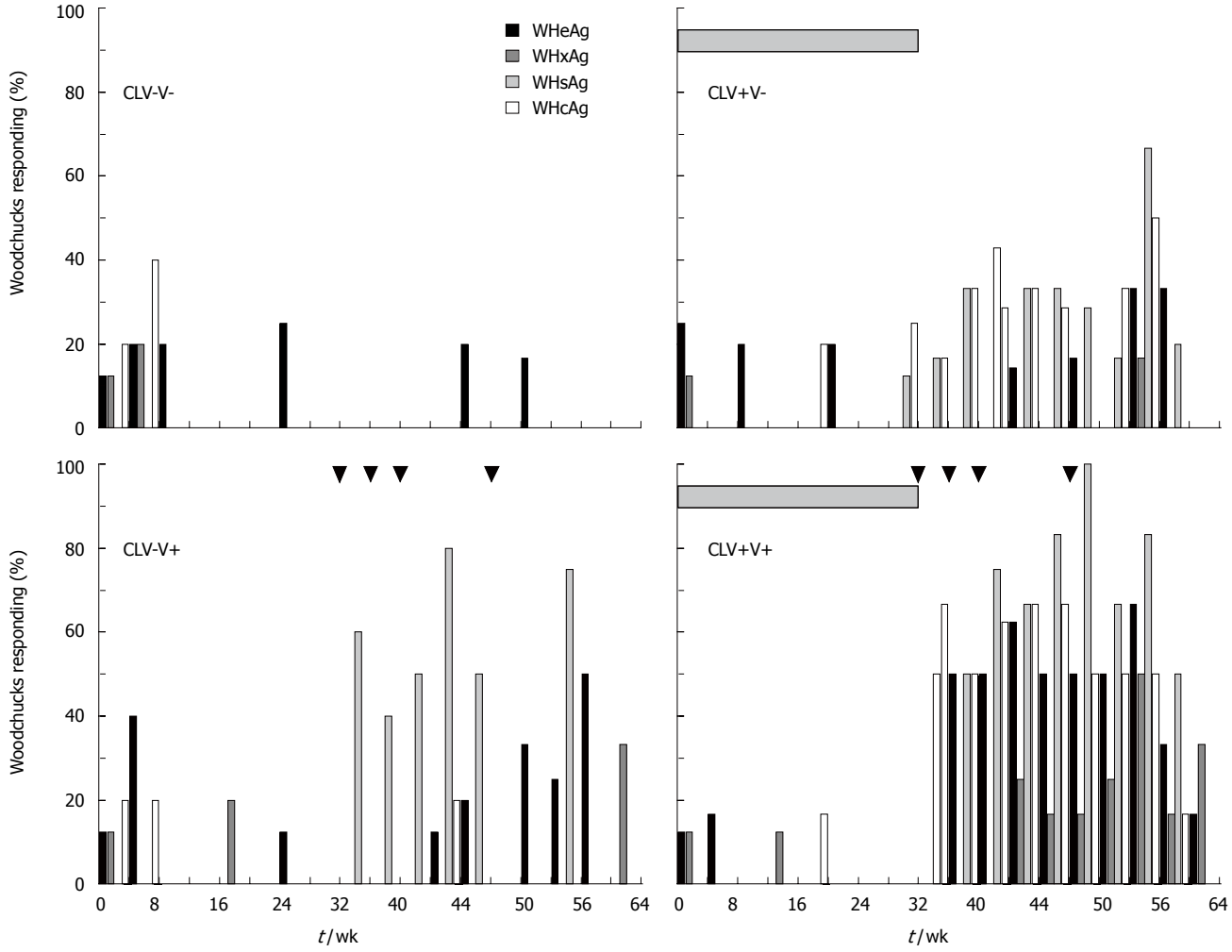

Figure 5 Combination treatment with clevudine and WHsAg vaccine enhances and expands the pattern of cell-mediated immune responses to WHV antigens in chronic WHV carrier woodchucks. Changes in the PBMC responses to WHsAg, WHcAg, WHeAg, and WHxAg of chronic carriers in response to treatment with placebo (CLV-V-), vaccine (CLV-V+), drug (CLV+V-), and combination of drug and vaccine (CLV+V+) are shown. Horizontal bars denote the period of clevudine (CLV) administration for 32 wk. Arrowheads represent the 4 immunizations (V) using 50 μg doses of an alum-adsorbed, formalin-inactivated WHsAg vaccine at wk 32, 36, 40, and 48.

increased expression of these cytokines can occur in established chronic WHV carriers with progressing chronic hepatitis and liver injury[45,96,97,178], but with little concurrent reduction in viral replication. This indicates that additional responses would be important to developing a more complete therapeutic effect resembling recovery. Recent studies have shown that woodchuck IFN-γ (and TNF-α) does not significantly deplete WHV RNA or WHV DNA replicative intermediates *in vitro* in virus-infected primary hepatocytes from chronic carriers[179]. Other studies in primary hepatocyte cultures from established WHV carriers suggest that expression of IFN-γ from a transfected plasmid (and also of TNF-α) can induce partial host response profiles with similarity to recovering liver, and also impair a later step in viral replication by a non-cytolytic mechanism that is probably mediated by TNF-α[180].

The effects of woodchuck IFN-α and IFN-γ on WHV replication were determined *in vivo* in a recent study in chronic WHV carrier woodchucks using an adenoviral vector for the expression of these cytokines[181]. Following vector administration directly into the liver, a slight but transient reduction in intrahepatic WHV DNA replication and in serum WHV DNA of about 1 log was obtained with the IFN-α expressing vector. The intrahepatic expression of IFN-γ, however, had no effect on WHV, thus leading to the conclusion that hepatocytes of chronic WHV carrier woodchucks may be functionally altered in their response to IFN-γ or resistant to this cytokine. In another study, the administration of woodchuck IFN-α using an adeno-associated viral vector for intrahepatic delivery of this cytokine into chronic WHV carrier woodchucks had a significant antiviral effect in that serum WHV DNA was reduced by 2 logs on average (range 1.5 to 4 logs)[182]. The antiviral effect observed was transient in the majority of woodchucks, but two woodchucks appeared to have sustained suppression in serum WHV DNA concentration. The results from these studies indicate that *in vivo* therapeutic gene delivery to augment the deficient Th-type 1 cytokine responses in liver may restore some of the failed antiviral and immunologic functions in human chronic HBV infection.

Another approach to immunotherapy of chronic HBV infection involves the restoration and stimulation

of higher-affinity Th and CTL clones in the periphery (or locally in the liver). Rather than to supplement a specific cytokine deficiency, it may be possible to reconstitute a complete and successful cellular immune response to acute infection by transfer of autologous or histocompatible T cell clones. The efficacy of the latter approach has been demonstrated in recent clinical studies of lymphocompatible bone marrow or PBMC transplantation from HBV-recovered or anti-HBV immunized donors into chronic carrier recipients (e.g.,[183-187]). Studies of cell-based therapies in chronic WHV carrier woodchucks involving adoptive lymphocyte transfer from vaccinated or WHV-resolved donors are in progress using neonatal-infected carrier woodchucks made lymphocompatible with their sires by co-injection of parental bone marrow and/or lymphocytes at birth. Later, after the neonates become established WHV carriers, they are re-administered parental lymphocytes therapeutically that were primed in the parent by immunization or recovery from acute WHV infection (Menne *et al*, unpublished data). Recently, another approach involving adoptive immunotransfer *via* liver transplantation from vaccinated WHV-naïve woodchucks into chronic carrier woodchucks was tested[188]. Following vaccination of donor woodchucks with DNA plasmids encoding WHcAg, WHsAg, and WHsAg in combination with a plasmid expressing IFN-γ livers were transplanted into recipient woodchucks, and the therapeutic effect determined. Two of 3 recipient carriers demonstrated a reduction in serum WHV DNA below the limit of detection by Southern hybridization analysis immediately following transplantation that lasted for up to 7 wk. WHV DNA in serum samples was detected when a more sensitive PCR assay was used. Nevertheless, the reductions in serum viremia were consistent with parallel reductions in intrahepatic levels of WHV RNA and DNA replicative intermediates.

Combination therapy

The high viral and antigen loads in serum during the chronic phase of infection are believed to maintain immunologic tolerance in established carrier woodchucks[55,56]. In some cases, treatment with antiviral drug alone may unmask host immune responses as seen during treatment of adult-acquired chronic HBV infection with lamivudine[189,190]; however, such responses appear sub-optimal for bringing about a complete recovery phenotype. To facilitate the emergence of the host immune response from a tolerant state maintained by high antigen load, combination therapy with a nucleoside analogue followed by modulation of the deficient immune responses represents a promising approach. Such an approach might even be able to improve upon natural recovery by creating optimal conditions for more rapid and complete eradication of viral cccDNA from the system.

In one study, chronic WHV carrier woodchucks were treated with lamivudine at a relatively high daily dose of 200 mg/kg given orally for 23 wk[143]. At the time, WHV DNA and WHsAg serum levels had declined by 3 to 5 logs or 1 log, respectively, woodchucks were vaccinated with three doses of a serum-derived WHsAg in combination with a peptide carrying a Th epitope from sperm whale myoglobin. In contrast to a previous study[174], therapeutic vaccination did not induce detectable anti-WHs antibody responses in carriers; the levels of viremia and antigenemia remained nearly unchanged from that achieved by drug treatment, and they returned to pretreatment levels following drug withdrawal. One important finding of this study was that the combination of lamivudine and vaccine, but not treatment with drug alone, induced CMI to WHsAg and WHcAg, presumably by shifting the cytokine profile from Th-type 2 to that of Th-type 0/1.

In another study chronic WHV carrier woodchucks received lamivudine treatment for 6 mo, again at a relatively high dose (200 mg/kg per day, oral) in order to reduce serum WHV DNA by 1 to 3 logs, and were then superinfected with an adenoviral vector expressing β-galactosidase[191]. Compared to control woodchucks, combination treatment resulted in further reductions of serum WHV DNA (10-20-fold) in the majority of woodchucks. The vector itself induced local immune responses in liver, and a bystander antiviral effect was observed on intrahepatic WHV DNA, WHV cccDNA, and WHV RNA that correlated with the inflammatory responses involving increased intrahepatic expression of woodchuck leukocyte markers and cytokines. The suppression of WHV replication was transient, but prolonged compared to woodchucks receiving lamivudine monotherapy. Similar results were obtained following superinfection of chronic WHV carriers with adenoviral vectors expressing IFN-γ, TNF-α, or β-galactosidase in combination with orally administered clevudine at 10 mg/kg per day[156]. Adenovirus superinfection led to declines in the intrahepatic WHV DNA levels, but a long-term benefit of combination treatment over clevudine alone was not observed. However, in contrast to monotherapy with lamivudine[191], recrudescence of WHV replication was deltayed until 14 wk after withdrawal of clevudine.

The antiviral effect of a combination of two nucleoside analogues in addition to an adenoviral vector expressing IFN-γ also has been tested in chronic WHV carrier woodchucks[150]. Woodchucks received clevudine and emtricitabine simultaneously at daily oral doses of 10 mg/kg and 30 mg/kg, respectively, for 8 wk, with two intravenous injections of the vector at wk 4 and 8. Combination treatment with clevudine and emtricitabine resulted in an antiviral effect on WHV replication, with reductions in serum viremia by 4 logs, and associated declines in intrahepatic levels of WHV DNA replicative intermediates and WHV cccDNA. The antiviral effect was sustained in a few woodchucks following drug withdrawal. The additional administration of the adenoviral vector led to increased liver inflammation, but enhancements of the antiviral effect compared to combination treatment with clevudine and emtricitabine were not observed.

In our study in chronically WHV-infected woodchucks described above[55,56,155], combination therapy with clevudine (10 mg/kg per day, oral, 32 wk), followed by 4 doses of a conventional WHsAg vaccine (alum-adsorbed, formalin-inactivated WHsAg), enhanced the virus-specific CMI to WHsAg, and resulted in additional

collateral responses to other viral antigens (Figures 4 and 5). Vaccination alone elicited low-level antibody responses to WHsAg in most woodchucks but did not affect serum WHV DNA or WHsAg levels compared to placebo-treated control woodchucks. Chronic WHV carrier woodchucks treated first with clevudine to reduce serum WHV DNA (> 6 to 8 log reduction) and WHsAg (> 50- to 500-fold reduction), and then vaccinated, developed a more robust anti-WHs antibody response. After vaccination, WHsAg-specific CMI was shown in both vaccinated groups, but was significantly enhanced in woodchucks treated initially with clevudine, and was broadened to include responses to WHcAg and to selected peptide epitopes of WHcAg and WHsAg.

Thus, the long-term drug treatment combined with therapeutic vaccination was shown to break humoral and cellular immune tolerance in treated WHV carrier woodchucks better than the component monotherapies, and to produce a more complete immune response profile resembling that in recovery from acute WHV infection, including an associated and marked reduction in the concentration of WHV cccDNA in liver. While the inclusion of vaccine after clevudine treatment did not result in a significant further antiviral effect beyond that of clevudine alone (i.e., clevudine is so potent that further antiviral effects would be difficult to measure), the combination therapy did have an additive benefit over the monotherapies in delaying the onset and occurrence of disease progression, including chronic hepatitis and HCC[30,155]. The results of this study suggest that the delay in the onset of chronic hepatitis and HCC is due to the uniformly high degree of suppression of viral load, especially the expression of viral antigens in serum and liver, any of which could act to maintain immune tolerance during chronic carriage. Longer term protection against the onset or development of HCC then appeared to be a function of the improved cellular and humoral immune responsiveness to viral antigens, which could no longer serve as endogenous tolerogens after reduction by drug.

CONCLUSIONS

The woodchuck animal model of chronic HBV infection has been valuable in determining the mechanisms of hepadnavirus replication and for studies of viral pathogenesis including associated disease sequelae and host immune responses. Continued modeling of early acute phase immune responses leading to resolution versus chronicity in the neonatal woodchuck may help to identify useful predictive markers of outcome that will facilitate the early identification of the carrier state, and the rational development of antiviral and/or immunotherapies for established chronic HBV infection. Colony-born woodchucks infected as neonates with well-characterized inocula also enable the evaluation of efficacy and toxicity of new types of prophylaxis or therapy under controlled experimental conditions in a relevant animal model within a reasonable time frame. Continued testing of new therapeutic approaches empirically and rationally in the woodchuck model will ultimately improve the chances for successful therapeutic eradication of established chronic HBV infection and its disease sequelae.

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TOPIC HIGHLIGHT

Dieter Glebe, PhD, *Series Editor*

Antiviral therapy and resistance with hepatitis B virus infection

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Abstract

Hepatitis B virus (HBV) infection is still the most common cause of hepatocellular carcinoma and liver cirrhosis world wide. Recently, however, there has been quite dramatic improvement in the understanding of HBV associated liver disease and its treatment. It has become clear that high viral replication is a major risk factor for the development of both cirrhosis and hepatocellular carcinoma. Early studies have shown lamivudine lowers the risk of HBV associated complications. There are currently three nucleos(t)ides licensed, in addition to interferon, and there are more drugs coming to the market soon. Interferon or its pegylated counterpart are still the only options for treatment with defined end points, while nucleos(t)ides therapy is used mostly for long term treatment. Combination therapies have not been shown to be superior to monotherapy in naïve patients, however, the outcome depends on how the end point is defined. Interferon plus lamivudine achieves a higher viral suppression than either treatment alone, even though Hbe-seroconversion was not different after a one year treatment. HBV-genotypes emerge as relevant factors, with genotypes "A" and "B" responding relatively well to interferon, achieving up to 20% HBsAg clearance in the case of genotype "A". In addition to having a defined treatment duration, interferon has the advantage of lacking resistance selection, which is a major drawback for lamivudine and the other nucleos(t)ides. The emergence of resistance against adefovir and entecavir is somewhat slower in naïve compared to lamivudine resistant patients. Adefovir has a low resistance profile with 3%, 9%, 18%, and 28% after 2, 3, 4, and 5 years, respectively, while entecavir has rarely produced resistance in naïve patients for up to 3 years.

Key words: Hepatitis B; Antiviral therapy; Resistance; Interferon; Nucleosides; Nucleotides

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INTRODUCTION

Even though hepatitis B virus (HBV) infection is a preventable disease through vaccination, an estimated 2 billion people are HBV infected, with more than 350 million HBsAg positive and considered as carriers or actively infected[1]. As with hepatitis C, only about one third of the patients that are HBsAg positive require antiviral therapy because of active HBV replication and associated liver disease determined by elevated liver enzymes. Recently, it was demonstrated that, at least in Asian males older than 30 years of age, there is a viral load related risk of hepatocellular carcinoma[2]. This would suggest that antiviral therapy might be indicated even in the absence of active liver disease to decrease the risk of developing hepatocellular carcinoma.

There are different potential mechanisms for how HBV can be inhibited. Mechanisms include an antiviral and immune modulating approach with substances like interferon, a purely antiviral approach inhibiting the HBV polymerase with substances like acyclovir, ganciclovir, and more recently lamivudine, adefovir, entecavir, telbivudine, and several not yet licensed drugs. Interestingly, most of the antivirals studied for HBV were derived from herpes virus or HIV drug development. Thus many of these substances are active against both HBV and HIV, as HBV likewise to HIV replicates *via* an error-prone viral reverse transcriptase. This cross-reactivity must be kept in mind because patients with an HIV infection must never be treated with a monotherapy for HIV, i.e. lamivudine, tenofovir. The reverse transcriptase of HBV and HIV displays similarities, including a so called YMDD motif. Thus, in any patient undergoing HBV-specific treatment, which might also act against HIV, an HIV test should be recommended. Furthermore, if HBV needs to be treated while HIV does not need to be treated, entecavir, telbivudine, and interferon are the primary options. Future options also might include new approaches such as the inhibition of

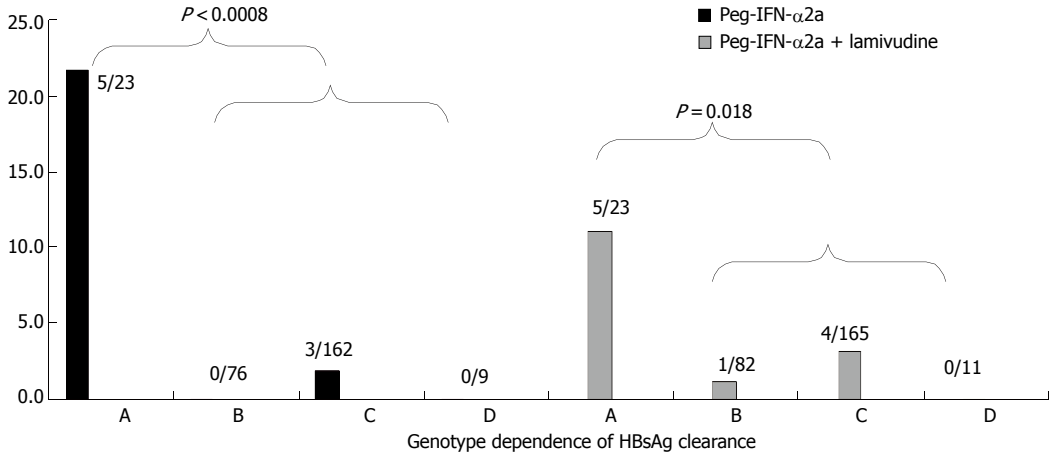

Figure 1 HBsAg seroconversion occurs more frequently on HBV gentoype "A" compared to the other Genotypes. Date given in percent (%). Adapted from Lau GK, *et al*. *N Engl J Med* 2005.

viral entry by using a peptide that is competitive with HBV binding.

Among the earliest substances to be used in man were interferon, acyclovir (previously shown to ameliorate Herpes Simplex Virus I associated encephalitis), as well as vidarabine and ARA-A[3-5]. Even though promising, these agents, except for interferon, were subsequently shown to have minor potency in controlled trials and/or had significant side effects prohibiting further development[6,7]. Similarly, a combination of interferon with these "antiviral" agents did not improve the efficacy of interferon[8]. Ganciclovir, which is effective against cytomegalovirus, has also been evaluated for effectiveness against HBV[9], but was subsequently not developed further because more potent drugs were emerging.

GOALS OF THERAPY FOR HBV INFECTION

The main goal of antiviral therapy is to prevent the development of liver failure, due to either acute fulminant hepatitis or chronic hepatitis B with subsequent liver cirrhosis, the emergence of hepatocellular carcinoma, and HBV transmission. All of these can likely be achieved by suppressing HBV replication, which thereby leads to the remission of liver disease activity and infectivity.

In patients with wild type virus infection, the primary goal of antiviral therapy is to achieve seroconversion from HBeAg to the corresponding anti-HBe antibody (i.e. HBe seroconversion) because this immunologic event is associated with reduced risk for progression of liver disease[10]. Noteworthy, a prior decline in viral load is mandatory to obtain HBe seroconversion, which is subsequently required to also achieve seroconversion from HBsAg to the homologous anti-HBs antibody (i.e. HBs seroconversion). This, however, is achieved less frequently and its likelihood, as that of HBe-Seroconversion, might be genotype related (Figure 1)[11].

HBeAg can be negative in the presence of ongoing high viral replication. In patients with HBeAg negative chronic hepatitis B, pre-core mutants can be detected, which are characterised by an inability to produce HBeAg in detectable quantities (Core-promoter mutations) or show a failure to produce HBeAg (start codon mutations or mutations towards a stop codon typically in the second to last codon of the pre-core region). Available antiviral agents are effective in suppressing HBV replication but in many cases they are not capable of inducing a sustained response after treatment cessation. Therefore, the main objective of therapy is to control viral replication to prevent ALT flares and/or induce remission of disease.

TREATMENT OUTCOME PARAMETERS

Treatment responses have been poorly defined in the past and different studies use different endpoints, thereby making clear comparisons troublesome. In an approach to unify treatment outcome measurements, the European consensus conference in 2002 defined different types of responses[12]; i.e., an initial response, an on-treatment or maintained response, and the sustained response when antiviral treatment has been stopped. The virological response is defined by the decline in HBV DNA below 10^4 or 10^3 copies/mL, the biochemical response by the normalization of ALT levels, and the histological response (HAI score) by the improvement in the inflammatory activity or fibrosis indices. The combined response is defined by the improvement in ALT levels and decrease in viral load while the complete response is characterized by the combination of the decrease in viral load, the normalization of ALT levels, the occurrence of an HBe- or HBs-seroconversion, and an improvement of liver disease at histology.

The treatment response is also defined based on the duration of therapy. An initial response is characterized by at least 1 $\log_{10}$ copies/mL decrease in viral load compared to the baseline value at wk 12 of therapy. The maintained response is defined by a low viral load during therapy. Depending on the use of nucleoside analogue or interferon, there is no agreed threshold to define the maintained response. Usually, a decrease of viral load below 10^4 copies/mL is associated with an improvement of liver histology. However, with nucleoside analogs, the lower the viral load, the lower the risk to develop viral drug resistance. It seems to emerge that viral load shall decrease to $< 3 \log_{10}(10^3)$ copies/mL. The end of treatment response is defined by the response observed at the end of therapy, if there was a decision to stop treatment. A relapse is defined by the increase in viral load after treatment

cessation. The sustained response is conventionally defined by the maintenance of the response 6 mo after drug withdrawal. Finally, a breakthrough is an increase of the viral load of at least 1log after initial response (see also resistance).

To enable better comparison of different studies in the future the following data should always be reported within a given study: HBeAg loss & HBeAg seroconversion to anti-HBe; HBsAg loss & HBsAg seroconversion to anti-HBs; End of treatment results, and if applicable at 6 mo follow-up; HBV-DNA log reduction within defined time points e.g. at wk 12 and 24; Number of patients not achieving a 1 or 2 log reduction within 12 and 24 wk; Mean and median log reduction; Achieved HBV DNA reduction to absolute values, such as below 400 copies (100 IU/mL)and below 50 copies (12.5 IU/mL); HBV-Genotypes.

If new assays become available, the studies should report data in a way that is comparable to former studies.

INDICATION OF ANTIVIRAL THERAPY

Treatment goals and knowledge of the natural history of disease are important for deciding who needs treatment. Two studies have shown that Asian males who are older than 30 years and HBeAg positive[13] or have a high viral load have a 10% risk of developing a hepatocellular carcinoma or cirrhosis[2,122]. In these patients, antiviral strategies seem justified even in the absence of liver disease. In contrast to HCV, HBV can lead to hepatocellular carcinoma in absence of advanced fibrosis/cirrhosis. However, whether these data that were derived from an Asian population can be translated to other regions of the world with different HBV genotypes and ethnic backgrounds appears questionable. There was no difference in survival and liver related death in European HBsAg positive blood donors *vs* HBsAg negative blood donors[14]. Based on the present knowledge of the natural history of chronic HBV hepatitis and on the efficacy of antiviral drugs, antiviral therapy of chronic HBV infection is indicated in patients with chronic hepatitis B in the immunoactive phase characterized by high levels of viral replication and elevated serum ALT levels (Table 1). Liver histology usually shows inflammatory activity and variable degrees of liver fibrosis depending on the duration of the disease. Since continuing HBV replication and elevation of ALT levels reflect a significant risk of disease progression towards liver cirrhosis and hepatocellular carcinoma[15,16], antiviral therapy is indicated to decrease viral load, normalize ALT levels and induce a remission of the liver disease.

There are two main forms of chronic HBsAg positive hepatitis, which are distinguished by their HBeAg status. The HBeAg positive form is associated with a so-called wild type virus infection, HBsAg and HBeAg positivity, high HBV DNA levels, usually > 10^6 copies/mL, and elevated ALT levels. The HBeAg negative form is associated with core promoter and/or pre-core mutant virus infection, HBsAg positivity and HBeAg negativity (most patients have anti-HBe antibody), HBV DNA levels that are fluctuating but are usually > 10^4 copies/mL, and elevated ALT levels that may also fluctuate over time.

Table1 Indication for observing and treating HBV

	HBV-DNA levels	ALT status	Therapy/ Observation	
HBeAg +	> 10^5	Normal	Observe	
HBeAg +	> 10^5	Elevated	Yes	IFN, Antivirals
HBeAg +	> 10^4	Normal	Observe	
HBeAg -	> 10^4	Elevated	Yes	Antivirals, IFN
HBeAg -	> 10^4	Normal	Observe	
Pregnancy	> 10^9 geq/mL	Irrelevant	Yes	Antivirals
HBsAg + chemotherapy	Irrelevant	Irrelevant	Yes	Antivirals
HBsAg -				
Anti-HBc + chemotherapy	Positive	Irrelevant	Observe, treat in case of HBs-Ag appearance	Antivirals
Advanced fibrosis	irrelevant	Elevated	Always	Antivirals
Transplanted patients	> 10^4		Consider Treatment	Antivirals
Asian male	> $10^{5/6}$	Irrelevant	Yes	Antivirals

Treatment endpoints differ depending on the form of chronic hepatitis B.

It is currently not recommended to treat patients who are in the immunotolerant phase. They are defined serologically by HBsAg positivity, HBeAg positivity, high HBV DNA levels (usually higher than 10^8 copies/mL), and normal serum ALT levels. They usually have no liver damage or only minimal liver disease at liver biopsy examination, but they are highly infectious. The results of clinical trials for interferon alpha or nucleoside analogs indicate that patients with high HBV DNA load and normal ALT levels have almost no chance of HBeAg seroconversion. However, patients should be monitored on a regular basis to diagnose a break in immune tolerance characterized by an elevation in ALT levels and a decline in viral load, which may reflect the onset of liver damage and represent an indication for antiviral therapy. In addition, being a 30 years old Asian male with a viral load above 10^6 might also serve as an indication because of the 10% change of developing a HCC or liver cirrhosis in ten years[2,121]. However, this prediction probably cannot be transferred to women and to European patients.

The other category of patients with chronic HBV infection who should not be treated are HBsAg inactive carriers. Their virologic profile is characterized by HBsAg positivity, HBeAg negativity, anti-HBe antibody positivity, persistently low HBV DNA levels (< 10^4 copies/mL), and normal ALT levels. Liver histology usually shows no or minimal damage and the risk of progressing liver disease is considered to be minimal as long as ALT levels remain normal and viraemia is below 10^4 copies/mL. It is currently recommended that these patients should not be treated but should be followed carefully every 3 to 6 mo to promptly diagnose reactivation of viral replication and ALT exacerbations. When their values have been stable for 2 years, one can consider extending their monitoring intervals to 12 mo intervals.

In the case of advanced fibrosis, the recommendation

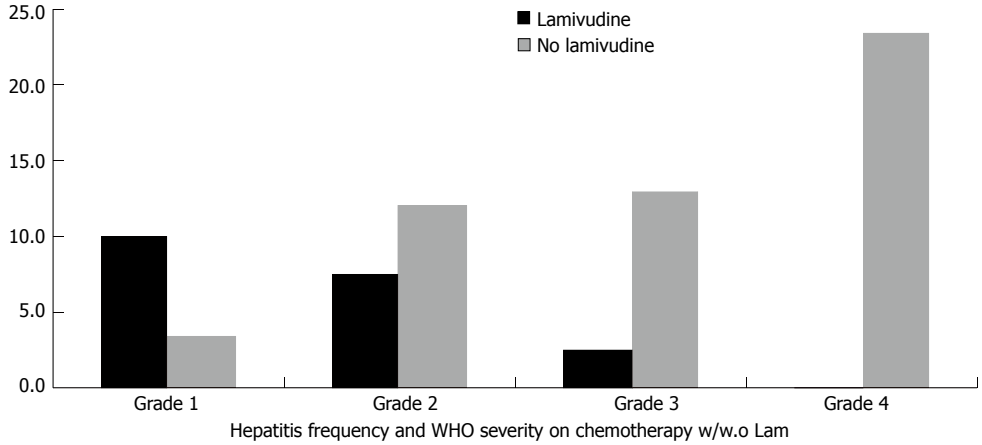

Figure 2 Pre-emptive lamivudine is associated with lower frequency and lower severity of hepatitis during chemotherapy. Date given in percent (%). Adapted from Li YH, *et al. Cancer* 2006.

is to initiate every patient on antivirals to prevent further deterioration of their liver disease independent of disease activity at least in the presence of HBV-DNA. Certainly, when fibrosis regresses during treatment an interruption might be considered.

Another clear indication for antiviral treatment is prevention of reactivation on chemotherapy, where lamivudine has been associated with lower frequency and lower disease severity of hepatitis (Figure 2)[17]. To a lesser extent, antiviral therapy seems indicated in late pregnancy in women with a high viral load[18,19], but formal clinical studies have not yet been published.

General outcome predictors

Some pre-treatment factors have been identified that predict responses to therapy. They may be useful in treatment decisions and drug choices. The results of clinical trials have shown that high ALT values (> 3 × ULN) are always predictive of a higher chance of HBeAg-seroconversion. In addition, a low viral load (< 10^7 copies/mL equivalent to 35 pg/mL) is predictive of a favourable response to standard or pegylated interferon. In addition, likewise to HCV though to a lesser extent, there is emerging evidence that HBV-genotypes are associated with treatment responses. While genotypes seem to be of no relevance for nucleos(t)ide therapy, there is ample evidence that the HBV genotypes A (versus D) and B (versus C) are associated with a better response to interferon therapy. HBsAg-seroconversion might be strongly associated with genotype A[20].

STANDARD INTERFERON ALPHA

A sustained response, defined by HBe seroconversion 24 wk post-treatment, is induced by subcutaneous administration of standard interferon in 20% to 40% of patients depending on patient characteristics; while only 5% to 10% of patients seronconvert in the placebo group[21,22]. Spontaneous HBe seroconversion is part of the natural history of the disease and is believed to be driven by the host immune response; in all clinical trials the spontaneous rate of HBe-seroconversion ranges from 5% to 10% per year. Patients with high ALT levels, a high HAI score, and low HBV DNA levels have a higher chance of HBe seroconversion (> 40%). While responses to HCV associated interferon therapy are usually associated with an immediate drop in both HCV-RNA and ALT, response to interferon with HBV is, especially in responders, associated with a marked increase of ALT in conjunction with a decrease of serum HBV DNA during the second or third month of therapy. The former reflects the immunological response leading to clearance of the virus and might also be associated with the vanishing immunosuppression caused by HBV itself. Clearance of HBsAg and seroconversion to anti-HBs is a late event; the percentage of patients who became HBsAg-negative after seroconverting to anti-HBe varied widely (7%-65%) for follow-ups of 3-4 years[23,24]. The European consensus conference recommended using a regimen of 5 MU daily or 10 MU thrice weekly for 24 wk[12]. However, due to the frequency of side effects at these high doses of interferon, 5-6 MU interferon thrice weekly may be an optimal choice to allow the continuation of therapy. Side effects are frequent and numerous but usually mild and reversible after treatment withdrawal.

HBeAg negative patients with active hepatitis B are mostly infected with the so called pre-core mutant. Trials using 6-12 mo of interferon therapy in that patient population showed that, regardless of interferon dosage, there was a good response while on therapy (inhibition of HBV-DNA, normalization of ALT) but relapses post-therapy were common and observed in a majority of patients. These initial studies indicated that therapy, therefore, should not rely on courses of interferon less than 1 year. Long-term administration for at least 2 years showed clinical benefit in terms of viral suppression and ALT normalization. Approximately 30% of patients may present a sustained response after treatment withdrawal when the interferon course was sufficiently long to maintain the suppression of viral replication[25]. However, side effects and poor tolerance to interferon administration limit its prolonged use in this form of chronic hepatitis B.

RESULTS OF PEGYLATED INTERFERON ALPHA

Pegylation is binding a pegylated side chain to interferon leading to a 12 or 40 kD molecule, i.e., PEG-IFN-α2b, and Peg-IFN-α2a respectively, which increases the half life of interferon making a once weekly application feasible and sufficient. These pegylated interferons have proven

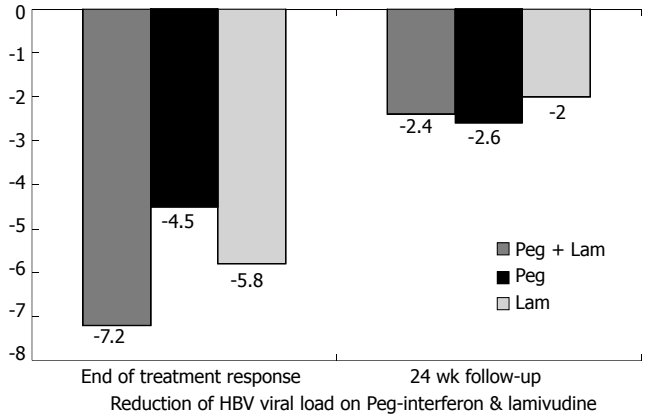

Figure 3 Viral load reduction at the end of treatment and at 24 wk follow-up in the different treatment arms. Adapted from Lau GK, *et al. N Engl J Med* 2005.

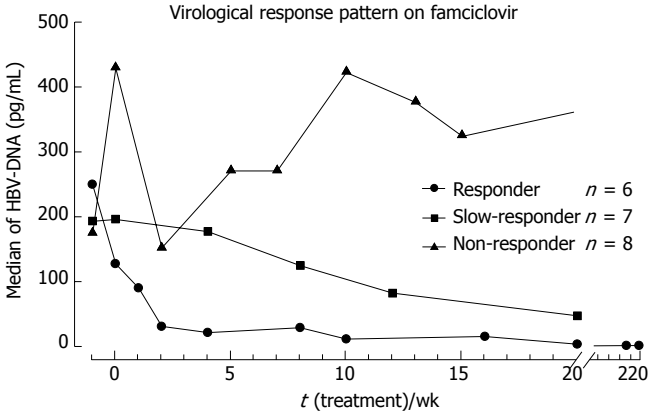

Figure 4 Different response pattern on famciclovir. Adapted from Tillmann HL *et al. Hepatology* 1998.

to not only increase convenience by enabling once weekly dosing but they have also improved efficacy dramatically in hepatitis C. Thus, it was a logical consequence to also evaluate them in Hepatitis B. A phase II study evaluated the efficacy of 90, 180 and 270 μg of PEG-IFN-α2a for 24 wk in comparison to a standard interferon-α2a 4.5MU three times per week[26]. This dose of standard interferon 3 times per week, however, has to be considered to be inappropriate at that time.

In the subsequent phase Ⅲ trials the antiviral effect of pegylated IFN-α2a (40 kDa) or -α2b (12 kDa) administration was evaluated for 48 wk (versus 24 wk used previously and considered as standard care). These trials have shown a HBe seroconversion rate of approximately 30% 6 mo post-treatment[11,27]. However, standard interferon was not a comparator in that study; only lamivudine was tested. Interestingly, a HBs seroconversion rate of 3%-5% was observed at the end of follow-up, while clearance of HBsAg was observed in up to 7% of patients with high genotype dependence (Figure 1). Tolerance and the nature and frequency of side effects for pegylated interferon alpha were generally similar to that of standard interferon in historic controls. Flu like syndrome, inflammatory skin reaction at the injection site and neutropenia were more frequent with pegylated than with standard interferon. Interestingly, depression, which occurs in about 30% of HCV patients during treatment, was reported to be lower than 3%. Even though viral suppression at the end of follow-up was similar for Peg-IFN-α2a and Peg-IFN-α2a plus lamivudine, the end of treatment viral suppression was significantly more pronounced in combination therapy (Figure 3) based on the given confidence intervals.

NUCLEOS(T)IDE ANALOGUES

While interferon usually leads to some side effects, such as flu like symptoms, the various nucleos(t)ides are characterised by few side effects, at least at the licensed dose.

Famciclovir

The first nucleos(t)ide studied in a larger trial was famciclovir, which was developed as a treatment for acyclovir resistant herpes simplex virus I infection. Famciclovir was subsequently shown to also have some HBV-activity *in vitro* and was thus developed for HBV therapy. In both liver and heart transplant patients, famciclovir has proven to ameliorate liver disease, despite only moderate virological response in most of the patients[28-30]. Basically three different patterns of response were determined in our transplant patients. A third of the patients responded well, a third showed slow response and another third did not show any response (Figure 4). Interestingly, the responders showed a clear virological response, which rarely exceeded one log reduction within three months, while transaminases and liver function deterioration were ameliorated.

A controlled trial also proved efficacy[31], but the drug is relatively expensive to produce and lamivudine was emerging as a more potent and less expensive drug. Nevertheless there was a clinical response despite relatively moderate viral suppression of 70%, which is equivalent to less than a 1 log reduction. Some of the patients showed marked and clear improvement in liver function after having had a continuous decrease in liver function prior to initiation of famciclovir (Figure 5).

Despite promising results in liver[28,29] and heart transplant[30] patients, famciclovir was not developed further after the potency of lamivudine became evident. All of the patients responding slowly or not responding to famciclovir showed immediate and more marked response to lamivudine, as did the patients developing resistance on famciclovir[32].

Lamivudine

Lamivudine (3TC) has been developed for inhibiting the reverse transcriptase of HIV[33], but as HBV's life cycle also requires a functioning reverse transcriptase, lamivudine was investigated and proven to be effective in inhibiting HBV as well, both *in vitro* and *in vivo*[34]. Several phase Ⅲ trials have demonstrated the antiviral efficacy of lamivudine administration in patients with HBeAg positive[35,36] and HBeAg negative chronic hepatitis B in doses of 100 to 300 mg/d[37]. The higher doses used in HIV have not proven to be more efficacious[38-40], even though their potential in preventing resistance has not been determined. Advantages of lamivudine are oral administration, an excellent safety

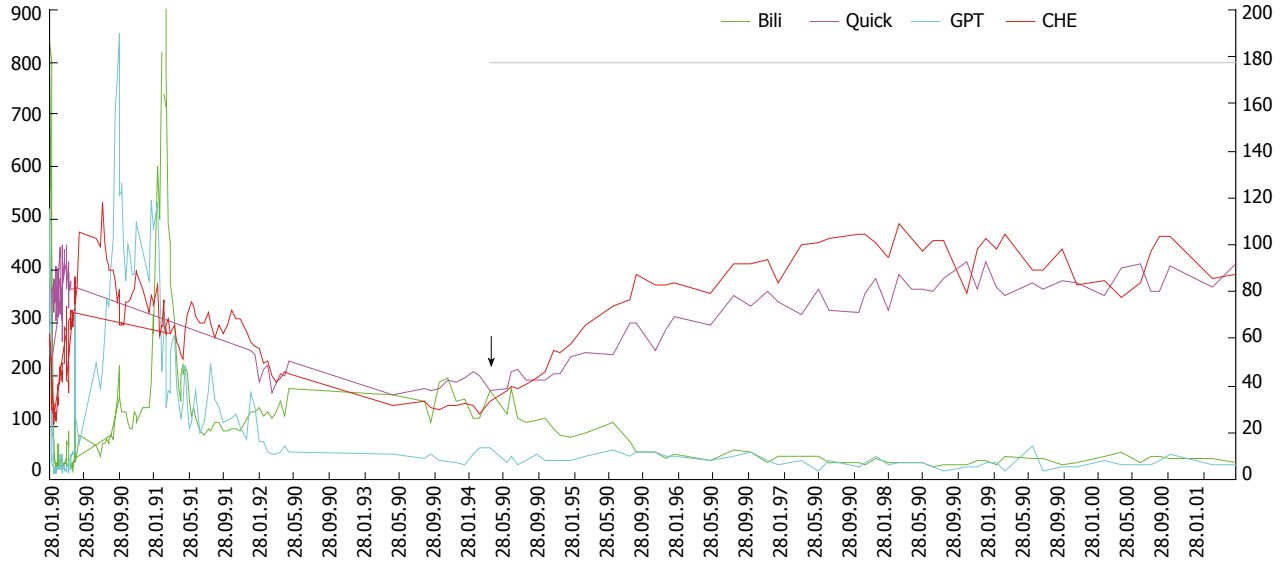

Figure 5 Improvement on famciclovir in a patient with continuous deterioration prior to initiation of famciclovir therapy. Arrow indicates start of famciclovir, where after Bilirubin slowly normalised, and both Quick test (Marker for impaired clotting function) and cholinesterase (CHE) stabilised indicating stabilisation of liver function. (unpulished).

profile, a rapid antiviral effect, and a relatively low cost of therapy. Viral load declines by 3-5 $\log_{10}$ copies/mL after a year of therapy compared to baseline values. The antiviral effect is accompanied by a significant decrease in ALT levels, and an improvement in the histology activity index. An improvement of liver fibrosis has also been observed during lamivudine therapy[41]. However, the primary goal of therapy, i.e. HBe seroconversion, is obtained only in approximately 20% of patients after 1 year of treatment, which was nevertheless significantly higher than in patients receiving placebo (5%-10%). Continuous lamivudine therapy is indicated in patients who do not seroconvert. It avoids a rebound of viral replication and exacerbations of liver disease. Continuing lamivudine therapy is associated with a progressive increase in the number of patients who undergo HBe seroconversion, reaching approximately 50% after 4 years of therapy[42]. A factor influencing the durability of HBe seroconversion is the duration of lamivudine therapy after seroconversion. In HBeAg negative patients, long-term lamivudine treatment is required because rebound is immediate after cessation[37].

Emtricitabine

Emtricitabine (FTC) is a L-deoxycytidine analogue as is lamivudine. Emtricitabine was also developed for HIV therapy, where it is often used in a fixed combination with tenofovir. Emtricitabine was evaluated in phase Ⅱ and phase Ⅲ trials. In a study randomising 98 patients to receive emtricitabine at 25, 100, or 200 mg daily for 48 wk and then 200 mg until wk 96, the dose of 200 mg daily provided the best results. After 2 years, 53% of the patients had serum HBV DNA below 4700 copies/mL, 33% seroconverted to anti-HBe and 85% had normal ALT levels. Resistance mutations were observed in 18% of patients after 96 wk of therapy[43].

A 200 mg dose of emtricitabine has been shown to be superior to placebo for histologic improvement (103 of 167 (62%) patients receiving FTC *vs* 20 of 81 (25%) receiving placebo; $P < 0.001$). Serum HBV DNA less than 400 copies/mL was achieved in 91 of 167 (54%) patients in the FTC group *vs* 2 of 81 (2%) in the placebo group ($P < 0.001$). Resistance towards FTC was detected in 20 of 159 FTC treated patients (13%, with a 95% confidence interval of 8%-18%). The safety profile of emtricitabine was found to be similar to that of placebo during treatment[44]. Being an L-nucleoside, FTC shows cross resistance to Lamivudine[45].

Telbivudine

Telbivudine is also an L-analogue, such as lamivudine, and it shares a similar resistance profile to lamivudine. However, resistance to telbivudine is associated with the YIDD mutation, leaving entecavir fully active. The safety, antiviral activity, and pharmacokinetics of telbivudine have been assessed in 43 adults with hepatitis B and antigen-positive chronic hepatitis B[46]. This placebo-controlled dose-escalation trial investigated six telbivudine daily dosing levels (25, 50, 100, 200, 400, and 800 mg/d); treatment was given for 4 wk. There was more than a 2 log reduction in all dose groups within one week, with disclosing higher potency of the > 400 mg dose only in the second phase. Telbivudine was well tolerated at all dosing levels, with no dose-related or treatment-related clinical or laboratory adverse events. Antiviral activity was dose-dependent, with a maximum at doses of 400 mg/d and or more. In the 800 mg/d cohort, the mean HBV DNA reduction was 3.75 $\log_{10}$ copies/mL at wk 4, comprising a 99.98% reduction in serum viral load. Subsequently, large phase Ⅲ studies have shown the superiority of telbivudine compared to lamivudine in the suppression of viral load (by 6.5 $\log_{10}$ versus 5.5 $\log_{10}$) and improvement of liver histology[47]. A 24 wk study also showed telbivudine to be more active than adefovir with a 6.3 *vs* 4.97 log reduction of HBV-DNA[48]. Telbivudine resistance was observed in

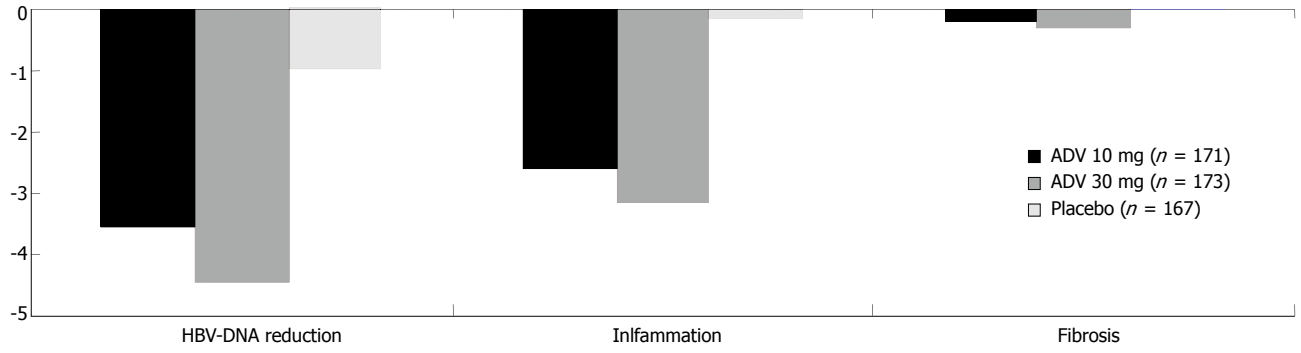

Figure 6 Reduction in HBV-DNA, as well as histological improvement in inflammation and fibrosis according to the Knodell score after 48 wk therapy with 10, 30 mg ADV. Adapted from Marcellin P, *et al*. *N Engl J Med* 2003.

approximately 5% of patients after 1 year of therapy and associated with a M204I mutation, as expected, within the "YMDD"-motif in the viral polymerase[49]. However, the M204V mutation, which is frequently associated with additional mutations at 180 and 173, has not been detected with telbivudine[50]. This might be another advantage in addition to its higher antiviral activity. Importantly, pharmacokinetics indicate no alteration with impaired hepatic function[51].

Adefovir

In the early 1990s, adefovir was shown to inhibit HBV and HIV in cell cultures[52,53]. Its development for HIV was halted because the dose required for HIV inhibition was associated with significant nephrotoxicity beyond 24 wk of treatment[54]. However, HBV was inhibited with lower doses of adefovir and it could even be used safely in renal impaired patients[55]. It was shown that a 10 mg dose provided a smaller decrease in viral load than a 30 mg dose (Figure 6) but there was a higher creatinine increase with the 30 mg dose and therefore only the 10 mg dose was developed further[56].

In a large phase Ⅲ trial, 515 patients with HBeAg positive chronic hepatitis B were treated with adefovir 10 mg (n = 171), adefovir 30 mg (n = 173) or placebo (n = 167) for 48 wk. HBe seroconversion was achieved only in a minority of patients, i.e. 14% in the 30 mg daily and 12% in the 10 mg daily dosing group of patients receiving adefovir dipivoxil versus 6% in the placebo group. ALT levels normalized in 48% and 55% of patients receiving adefovir 10 and 30 mg adefovir respectively, versus 16% in the placebo group. Reduction of HBV-DNA and liver inflammation and fibrosis improved significantly in patients given adefovir (Figure 6)[57]. Tolerance for the daily dose of 10 mg adefovir was comparable to placebo. Extended administration of adefovir dipivoxil showed an increased rate of HBe seroconversion over time: 14% of 296 patients, 33% of 231 patients, and 46% of 84 patients after 1, 2, and 3 years of therapy, respectively[57,58].

Similar to HBeAg positive patients, adefovir administration for 48 wk in HBeAg negative patients induced histologic improvement more frequently in adefovir treated (64%) *vs* placebo treated patients (33%, $P < 0.001$), and reduced serum HBV DNA below < 400 copies/mL [51% (63 of 123) *vs* 0%; $P < 0.001$][59]. HBV DNA was below 1000 copies/mL in 51%, 71% and 79% patients after 48, 96 and 144 wk, respectively[59-60]. Interestingly, in the majority of patients who were switched from adefovir to placebo, the benefit of treatment was lost, indicating that antiviral therapy with nucleoside analogs must be prolonged in this patient population to avoid viral reactivation and ALT flares. Side effects after 144 wk were similar to those observed at wk 48. It has been presented recently that 22/33 anti-HBeAg negative patients showed sustained response when adefovir was stopped after 4 to 5 years of continues adefovir therapy[122].

Entecavir

Entecavir was developed as an anti-herpes drug, but proved to display only moderate activity, which eventually led to discontinuation of development for this indication. However, Bristol-Myers Squibb discovered that entecavir was extremely potent against HBV through inhibition of HBV-DNA polymerase, with relatively low toxicity. Entecavir is the first HBV-specific antiviral to be licensed that seems to lack both HIV and herpesvirus cross-reactivity[61], which is especially attractive for HIV-HBV co-infected patients not yet requiring HIV-treatment. Entecavir has been evaluated for naïve patients in two controlled phase Ⅲ trials involving 715 HBeAg positive and 648 HBeAg-negative patients with chronic HBV infection, detectable HBV DNA, persistently elevated ALT levels and chronic inflammation on liver biopsy. Entecavir administered 0.5 mg orally once daily for 52 wk was shown to be superior to lamivudine (100 mg orally once daily for 52 wk) for the primary efficacy endpoint of histological improvement and for secondary endpoints, such as the reduction in viral load (6.9log *vs* 5.4log, $P < 0.001$ for HBeAg+; 5.0 *vs* 4.5log, $P < 0.001$ for HBeAg-) and normalization of ALT (68 *vs* 60%, $P = 0.02$ for HBeAg+; 78 *vs* 71%; $P = 0.045$ for HBeAg-)[62,63]. After 2 years of treatment, 81% of patients receiving entecavir had a viral load below 300 copies/mL versus only 39% of patients receiving lamivudine, 31% seronconverted to anti-HBe versus 26% in the lamivudine group, and 5% showed a clearance of HBsAg versus 3% in lamivudine treated patients[64]. The second year, however, was limited to 307 of the initial 709 patients. In lamivudine refractory

patients, entecavir administered at 1 mg once daily induced a significant viral load reduction and histological improvement, by comparison with the control group treated with lamivudine[65]. Entecavir was approved in 2005 by the US FDA for the treatment of chronic HBV infection in adults with evidence of active viral replication and either evidence of persistent elevation in serum ALT or histologically active disease. Entecavir resistant mutants have been described mainly in patients with lamivudine resistance[66]. Approximately 9% of lamivudine resistant patients treated with entecavir develop resistance to entecavir after 2 years of therapy. The resistant mutants are then resistant to both lamivudine and entecavir.

Tenofovir

Tenofovir is licensed for the treatment of HIV infection but has known activity against HBV as well. It is less nephrotoxic and therefore it can be used in a higher dose (300 mg) unlike adefovir, which is licensed for a 10 mg dose.

Tenofovir's anti-HBV activity has been studied *in vitro* and *in vivo* mostly in HIV infected patients coinfected with HBV. In this patient population, tenofovir administration decreased HBV load significantly both in lamivudine naïve and lamivudine resistant patients[67-69]. There is good evidence from non-randomized[70], but also a small randomised study, that tenofovir is more potent than adefovir in reducing HBV load[71]. Phase Ⅲ trials are ongoing to compare the anti-HBV activities of tenofovir and adefovir in HBV mono-infected patients and in HIV-HBV co-infected patients. Currently, even though it has higher potency and lower cost compared to adefovir, tenofovir cannot be prescribed. This can be considered as a drawback of modern bureaucratic medicine, which prohibits using a drug that has a better safety profile and higher activity at lower costs, but has not been specifically evaluated for that indication.

Clevudine

Clevudine is an artificial beta-L nucleoside analogue that shows cross-resistance to lamivudine[45]. It seems to have an advantage in that viral load rebound after therapy cessation is not immediate[72]. A specific attractive aspect of clevudine is it's activity against delta virus infection, at least in the woodchuck model[73].

Pradefovir (Remofovir)

There is evidence that the safety of ADV could be improved if liver-specific targeting could be achieved, thereby allowing higher liver-associated concentration without increase of systemic exposure with nephrotoxic consequences. One such prodrug is pradefovir, formerly remofovir[74], which is under clinical development. 10, 20 and 30 mg of pradefovir seem to be more potent than 10 mg of adefovir (-4.22, -4.33 , -5.06log *vs* -3.66) [75]. However, it needs to be determined whether it is more effective than tenofovir.

ANA380

ANA380 is a prodrug of ANA317, another recently reported substance with activity against lamivudine resistant HBV. Patients treated with ANA 380 at 30 mg, 60 mg, 90 mg, 150 mg and 240 mg dose levels experienced reduction in plasma HBV viral DNA at 12 wk of 2.8 log. 3.2 log, 3.9 log10, 3.9 log10 and 4.1 log10 units, respectively[76].

Myrcludex B: an acylated PreS1 peptide

It was found that the preS1 amino acids 2-48 mediate attachment of the virus to its target cells. Furthermore amino-terminally acylated peptides containing amino acids 2-18, and even more efficiently with 2-48 of the PreS1 domain, can be used to block hepatitis B virus infection[77]. Using this concept, Urban *et al,* developed a peptide that was shown to inhibit HBV-infection *in vitro* and in animal models and is currently being developed as an antiviral approach. It is currently not clear whether it will inhibit infection in a post exposure approach, i.e. after needle stick injury or liver transplantation of HBV-positive patients, to prevent re-infection or whether it might have antiviral activity in chronic hepatitis B.

COMBINATION THERAPY

Several studies have evaluated the efficacy of a combination of interferon alpha 2a or 2b with lamivudine and more recently a combination of Peg-IFN alpha 2a or 2b with lamivudine in comparison with pegIFN alone and/or lamivudine alone[11,27]. It was concluded that the efficacy of combined Peg-IFN plus lamivudine is not different from Peg-IFN alone if both are given for 48 wk. However, this depends on what you are observing. HBeAg seroconversion actually was even lower, though not significantly different, 24 wk post-treatment. Similarly the viral load reduction and normalisation of transaminases was similar between Peg-IFN plus lamivudine *vs* Peg-IFN monotherapy 24 wk after the end of therapy[78].

The decline of viral load was higher, however, in the combination group than in the single treatment group during therapy (Figure 3). The rate of lamivudine resistance was lower in patients who received a combination of lamivudine with pegIFN compared to lamivudine monotherapy, and following the state of art, one would not have stopped lamivudine therapy at 48 wk. Thus, it may be premature to state that Peg-IFN should not be combined with lamivudine, but this certainly would need further study.

Likewise, a very small study suggested that ccc-DNA reduction was augmented when adefovir is combined with Peg-IFN *vs* adefovir alone[79]. Whether this can eventually lead to higher HBsAg seroconversion rates needs to be determined in future studies.

A combination therapy approach is also suggested with use of some of the more recently developed antivirals. It had been shown that adefovir plus emtricitabin is superior to adefovir alone[80]. Given the similarity but superiority of tenofovir versus adefovir, the combination of tenofovir with emtricitabine appears especially promising, and since this combination is one of the backbones of HIV-antiretroviral therapy and there is already an excellent track record.

Recently, emtricitabine was also combined with 10 mg clevudine (1.8 to 2.3 log reduction) and results showed a superiority for the combination versus emtricitabine alone

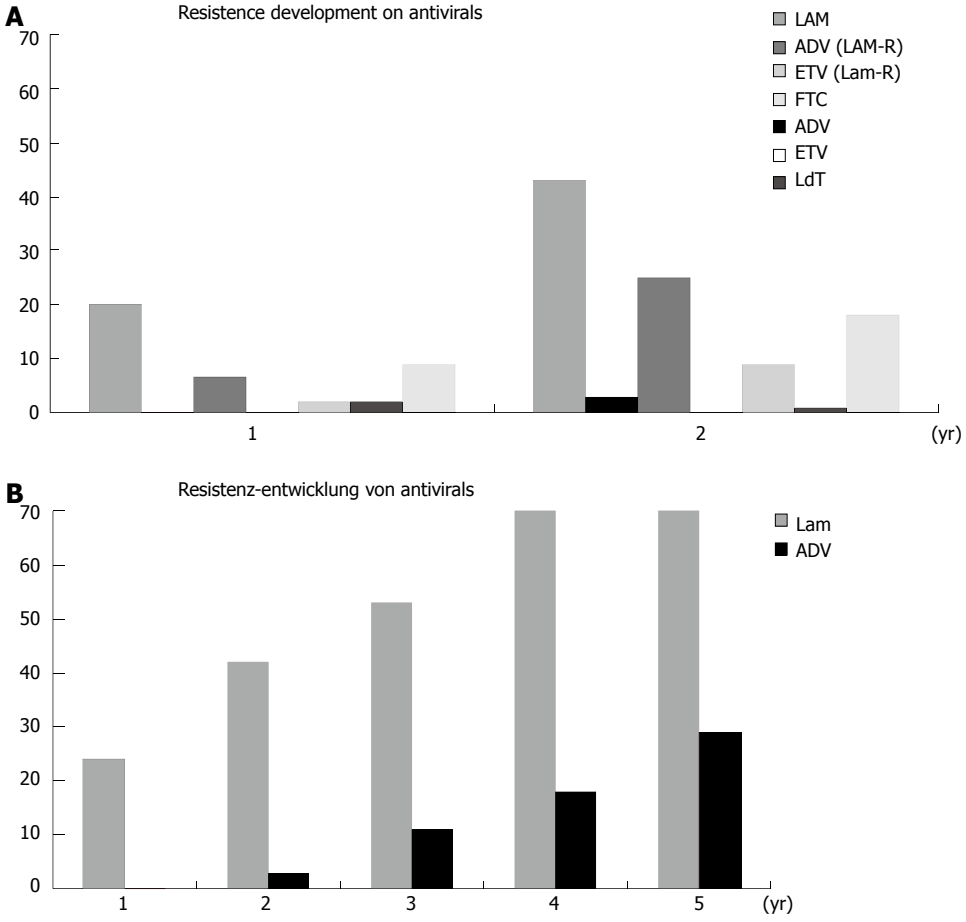

Figure 7 Prevalence of resistance on different antivirals, up to 2 (**A**) and up to 5 (**B**) year data.

(1.4 to 2.0 log reduction)[81]. These viral load reductions, however, were still lower than those reported on 50 mg clevudine monotherapy (s. clevudine above). Thus, in the combination trials each drug should also be tested as monotherapy to exclude the "combination effect" of higher potency for one of the drugs.

DRUG RESISTANCE

HBV replicates *via* an error prone viral reverse transcriptase resulting in a large pool of quasispecies with mutations interspersed throughout the genome. During antiviral drug selection pressure (e.g., lamivudine, adefovir, or entecavir), HBV mutants are selected from the preexisting pool of quasispecies and over time become the dominant species.

Drug resistant mutants emerge as a function of at least six factors: (1) the viral mutation frequency (annual error rate), (2) the intrinsic mutability of the antiviral target site (some mutants are lethal and cannot replicate), (3) the selective pressure exerted by the drug (the stronger the more likely a resistance emerges), (4) the magnitude and rate of virus replication (the higher the viral load, the more likely resistance emerges), (5) the overall replication fitness of the mutant (some mutants are replicating very poorly and some require addition compensatory mutations), and (6) the availability of replication space (the amount of cccDNA harboured in a cell is limited; if there is no space for new cccDNA the likelihood of resistance is reduced).

HBV resistance to antivirals can be defined at different levels, which usually develop sequentially: (1) genotypic resistance is the detection of polymerase gene mutations known to confer resistance to the drug, (2) virologic breakthrough has been defined as an increase of at least one $\log_{10}$ copies/mL compared to the lowest value during treatment, associated with the presence of resistance mutations following genotypic resistance, (3) clinical failure is defined as viral breakthrough and increase in ALT levels and subsequently progression of liver disease usually following the virological breakthrough. Very rarely increased replication of viruses can be observed after emergence of resistance[82], which was first described 40 years ago for enteroviruses[83], and some years ago for HIV[84,85], which has, however, minor clinical relevance because of the multiple drug approach in HIV.

One of the clear advantages of interferons is their inability to significantly induce mutations, which would subsequently abolish interferon activity. In contrast, all nucleos(t)ide given for more than 48 wk have been shown to induce mutations with various frequencies after 1 to 2 years and during longer treatment (Figure 7A and B), which leads to impaired sensitivity towards the appropriate antiviral. The first antiviral leading to some clinical improvement but also to mutations was famciclovir. It's signature mutation was L528M[32] (now corresponding to rtL180M, as numbering was changed to start at the start of the reverse transcriptase of HBV-DNA polymerase with the highly conserved EDWGPCDEHG motif[86]) thereby eliminating different numbering for different HBV

genotypes. Patients with such a L528M/rtL180M mutation were shown to be sensitive to lamivudine, at least until YMDD mutations due to lamivudine became known as well[32].

New drugs have become available and knowledge of the *in vitro* cross-resistance profile has provided the rationale for their use in patients with treatment failure. The rescue treatment of patients with drug resistance has improved significantly in recent years.

The major problem of long-term lamivudine therapy is the occurrence of drug resistance. The spontaneous variability of the HBV genome and the slow kinetics of viral clearance, are the biological basis for the selection of drug resistant mutants. The results of phase III clinical trials and of cohort studies have shown an incidence of lamivudine resistance of approximately 20% per year[87]. Lamivudine resistance develops in up to 70% of patients after 4 years of therapy[88,89], leading to an increase in viral load (viral breakthrough) that is followed by an increase in ALT levels (biochemical breakthrough), a reduced HBe seroconversion rate in HBeAg positive patients, and a progression of liver disease[87]. In some patients, especially those with liver cirrhosis or severe fibrosis, the biochemical breakthrough that follows lamivudine resistance may cause a severe and acute exacerbation of liver disease that may precipitate liver failure[88,90-92].

Long-term studies have shown that antiviral efficacy and histological improvement is progressively lost with time because the prevalence of resistance mutations increases as liver disease continues. This was observed in some patients with YMDD mutations but none without those mutations[93]. ALT levels increase progressively with the duration of infection with the YMDD mutants and it was reported that no patient who developed lamivudine resistance mutation for 24 mo had normal ALT levels[94]. It is therefore necessary to make an early diagnosis of drug resistance to adapt rescue antiviral therapy prior to the degradation of liver functions[92,95].

In a retrospective nationwide analysis of lamivudine therapy in Italy, the development of clinically important events after virological breakthroughs depended on the severity of the underlying liver disease; severe hepatitis flares at the emergence of YMDD were noted in patients with child B and C cirrhosis but not in patients with non-cirrhotic chronic hepatitis[96], which is in agreement with previous studies[91,92]. Interestingly, the rate of HCC was diminished even in multivariate analysis in patients with maintained response *vs* those with breakthrough[96].

Mutations conferring resistance to lamivudine are mainly located in the C domain of the reverse transcriptase within the YMDD motif, i.e. M204V or M204I, and may be associated with compensatory mutations in the C domain, i.e. V173L or L180M. After 1 year of treatment, lamivudine resistant mutants emerged in 22% of patients, increasing to 38% after 2 years, 53% after 3 years, 66% after 4 years, and 69% after 5 years[87,89,97]. However, this also means that approximately 30% of patients seem to never develop resistance against lamivudine. *In vitro* and *in vivo* studies showed that the main lamivudine resistant mutants remain sensitive to adefovir[98,99], tenofovir[70], and entecavir[62,100], even though susceptibility to entecavir was reduced *in vitro*[101].

Comparing the addition of adefovir to ongoing lamivudine and the switch from lamivudine to adefovir did not reveal any difference in viral load decline in these two treatment groups. However, recently Lampertico *et al*[102] presented data showing very pronounced viral load reduction if adefovir was added when viral resistance emerged instead of when clinical resistance with elevated liver enzymes was evident. In addition, the risk of resistance to adefovir was significantly less frequent in patients receiving adefovir in addition rather than as a substitute for lamivudine[103]. Thus, because of the lack of cross-resistance between the two drugs, there is now a consensus among experts that adefovir should be added to lamivudine in patients with lamivudine failure to prevent or delay the subsequent selection of new resistant mutants.

Because of the reduced susceptibility of the lamivudine resistance mutant to entecavir *in vitro*, entecavir was given to patients with lamivudine failure at a dose of 1 mg daily instead of 0.5 mg, which was given to naïve patients. Entecavir induced a significant decline in viral load in these lamivudine refractory patients[65]. Noteworthy, cases of entecavir resistance were described so far only in lamivudine resistant patients, suggesting that some level of cross-resistance between these two drugs is responsible for the selection of mutants resistant to both drugs. Based on these findings, follow-up studies are required to better determine the indication of entecavir in patients with prior lamivudine resistance.

Telbivudine and emtricitabine share the same resistance mutations as lamivudine except that telbivudine seems to not induce mutations at L180M and 173 as frequently. The year one resistance data within the GLOBE-study indicate that the telbivudine resistance is associated with a M250I mutation and not with a M250V mutation.

In patients treated continuously with adefovir 10 mg/d as a monotherapy, drug-resistant mutants emerged in 2%, 5.9%, 18%, and 29% of patients after 2-5 years, respectively. Resistance to adefovir is most frequently conferred by the selection of a rtN236T mutation in the D domain of the HBV polymerase or a rtA181V mutation in the B domain of the polymerase[104-106]. This may be accompanied by liver failure[107]. *In vitro*, the rtN236T mutation is sensitive to both lamivudine and entecavir and the rtA181V showed a decreased susceptibility to lamivudine, which can be confirmed *in vivo*[106,107]. Adefovir resistance can probably be significantly reduced if treatment is combined with lamivudine. In addition, it has been suggested that resistance to adefovir is more likely to develop in lamivudine resistant patients with 10% *vs* 0% after one year adefovir [108], which is in agreement with another Korean study reporting a resistance rate of 6.4% and 25.4% after 1 and 2 years adefovir therapy, respectively[109]. Mutant HBV with resistance against both adefovir and lamivudine can emerge[106].

Recently, it was suggested that a mutant/variant rtI233V is naturally occurring even before therapy in some HBV patients[110] and might be associated with reduced susceptibility to adefovir. Resistance was proven for that

mutation *in vitro* after it had been observed in three patients not responding to adefovir[111]. Surprisingly, this mutation has not been observed in patients developing virological breakthrough. This mutation has not, however, been seen in any of the 20 patients with insufficient response to adefovir from another institution[112]. Since 10% of patients with normal rt sequence have an insufficient response to adefovir, this may be more related to drug transporter polymorphisms, since they have been related to nephrotoxicity, but could also account for the insufficient response.

Entecavir resistance was observed mainly in the therapy of lamivudine refractory patients. The resistance rate appears to be approximately 10% after 2 years and 25% after 3 years in patients with lamivudine failure and 0.8% in naïve patients over 3 years[123]. The main resistance mutations are rtT184G, rtS202I, rtM250V on a background of lamivudine resistance mutations[113]. These mutants are resistant to lamivudine but appear to be susceptible to adefovir *in vitro*[114]. Clinical data are awaited to provide recommendation for the treatment of entecavir resistant patients. The emergence of entecavir resistance seems to be bound to the presence of a M250V mutation, thus leaving entecavir as a full option in case of telbivudine resistance.

Monitoring of antiviral therapy

Monitoring during antiviral therapy could serve different purposes: (1) estimation of the response based on early viral kinetics; and (2) early recognition of the development of viral resistance with an increase in the viral load after initial reduction or by mutation monitoring.

It was initially shown by Puchhammer-Stockl *et al*[115] that patients showing an early viral response are less likely to develop resistance with lamivudine, which was recently confirmed prospectively for telbivudine[116]. Nevertheless monitoring viral resistance by observing the emergence of mutations known to confer resistance would be the most sensitive way to monitor patients who remain viraemic on current treatments. Based on experience, it is not recommended to wait until an increase in viral load associated with ALT-elevation is evident, since these patients are less likely to respond as well as those placed on an alternative additional antiviral therapy earlier. In addition, this approach harbours the risk of hepatic decompensation. With any nucleos(t)ide there will always be a risk for the emergence of drug-resistance, which mandates monitoring patients on antiviral therapy. The rationale for the timing of monitoring derives from the consideration that the biochemical breakthrough usually occurs with a delay of several weeks after the virological breakthrough and that the clinical impact is usually different in non-cirrhotic than in cirrhotic disease. In the former, the ALT breakthrough most often has no major clinical consequences and in the latter it may precipitate liver failure and death. Monitoring should be performed by measuring the viral load with quantitative HBV DNA testing and certainly transaminases. If the residual viraemia remains high (see below) treatment should be switched to alternative therapy, if possible.

The antiviral response at wk 24 of therapy was found to be a predictor of subsequent efficacy (HBeAg loss, HBV DNA < 200 copies/mL, ALT normalization, and viral breakthrough) in patients treated with lamivudine or telbivudine[117]. In the 5-year study of adefovir administration in HBeAg negative chronic hepatitis, patients with a viral load lower than 3 $\log_{10}$ copies/mL after 1 year of therapy had a significantly lower risk of developing resistance by year 3 of treatment (< 3%) compared to a risk of 26% and 66% for those having a viral load between 3 and 6 $\log_{10}$ copies, and > 6 $\log_{10}$ copies/mL, respectively[118]. On the other hand, this suggests that those who do not achieve a viral load reduction should be given rescue therapy before the development of true resistance.

During long-term treatment, a 3 or 6 monthly assessment of viral load and serological markers is required to monitor antiviral treatment efficacy and determine whether the response is maintained or whether drug resistance is developing. Certainly drug compliance is important, as any drug interruption may lead to a rebound of viral replication and ALT flares. The detection of polymerase mutations can be performed by sequencing, line probe assay, and DNA chip technologies. Detection may become more complex when additional treatment options become available, since there is emerging evidence that the cross-resistance profile is different from one mutant to another[119,120]. The line probe assay is more sensitive than sequencing of PCR products but cannot detect new mutations.

New tools may become available in the future to monitor the efficacy of antiviral therapy, such as the quantification of intrahepatic cccDNA or the quantification of serum HBsAg as a surrogate marker. Furthermore, with the development of new drugs and the increasing complexity of the resistance profile, phenotypic assays to determine drug susceptibility of the clinical isolates may prove useful in tailoring antiviral therapy to the virological situation of the patient, as already shown in HIV.

CONCLUSIONS

Patients with minimal disease, whether in the immuntolerant phase or with inactive infection, should not be treated. However, if it is confirmed that the risk of HCC is 10% within 10 years for patients with more than 10^6 viral load, these patients should receive antiviral treatment irrespective of the activity of their liver disease. In patients with chronic hepatitis proven by ALT elevation and abnormal liver histology, antiviral therapy is indicated because all studies have shown that antiviral therapy decreases the risk of liver disease progression compared to the natural history of the disease. In patients who are HBeAg positive, the primary goal of antiviral therapy is to obtain HBe seroconversion. If the patient is young and has predictive factors of favourable response, a finite course of pegylated interferon should be tried as a first line option in genotype A and B patients. In other cases (including non-responders to IFN, patients intolerant to interferon and those with factors of poor response to interferon), long-term therapy

Table 2 Treatment options for chronic hepatitis B and their profile

	Standard Interferon PegIFN	Lamivudine	Adefovir	Entecavir
Viral suppression	4.5 log10 copies/mL	5-6 log10 copies/mL	3-4 log10 copies/mL	6-7 log10 copies/mL
Long term therapy	(6 to) 12 mo in HBe pos. Patients 24 mo in HBeAg neg. patients	+ (continuous)	+ (continuous)	+ (continuous)
Side effects	+	- to very low	- to very low	- to very low
HBe seroconversion	30%	15%-20% at 1 yr, 25%-30% at 2 yr	10%-15% at 1 yr	20% at 1 yr, 30% at 2 yr
Predictive factors for seroconversion	High ALT, low HBV DNA levels	High ALT	High ALT	?
Clearance of HBsAg	+	-	-	?
Cost	++ per months	+	++	(++)
Sustained response	< 30% HBeAg - ca. 30% in HBeAg +	Maintained response 30%-35% (3 yr)	Maintained response ⩾ 70% (5 yr)	Maintained response?
Maintained response	Not applicable	Maintained response 30%-35% (3 yr)	Maintained response ⩾ 70% (5 yr)	Maintained response?
Clearance of HBsAg	+ (Genotype dependent)	low	low	low
Resistance	No resistance but non-response	20% per year	0% at 1 yr up to 29% at yr 5	0% at 1 & 2 yr (2% at 1 and 10% at 2 yr in lam resistant patients)

with nucleos(t)ide analogues is usually needed.

Long-term therapy is probably required in patients who are HBeAg negative. Nucleoside analogues are better tolerated than pegylated interferon, but the therapeutic choice must take into account the risk of drug resistance (Table 2).

Likely future therapy is to begin with an inexpensive antiviral and then adding or switching to another in the case of insufficient response. In patients with severe liver disease, i.e. decompensated liver cirrhosis or HBV recurrence on the liver graft, one might consider combining nucleoside analogues lacking cross-resistance from the start to provide the best chance of long-term control of viral replication and disease progression.

Finally, it is recommended that physicians should be brought back into the position of prescribing licensed drugs, even if they are only licensed for another treatment, when there is evidence for superiority of such an approach. One such example is tenofovir, which has been licensed for HIV and displays higher efficacy and a better safety profile than adefovir.

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BASIC RESEARCH

Effects of nociceptin/orphanin FQ on rats with cathartic colon

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Abstract

AIM: To demonstrate the change and effect of nociceptin/orphanin FQ in the colon of rats with cathartic colon.

METHODS: The cathartic colon model was established by feeding rats rhubarb for 3 mo, the changes of colonic electromyography were investigated by both suspension muscle strips test and serosal recordings of colonic myoelectrical activity. Immunohistochemical staining (S-P methods) and image analysis were used to determine the changes of nociceptin/orphanin FQ in the proximal colon and distal colon of rats with cathartic colon.

RESULTS: Suspension muscle strips test *in vitro* showed OFQ (10^{-9}-10^{-6} mol/L) concentration dependently caused an immediate tonic contraction in the isolated colon. But the increase of tension in cathartic colon was less than control groups ($P < 0.01$). Intravenous administration of OFQ (1 μg/kg) caused phasic contractions in the proximal colon, while the amplitude of phasic contractions caused by OFQ in cathartic colon was much lower than that in the control groups (2.58 ± 0.41 *vs* 4.16 ± 0.53, $t = -2.6$, $P = 0.012$). OFQ was highly expressed in the myenteric plexus of the rat colon but not in the muscle cells. The immunoreactivity of OFQ in the proximal colon in cathartic colon rats decreased significantly compared with the control group ($P = 0.001$).

CONCLUSION: Colonic smooth muscle of cathartic colon showed low sensitivity to the stimulation of OFQ, suggesting that it might be caused by the abnormal distribution of OFQ or the abnormalities of receptors, leading to the disorganization of dynamic and incoordinated contractions.

Key words: Nociceptin/Orphanin FQ; Opioid; Muscle tension; Colonic motility; Enteric nervous system; Cathartic colon

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INTRODUCTION

Slow transit constipation (STC) is a common syndrome in idiopathic constipation. It is a severe disorder of colonic motility. Because of lack of understanding of the etiology, current medical treatments for STC are often ineffective[1]. It has been shown that predominant symptoms in STC correlate with the enteric nervous system (ENS) abnormalities[2]. Animal experiments have shown that stimulant laxatives can damage the ENS and cause changes of some enteric neurotransmitters and thus slow gastrointestinal tract transit occurs[3].

The peptide nociceptin, also called orphanin-FQ (N/OFQ), is a 17-amino-acid peptide and was identified in 1995[4]. Because it binds an opioid receptor like 1 (ORL1)receptor with a high affinity, it has been reported to be an endogeneous agonist for ORL1 receptor. *In situ* hybridization studies have revealed the wide distribution of the ORL1 receptor mRNA in the central nervous system of rats[5], especially in the coli involved in pain control, so most of the researches focused on the role of N/OFQ in analgesics. However, by the activation of the ORL1 receptor, N/OFQ can also influence reward, anxiety, feeding and memory processes[6,7], cardiovascular and renal functions[8,9], gastric and intestinal motility[10,11] and secretions[12,13].

The gastrointestinal tract is an important model system for the research of pharmocological characterization of opioid receptors. Therefore, if N/OFQ can also act as a brain-bowel peptide like classical opioid, it may play a role in the regulation of gastrointestinal tract functions. In this study, with a rat model of cathartic colon, we determined the effects of OFQ on mechanical activity of the rat colon and conducted immunohistochemical studies on rat colon to assess the changes of OFQ.

MATERIALS AND METHODS

Experimental animals

Fifty Wistar rats of either sex, obtained from the

Experimental Animal Center, Lanzhou University (Lanzhou, China), weighing 290 ± 50 g, were divided randomly into control group (n = 25) and cathartic colon group treated with rhubarb (n = 25). Rats were housed in cage, one per cage under standard laboratory conditions (room temperature, 18-28℃, relative humility, 40%-80%). Control rats were given soft chows, while the rats in rhubarb group were given chows premixed with rhubarb powder. The initial rhubarb dosage was 200 mg/kg·d, and another 200 mg/kg was added every day until it reached 1000 mg/kg·d for several days until loose stool disappeared. Then, rhubarb was added at 200 mg/kg·d again to 3600 mg/kg·d for 3 mo.

Materials

Tetrodotoxin (TTX) and naloxone were obtained from Sigma Co. USA. OFQ (1-17) was obtained from Physical Laboratories, School of Life Science, Lanzhou University (Lanzhou, China). Rabbit antinociceptin antiserum (Chemicon Pharmaceuticals Inc, 1:500. Goat anti-rabbit biotinylated secondary antibody (Rocland Laboratories, USA, 1:15000).

Motility studies

Wistar rats (body weight, 290 ± 50 g) were starved overnight and killed by head-strike, longitudinal muscle strips were isolated from proximal colon, muscle strips (10 mm in length and 3 mm in width) were suspended between 2 platinum electrodes in a 30 mL organ bath filled with Krebs-Henseleit buffer containing 118 mmol/L NaCl, 4.8 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 25 mmol/L $NaHCO_3$, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 11 mmol/L glucose and 0.1% bovine serum albumin. Krebs-Henseleit buffer solution was continuously gassed with 95%O_2-5%CO_2 and maintained at 37℃ and pH 7.4. Mechanical activity was recorded on a polygraph through isometric transducers. Muscle strips were stretched in 1-mm increments and repeatedly exposed to 10^{-6} mmol/L carbachol to determine L_0, the length at which the maximal active tension response developed. The resting of tension was kept constant during the equilibration period, dose-response curves were constructed after applying OFQ (10^{-9}-10^{-6} mol/L) to longitudinal muscle stripes from proximal colon. Doses of OFQ were administered at 20-min intervals. To investigate the neural pathways responsible for the contractile action of OFQ, we examined the effects of TTX and naloxone on OFQ-induced contractions. Muscle strips were preincubated with each antagonist for 10 min followed by incubation with OFQ (n = 8).

Response of colon in anesthetized rats

Colonic motility studies in anesthetized rats were carried out according to the procedures described previously (Nagasaki *et al*, 1989.) In brief, rats were fasted for 20 h and anesthetized with urethane (1.2 g/kg s.c.). After midline laparotomy, a strain gauge force transducer (F-081S, Star Medical, Japan) was sutured on the serosal surface of the proximal colon to record the circular muscle contraction. For intravenous administration of drugs, the right femoral vein was cannulated. After the contractile response to bethanechol (30 mg/kg i.v) became stable, OFQ (1 μg/kg) was administered intravenously (n = 7).

Immunohistochemistry

The animals were deeply anaesthetized with 5% isoflurane and perfused transcardially with aerated calcium-free Tyrode's solution, followed by a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 mol/L phosphate buffer (pH 7.2-7.4). The proximal and distal colons were removed immediately after perfusion, post-fixed in the same fixative for 4h and then cryoprotected in 10% sucrose in phosphate-buffered saline (PBS, pH 7.4) overnight.

A series of sections were cut and mounted on gelatin-subbed slides. Elimination of endogeneous peroxidase activity was performed with 0.3% hydrogen peroxide in PBS at room temperature for 30 min. The sections were pre-blocked with 3% normal goat serum, 0.5% triton X-100, and 1% bovine serum albumin in PBS for 30 min. Then incubated in rabbit antinociceptin antiserum (Chemicon Pharmaceuticals Inc, 1:500) at 4℃ overnight. The sections were rinsed with PBS and incubated for 1h with goat anti-rabbit biotinylated secondary antibody (Rocland Laboratories, USA, 1:15000). Then they were incubated in an avidin-biotin complex coupled to horseradish peroxidase for 10 min at room temperature. Finally, after rinsing with PBS, the nociceptin-positive neurons and nerve fibers were visualized using 3, 3' -diaminobenzidine (DAB, Maixin Chemical Co. Fuzhou, China). The slides were then dried, dehydrated in ethanol (70%-100% gradually), cleared in xylene, and coverslipped with mounting solution. Immunohistochemical image analysis was made to assess the changes of OFQ both in proximal and distal colons in two group rats (n = 10).

All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Statistical analysis

Results were expressed as mean ± SE. Data were analyzed by SPSS10.0. Differences were analyzed by one-way analysis of variance ANOVA and independent t test. Probabilities of $P < 0.05$ were considered significant.

RESULTS

Motility studies

In vitro studies using longitudinal muscle revealed that OFQ (10^{-9}-10^{-6} mol/L) induced contractions in the colon. The threshold concentration of OFQ to induce contractions was 10^{-9} mol/L. In the proximal colon of two group rats, OFQ (10^{-9} to 10^{-6} mol/L) induced contractions in a dose-dependent manner (Figure1A), but the increase of tension in cathartic colon was lower than in the control groups (Figure1B). To determine if OFQ activity was mediated by neural pathways or if it was a direct myogenic effect, OFQ-induced contractions were examined in the presence of TTX. In the presence of TTX, OFQ failed to elicit additional contractions (Figure 1C). The contractions

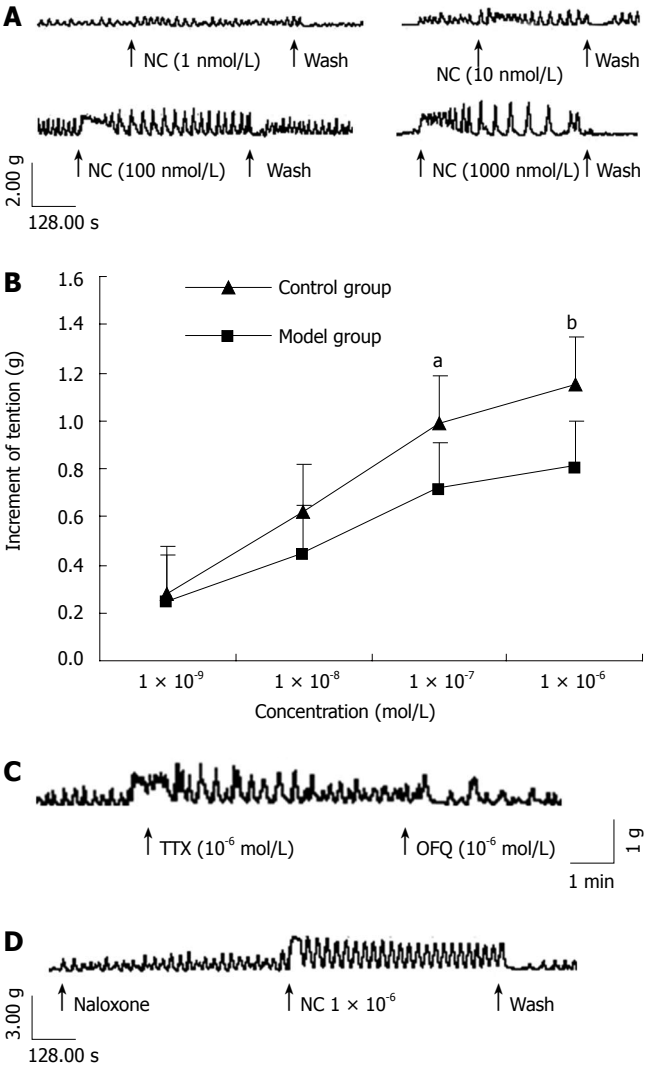

Figure 1 Effects of OFQ (10^{-9}-10^{-6}mol/L) on colon of rats *in vitro*. **A**: Effects of OFQ on the contractile activity of longitudinal muscle strips obtained from the rat proximal colon; **B**: Different effects of OFQ (10^{-9}-10^{-6} mol/L) on increment of tension in two groups of rats. $^{a}P < 0.05$ *vs* 1 × 10^{-9} mol/L; $^{b}P < 0.01$ *vs* 1 × 10^{-9} mol/L; **C**: In the presence of TTX (10^{-6} mol/L), OFQ (10^{-6} mol/L) failed to evoke additional contractions; **D**: The response to OFQ (1 × 10^{-6} mol/L) was not affected by naloxone (1 × 10^{-6} mol/L).

induced by OFQ (1 × 10^{-6} mol/L) were not affected by classical opioid antagonist, naloxone (1 × 10^{-6} mol/L) (Figure1D).

Intravenous administration of OFQ caused phasic contractions in the proximal colon (Figure 2A), it was not affected by classical opioid antagonist, naloxone (1 × 10^{-6} mol/L) (Figure 2B), but the amplitude of phasic contractions caused by OFQ in cathartic colon was much lower than control groups (Figure 2C, 2.58 ± 0.41 *vs* 4.16 ± 0.53, $t = -2.6$, $P = 0.012$).

Immunohistochemistry

Immunohistochemistry of OFQ in the rat gastrointestinal tract showed that Orphain FQ immunoreactive (OFQ-IR) neurons and nerve fibers were visualized in the myenteric plexus of the rat colon (Figure 3A). The immunoreactivity of OFQ in the myenteric plexus in the proximal cathartic colon of rats decreased significantly as compared with

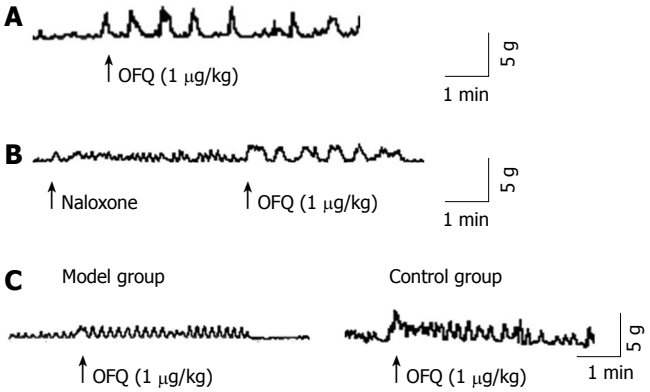

Figure 2 Effects of OFQ on colon of rats *in vivo*. **A**: Intravenous administration of OFQ (1 μg/kg) caused phasic contractions in the colon. **B**: Naloxone (300 μg/kg) has no effects on phasic contractions caused by intravenous administration of OFQ (1 μg/kg)in the proximal colon. **C**: Intravenous administration of OFQ (1 μg/kg) caused phasic contractions in the proximal colon in two groups.

the control group. (176.42 ± 5.792 *vs* 137.367 ± 25.508. $P = 0.001$) (Figure 3B, 3C). The negative control showed no staining of OFQ-IR in the myenteric plexus of the gastrointestinal tract.

DISCUSSION

Due to the diet and social-psychological changes, constipation has become an important factor affecting the quality of human life in modern society, therefore the morbidity is getting higher and higher. STC is a common syndrome in idiopathic constipation. It is a severe disorder of colonic motility characterized by a reduction in the frequency, amplitude and duration of propulsive contractions in the large bowel. Because of lack of understanding of the etiology, current medical treatments on STC are often ineffective. Animal experiments have shown that stimulant laxatives can damage ENS and cause the changes of some enteric neurotransmitters and thus the slow gastrointestinal tract transit occurs.

The innervation of the gastrointestinal tract is unique among the visceral organs. Neurons originating in the enteric ganglionated plexuses within the gut wall coordinate intrinsic reflexes associated with intestinal motility, epithelial secretion, and mucosal blood flow[14]. The myenteric plexus lies between the longitudinal and circular smooth muscle layers and regulates intestinal motor function. Bowel motility is controlled by a local network of intramural nerves[15]. Effects of opioid peptides on GI motility have been studied. Morphine can cause constipation due in part to slowing of GI transit and inhibition of active intestinal secretion, which was the normal side effect in clinical practice[16].

Despite its being homologous to classical opioids, dynorphin A, the OFQ/ORL1 system represents a new peptide-based signaling pathway, which is pharmacologically distinct from the opioid system[17]. Probing into the distribution and function of N/OFQ and ORL1 receptor in the gastrointestinal tract of the animal model will contribute to the overall understanding of the characteristics of this neuronic system, accelerating

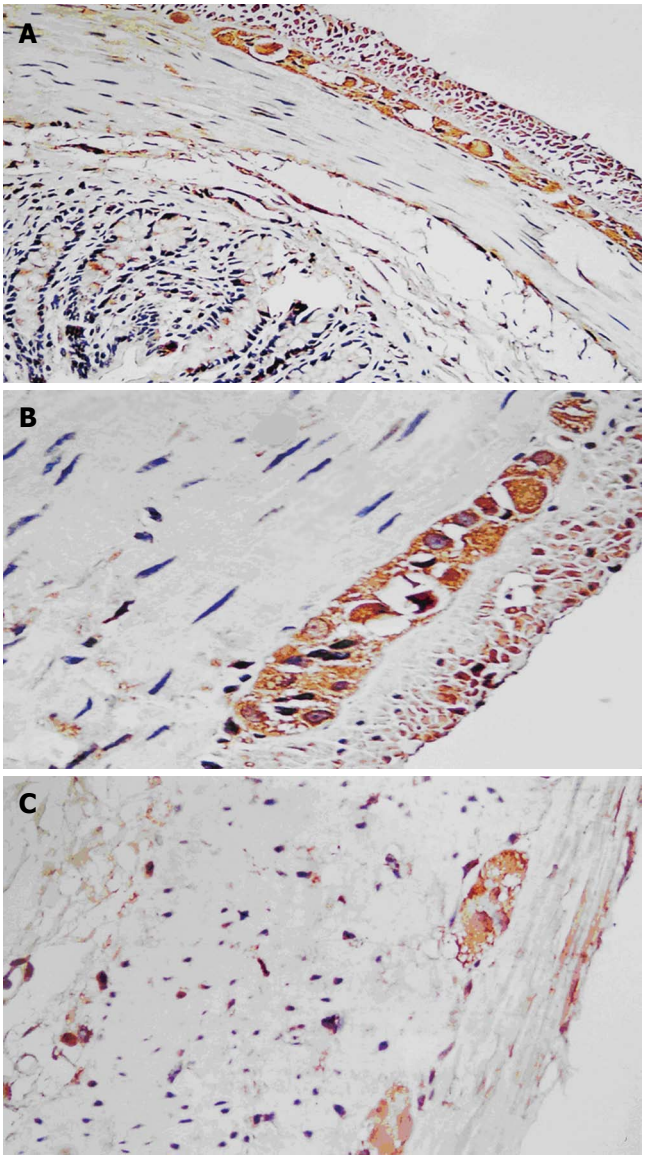

Figure 3 **A**: Expression of OFQ in the myenteric plexus of colon in control group. SP × 200; **B**: Strong positive expression of OFQ in colon of control group. SP × 400; **C**: Weaker positive expression of OFQ in colon of model group. SP × 400.

related researches of the gastrointestinal motility regulated by OFQ/ORL1 receptor system and promote the general comprehensivness of the opioid system. Thus, the model of rats with cathartic colon was established by feeding the rats with rhubarb for 3 months so as to illustrate the etiology of STC.

Our *in vitro* studies showed that OFQ induced contractions of colonic longitudinal muscle strips. In the proximal colon, OFQ (10^{-9}-10^{-6} mol/L) induced contractions in a dose-dependent manner (Figure 1). But the increase of tension in cathartic colon was lower than the control groups (Figure 2). The mechanisms underlying OFQ-induced contractions were further investigated in the proximal colon. To determine if OFQ activity was mediated by neural pathways or if it was a direct myogenic action, OFQ induced contractions were examined in the presence of TTX, it has been supposed that TTX can abolish neuronal transmitter transition[18]. In the presence of TTX, OFQ failed to evoke additional contractions, suggesting that action of OFQ is mediated by neural pathways. It was coincidental with the present study that OFQ was highly expressed in the myenteric plexus of the rat colon, but not in the muscle cells. The *in vitro*, inhibitory effects of OFQ on EFS-evoked muscle contractions has been reported for mouse vas deferens and guinea pig ileum[19]. EFS-evoked muscular contractions were significantly reduced by 50% in guinea pig ileum by OFQ (10^{-7} mol/L) and was significantly reduced in mouse vas deferens by OFQ. Thus, the mechanisms and sites of action of OFQ may differ among different regions of gastrointestinal tract and different species.

Our study *in vivo* showed that intravenous administration of OFQ (1 μg/kg) significantly increased muscle contractions in the proximal colon. These observations indicate that OFQ also stimulates colonic motility *in vivo*. The effects cannot be affected by classical antagonist, naloxone, but can be abolished in the presence of TTX, suggesting that the action of OFQ is nerve mediated. Currently, the physiological role of OFQ in colonic motility is unknown. However, Takahashi *et al*[20] in an *in vivo* study showed that intravenous administration of OFQ (3 pmol/kg to 3 nmol/L) significantly increased muscle contractions in the proximal colon. Furthermore, OFQ (1 nmol/L, subcutaneously) accelerated the colonic transit by promoting migrating colonic contractions in rats[21]. These observations also indicate that OFQ can stimulate colonic motility *in vivo*.

The immunoreactivity of OFQ in the myenteric plexus in the proximal region of the cathartic colon of rats decreased significantly compared with the control group. It has been suggested that OFQ prefered to be located in the stimulating motor neuron[22]. Colonic smooth muscle of cathartic colon showed low sensitivity to the stimulation of OFQ and this suggested it might becaused by the abnormal distribution of OFQ or the abnormalities of receptors, leading to the disorganization of dynamic and uncoordinated contractions.

In conclusion, OFQ seems to modulate the colonic transit independently from the classical opioid peptides[23]. We confirmed that OFQ is present in the gastrointestinal tract and has an effect on colonic motility. The physiological role of the nociceptin-QRL1 system in the colon is not clear but it may be significant to pathophysiological processes that underlie motor dysfunction of the bowel[24]. These findings indicate that OFQ is a brain-gut peptide and plays a role in the control of gastrointestinal functions[25]. The abnormalities of enteric nervous system are responsible for slow transit constipation. It also indicates that chronic application of stimulant laxatives can induce disorganization and damage the enteric nervous system[26,27] and accelerate the pathological changes of STC. But the pathophysiology of STC is complex and not easily approached through the data from animals alone. Further studies according to the clinical characteristics of STC patients could provide additional insight into this issue. It is necessary to investigate the mechanism underlying the biological actions of N/OFQ on GI. Because OFQ seems to exert

its stimulatory action in the colon[28,29], in the future, synthesized analogues of OFQ may be used to treat constipation secondary to colonic inertia[30].

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CLINICAL RESEARCH

Malignancy and mortality in a population-based cohort of patients with coeliac disease or 'gluten sensitivity'

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Abstract

AIM: To determine the risk of malignancy and mortality in patients with a positive endomysial or anti-gliadin antibody test in Northern Ireland.

METHODS: A population-based retrospective cohort study design was used. Laboratory test results used in the diagnosis of coeliac disease were obtained from the Regional Immunology Laboratory, cancer statistics from the Northern Ireland Cancer Registry and mortality statistics from the General Registrar Office, Northern Ireland. Age standardized incidence ratios of malignant neoplasms and standardized mortality ratios of all-cause and cause-specific mortality were calculated.

RESULTS: A total of 13 338 people had an endomysial antibody and/or an anti-gliadin antibody test in Northern Ireland between 1993 and 1996. There were 490 patients who tested positive for endomysial antibodies and they were assumed to have coeliac disease. There were 1133 patients who tested positive for anti-gliadin antibodies and they were defined as gluten sensitive. Malignant neoplasms were not significantly associated with coeliac disease; however, all-cause mortality was significantly increased following diagnosis. The standardized incidence and mortality ratios for non-Hodgkin's lymphoma were increased in coeliac disease patients but did not reach statistical significance. Lung and breast cancer incidence were significantly lower and all-cause mortality, mortality from malignant neoplasms, non-Hodgkin's lymphoma and digestive system disorders were significantly higher in gluten sensitive patients compared to the Northern Ireland population.

CONCLUSION: Patients with coeliac disease or gluten sensitivity had higher mortality rates than the Northern Ireland population. This association persists more than one year after diagnosis in patients testing positive for anti-gliadin antibodies. Breast cancer is significantly reduced in the cohort of patients with gluten sensitivity.

Key words: Coeliac disease; Cancer; Mortality; Gluten sensitivity

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INTRODUCTION

Coeliac disease (CD) is an autoimmune disorder characterised by inflammation and villous atrophy of the small intestine, resulting in the malabsorption of vitamins and nutrients. It is caused by an immune response to wheat gluten (gliadin). Ireland is thought to have one of the highest incidences of CD in the world with a prevalence in Northern Ireland of at least 1 person per 122 in the population[1]. Diagnosis is normally confirmed by duodenal biopsy; however, highly sensitive and specific blood tests for CD are available including the transglutaminase antibody test (93% sensitive, > 99% specific) and the endomysial antibody (EMA) test (93% sensitive, > 98% specific)[2]. Another test the anti-gliadin antibody (AGA) test is not as sensitive or specific for CD. It is a measure of the immune response to gliadin and may detect people with gluten sensitivity who don't have clinically detectable CD. Patients testing positive for CD are advised to adhere to a strict gluten free diet for life[3,4] to avoid symptoms such as diarrhoea, anaemia and weight loss associated with this condition.

In addition to the significant morbidity that can be associated with this condition, CD is thought to be associated with an increased risk of malignancy and mortality. A recent European multi-centre study reported more than

a three-fold increased risk of non-Hodgkin's Lymphoma (NHL) in patients with clinically diagnosed CD[5]. Other studies have also reported an increased incidence and/or mortality of NHL in patients with CD[4,6-12]. Estimates of the standardised incidence ratio range from 3.3[5] to 18.0[10], although Card *et al*[7] suggest that the risk of NHL is at the lower end of these estimates.

CD has been associated with an increased risk of other cancers including cancers of the gastrointestinal tract[7-9,11], in particular cancers of the small intestine[7,9-11]. However, West *et al*[8] in a recent study including 4732 people with CD found that most cancers, except NHL, were reported within one year of diagnosis of CD.

Studies have also reported that CD may be associated with reduced risks of some cancers including breast cancer[8,11,13] although the reason for this association remains unknown. West *et al*[8] reported that lung cancer was less common in patients with CD with one possible explanation of the proposed protective effect of cigarette smoking[14].

Overall, mortality was higher in patients with CD compared to the population[8,10,15]. Metzger *et al*[15] reported a standardised mortality ratio (SMR) of all-cause mortality of 2.53 (95% CI 1.50-4.25) with an increased mortality from malignant neoplasms. West *et al*[8] observed that the association between all-cause mortality and CD remained significant more than one year after diagnosis.

The aim of this study was to identify the incidence of malignancy and mortality in patients with CD (positive EMA test) or patients with gluten sensitivity (positive AGA test) in a retrospective cohort study in Northern Ireland.

MATERIALS AND METHODS

Exposure measurement

A serological dataset of all patients investigated for suspected coeliac disease tested for IgA EMA and/or IgA AGA between 1993 and 1996 was obtained from the Regional Immunology Laboratory, Royal Group of Hospitals, Belfast. If patients had EMA/AGA tests on more than one occasion their date of inclusion in the study was the date of the first positive EMA or AGA result. Duplicates were removed and records with no date of birth excluded. Cases were defined as patients with 'coeliac disease' (positive EMA result) or 'patients with an intolerance to gliadin in the diet' (positive AGA, result more than 100×10^3 EU/L). No information was available on the EMA test results for 4585 patients who had an AGA test. These patients were excluded from the AGA positive, EMA negative analysis.

Outcome measurement - incidence

Researchers within the Northern Ireland Cancer Registry (NICR)-LAA, LJM, CF; who are experienced in the procedures involved in linking datasets, used forename, surname, date of birth and when available hospital number or General Practitioner name to match patients in the serological database to patients within the NICR database. EMA and AGA positive patients were followed-up for between 7 and 11 years until the end of December 2003. Exact and "fuzzy" matching algorithms were used to link patients in the two databases. "Fuzzy" matching used phonetic codes for the forename and surname to match similar sounding names which had different spellings (i.e. Smyth and Smith). If dates of birth were similar (i.e. 01/03/1975 and 03/01/1975) they were considered as potential matches. All "fuzzy" and potential matches were checked manually using all available information before being included in the study.

The main outcome measures were classified according to the International Classification of diseases (ICD) 9 before 2002 and ICD 10 thereafter: (1) Any malignant neoplasm (ICD 9: 140-208, 230-234; ICD 10: C00-C97); (2) NHL (ICD 9: 200, 202; ICD 10: C82-C85); (3) Gastrointestinal tract cancer (ICD 9: 150-154; ICD 10: C15-C21); (4) Small bowel cancer (ICD 9: 152; ICD 10: C17); (5) Lung cancer (ICD 9: 162-163; ICD 10: C34); (6) Breast cancer (ICD 9: 174-175; ICD 10: C50); (7) Prostate cancer (ICD 9: 185; ICD 10: C61); (8) Liver cancer (ICD 9: 155; ICD 10: C22).

Outcome measurement - mortality

The serological database was also linked by forename, surname and date of birth to death records held by the Registrar General's Office (GRO), Northern Ireland until the end of December 2003. These files contain cause-specific mortality data on all deaths occurring within Northern Ireland. Exact matching and "fuzzy" matching algorithms were used and potential matches were checked manually. Outcome measures included all-cause and cause-specific mortality. Ethical committee approval for this study was obtained from the Research Ethics Committee of the Queen's University Belfast to match the databases.

Statistical analysis

Total person-years of follow-up for the cohort of patients with a positive EMA test and for the cohort of patients with a positive AGA test were calculated by collating the person-years of follow-up for each patient from the date of entry into the cohort (1993 to 1996) until the date of death or 31st December 2003, whichever was earlier.

Indirect standardization was used to calculate standardised incidence ratios (SIRs) for all malignant neoplasms and for each cancer type (including NHL, gastrointestinal cancers, small bowel, breast, prostate, liver and lung cancer) for patients with a positive EMA test, for patients with a positive AGA test and for patients with a positive AGA test but a negative EMA test. The SIRs were calculated by taking the total number of observed cancers within the cohort of patients with a positive EMA or AGA test and dividing this by the expected number of cancers, calculated by applying age and sex-specific incidence rates within the Northern Ireland population to each cohort. CIs were estimated using the Poisson distribution. Standardised mortality ratios were calculated in a similar manner to the SIRs using all and cause-specific mortality figures obtained from the General Registrar Office, Northern Ireland.

RESULTS

Of the 13 338 patients in Northern Ireland who under-

Table 1 Number of EMA and AGA positive and negative patients

AGA	+ve EMA	-ve EMA	Not recorded	Total
+ve	275	456	402	1133
-ve	215	7807	4183	12 205
Total	490	8263	4585	13 338

Table 2 Standardised incidence ratio (SIR) of malignancies

	EMA +ve			AGA +ve			AGA +ve, EMA -ve		
	n	Expected *n*	SIR (95% CI)	*n*	Expected *n*	SIR (95% CI)	*n*	Expected *n*	SIR (95% CI)
All malignancies	24	25.41	0.94 (0.57-1.32)	96	86.13	1.11 (0.89-1.34)	68	37.28	1.82 (1.39-2.26)
All malignancies (> 6 mo after diagnosis)	20	24.26	0.82 (0.46-1.19)	75	86.05	0.87 (0.67-1.07)	52	39.84	1.31 (0.95-1.66)
All malignancies (> 12 mo after diagnosis)	18	24.24	0.74 (0.40-1.09)	67	85.94	0.78 (0.59-0.97)	44	37.15	1.18 (0.83-1.53)
Non-Hodgkin's lymphoma	2	0.27	7.47 (0.00-17.83)	6	2.59	2.32 (0.46-4.17)	3	0.52	5.76 (0.00-12.28)
Gastrointestinal cancer	4	2.97	1.35 (0.03-2.67)	14	11.70	1.20 (0.57-1.82)	9	5.24	1.72 (0.60-2.84)
Small intestine	1	0.04	23.33 (0.00-69.07)	1	0.14	7.28 (0.00-21.54)	1	0.06	15.51 (0.00-45.90)
Lung cancer	1	2.60	0.39 (0-1.14)	5	9.98	0.50 (0.06-0.94)	4	4.51	0.89 (0.02-1.76)
Breast cancer	4	3.92	1.02 (0.02-2.02)	5	10.53	0.47 (0.06-0.89)	1	4.09	0.24 (0.00-0.72)
Prostate cancer	2	1.12	1.78 (0.00-4.24)	8	6.30	1.27 (0.39-2.15)	3	3.13	0.96 (0.00-2.04)
Liver cancer	2	0.06	31.62 (0.00-75.45)	3	0.60	5.01 (0.00-10.69)	2	0.27	7.48 (0.00-17.86)

went EMA and/or AGA tests between 1993 and 1996 490 were EMA positive (5.6% of EMA tests), 1,133 AGA positive (8.5% of all AGA tests) and 456 patients were AGA positive and EMA negative (5.2% of those tested for both EMA and AGA) (Table 1).

The average age (age range) of patients with a positive EMA or AGA test were 45 (0-88) years and 50 (0-91) years respectively. Included in the database were 46 children (< 18 years) with a positive EMA test (9.2% of cohort) and 71 children with a positive AGA test (6.3% of cohort). In total 68.2% of EMA positives and 63.2% of AGA positives were male. The average AGA score in those testing positive was 215.4 × 10^3 EU/L (range 100-1163 × 10^3 EU/L).

Standardised incidence ratios-EMA

In total 24 (4.90%) of the EMA positive patients developed a malignant neoplasm during the follow-up period, 6 of which were diagnosed within 12 mo of the test. There were no significant associations between a positive EMA test and developing a malignant neoplasm (Table 2). Due to the small number of EMA positive patients developing malignancy the confidence intervals for the site specific cancers are large resulting in no significant associations despite a raised SIR being found for NHL [SIR 7.47 (95% CI 0.00-17.83)], small bowel cancer [SIR 23.33 (95% CI 0.00-69.07)] and primary liver cancer [SIR 31.62 (95% CI 0.00-75.45)]. Lung cancer appeared to be less common in EMA positive patients than in the Northern Ireland population [SIR 0.39 (95% CI 0-1.14)].

Standardised incidence ratios - AGA

In total 96 (8.47%) of the AGA positive population developed a malignant neoplasm. There were no significant associations between a positive AGA result and the development of malignant neoplasms (Table 2). However, malignancy was significantly raised in the AGA positive, EMA negative group [SIR 1.82 (95% CI 1.39-2.26)] (Table 2). This association was confined to males, SIR 2.90 (95% CI 2.11-3.69), and remained significant 6 and 12 mo after the AGA test, SIR 2.16 (95% CI 1.48-2.84) and SIR 1.91 (95% CI 1.27-2.55), respectively. Overall incidence of malignant neoplasms occurring 6 and 12 mo after the AGA test were significantly reduced in females SIR 0.60 (95% CI 0.27-0.92) and SIR 0.51 (95% CI 0.19-0.83) respectively. There was a significant inverse relationship between lung and breast cancer and a positive AGA result; however, only breast cancer was significantly reduced in the AGA positive EMA negative group. The SIR was raised for both small bowel and liver cancer but the results were not statistically significant.

Standardised mortality ratios - EMA

Overall mortality was significantly higher in the EMA positive population compared to the Northern Ireland population (Table 3). However, the statistically significant association did not remain when deaths within 6 mo or 1 year of the EMA test were excluded from the analysis. Although there were no other statistically significant association's mortality from NHL, endocrine deaths and urinary deaths appeared increased.

Standardised mortality ratios - AGA

Overall, mortality was higher in the AGA positive and in the AGA positive/EMA negative populations than in the Northern Ireland population (Table 3). This association

Table 3 Standardised mortality ratios (SMR) of all cause and cause-specific mortality

	EMA +ve			AGA +ve			AGA +ve, EMA -ve		
	n	Expected *n*	SMR (95% CI)	*n*	Expected *n*	SMR (95% CI)	*n*	Expected *n*	SMR (95% CI)
All cause mortality	46	26.01	1.77 (1.26-2.28)	234	101.25	2.31 (2.01-2.61)	114	47.25	2.41 (1.97-2.86)
All cause mortality (> 6 mo after diagnosis)	35	25.9	1.35 (0.90-1.80)	184	100.79	1.83 (1.56-2.09)	85	46.91	1.81 (1.43-2.20)
All cause mortality (> 12 mo after diagnosis)	32	25.91	1.24 (0.81-1.66)	165	100.09	1.65 (1.40-1.90)	75	46.59	1.61 (1.25-1.97)
Malignant neoplasms	9	7.05	1.28 (0.44-2.11)	52	32.28	1.61 (1.17-2.05)	27	14.55	1.86 (1.16-2.56)
NHL deaths	2	0.29	6.89 (0.00-16.44)	8	1.12	7.12 (2.18-12.05)	3	0.51	5.88 (0.00-12.53)
Endocrine deaths	1	0.16	6.10 (0.00-18.07)	2	0.24	8.19 (0.00-19.53)	1	0.58	1.73 (0.00-5.14)
Nervous system	1	0.25	3.96 (0.00-11.72)	2	2.92	0.69 (0.00-1.63)	1	2.92	0.34 (0.00-1.01)
Circulatory system	9	12.11	0.74 (0.26-1.23)	42	59.95	0.70 (0.49-0.91)	22	28.65	0.77 (0.45-1.09)
Respiratory system	3	2.67	1.12 (0.00-2.40)	35	25.18	1.39 (0.93-1.85)	2	1.45	1.38 (0.00-3.29)
Digestive system disorders	2	0.58	3.42 (0.00-8.17)	19	4.12	4.61 (2.54-6.68)	10	1.90	5.26 (2.00-8.52)

remained significant even when deaths occurring within 6 mo and one year of the test were excluded. Mortality caused by malignant neoplasms was also significantly raised in both groups as was mortality from digestive system disorders. Mortality from NHL was significantly higher and mortality from circulatory system disease was significantly lower in the AGA positive group but not in the AGA positive/EMA negative group.

DISCUSSION

In this retrospective cohort study the incidence of malignant neoplasms in patients with CD (positive EMA test) was similar to that of the Northern Ireland population. However, patients with gluten sensitivity (positive AGA test) had an increased incidence of malignant neoplasms within six months of diagnosis. In keeping with the findings of other studies all cause mortality was significantly raised in patients with CD; however, this was limited to the six month period following diagnosis. Patients with gluten sensitivity had a significantly raised SMR; this association remained significant more than 12 mo after diagnosis. Compared to the general population breast and lung cancer incidence were significantly lower in patients with gluten sensitivity. Mortality from malignant neoplasms, NHL and digestive system disorders was significantly increased.

The main strength of this study is its population-based design. All patients within Northern Ireland with clinical symptoms suspicious of CD and who also had a positive EMA and/or AGA test during the study period (1993 to 1996) were included. Since a wide spectrum of patients were included in the study the results should be more generalisable to patients with gluten sensitivity than the results of studies where CD was diagnosed in hospital or at referral centres[9-11,16,17]. It is likely that cases diagnosed as hospital in-patients or at referral centres have more severe disease and therefore cancer incidence and mortality may be higher in this group of patients than in all CD patients. CD may go undetected in the population and without screening it is difficult to generalise any of the results to all patients with CD within the population.

Population-based registers of cancer incidence and cause-specific mortality facilitated the matching process. Incomplete matching of patients on the serological database to the NICR and/or GRO database may result in an underestimation of the risk of malignancy and/or mortality compared to the general population. To minimize the possibility of observer bias the staff was blinded to the positive/negative result status of the patients during the matching process. Cancer incidence and mortality in EMA positive, AGA positive and AGA positive/EMA negative patients were compared to the rates in the Northern Ireland population. Other studies have used population-based controls[8] or population rates for comparison[7,10-12]; however, the study by Collin *et al*[17] used hospital outpatients undergoing upper gastrointestinal endoscopy as controls. These patients are likely to have been under investigation because of upper gastrointestinal symptoms and are unlikely to be representative of the general population. One of the proposed explanations for previous reports of increased gastrointestinal cancer incidence in CD patients[7-9,11] is that CD was diagnosed coincidentally when the patients were under investigation for tumour related symptoms. Several studies have reported cancer incidence and/or mortality rates with lag periods of between 1 and 5 years[7,10-12,16] after diagnosis of CD. We, therefore, included SIRs and SMRs excluding patients with an event in the 6-or 12-mo period post diagnosis.

There were 3908 person years of follow-up for malignancy and 3718 person years of follow-up for mortality in the cohort of patients with CD. Bias may have been introduced by loss to follow-up although emigration rates are low in Northern Ireland; data from the GRO (Northern Ireland) estimates that less than 0.1% of the population migrated from the province per year during the 1990s[18]. One of the potential weaknesses of this study was the lack of histological confirmation of CD. Although it is likely that jejunal biopsies were taken from patients with a positive EMA test gaining access to these records without patient consent would have required further ethical considerations. However, the sensitivity and specificity of the EMA test are high[2] and it is, therefore, likely to give a relatively accurate measure of the incidence of CD in the

cohort. The EMA test detects antibodies to an antigen (tissue transglutaminase) present in the endomysial lining of smooth muscle cells. The AGA test measures antibodies to gliadin which do not remain in patients who adhere to a strict gluten free diet. The assay will become negative if the patient is compliant with a gluten free diet. Therefore some patients with CD may not be detected within the cohort. The study by Logan *et al*[13] showed that cancer incidence was decreased in those who were diagnosed early and placed on a gluten-free diet. If this is the case then the incidence of malignancy in CD patients may be lower than reported. However, we had no way to determine whether or not the patients included in the study adhered to a strict gluten-free diet.

Another issue is that multiple comparisons may inflate the possibility of a Type 1 error occurring (i.e. the apparent association resulting by chance). However, only malignant neoplasms/causes of mortality where there had been a previous association or where there was an a priori hypothesis were investigated.

One issue with using these datasets and using a retrospective cohort design is that potential confounding variables were not collected at the time the cohort was established and therefore these could not be adjusted for in the analyses. Potential confounding variables could include co-existing medical conditions, smoking, body mass index, diet, age, sex, *etc*. For example, diabetes is associated with CD and possibly with endocrine deaths which were non-significantly raised and smoking which is less common in patients with CD[14] is associated with lung cancer incidence. There was a significantly reduced risk of lung cancer in patients with a positive AGA test and a non-significant reduced risk in patients with a positive EMA test. West *et al*[8] was the only study to attempt to adjust for potential confounding variables. Adjusting for BMI and smoking did not dramatically alter the observed associations; however, the authors suggest that data on potential confounders may be incomplete as it was obtained from routinely collected information.

Although some AGA positive/EMA negative patients may have CD there were a larger number that were AGA positive than expected according to the sensitivity of the EMA test. Therefore, some of these patients may be gluten sensitive but as yet have no damage to the endomycial muscle. If this is the case then this is the first study to investigate the risk of malignancy and mortality in this group of patients, who were excluded from the recent large study by West *et al*[8]. Mortality was significantly increased in this group of patients and remained elevated more than one year after the positive AGA test. Mortality from digestive system disorders was increased which was not surprising as a positive AGA test may be a marker for other gastrointestinal disorders. Interestingly, it is in this group of patients that breast cancer appears to be reduced. Other studies have reported reduced risks of breast cancer in patients with CD[8,11,13] although there were no significant associations between breast cancer and a positive EMA test in this study. Although the reason(s) for this association remain unknown Askling *et al*[11] suggest that the reduced risk of breast cancer may be a consequence of immune system dysfunction.

The size of the current study is smaller than the more recently published studies e.g. the study by West *et al*[8] who used the General Practice Research Database which had 4,732 CD patients. Malignancy was increased in patients in the first year after diagnosis but not beyond one year of follow-up. In the current study malignancy was not increased in patients with a positive EMA test but was in patients with a positive AGA test. Other studies have also reported no overall increased risk of malignancy in CD patients[7,16]. The size of the current study meant that there was insufficient power to detect any associations between cancer type and CD. However, the risk of NHL appeared to be increased which is in keeping with the results of other studies[3-5,7-12]. There was a significant increased risk of NHL deaths in patients with a positive AGA test. This group of patients is likely to include some patients with CD as there were a number of patients that had no EMA record in the database.

Malignancy and mortality were not significantly increased in patients with CD more than one year after diagnosis; however, overall mortality and mortality from malignant neoplasms were increased in patients who were gluten sensitive with a negative EMA test. If these patients do not have the histological characteristics of CD then it is important to determine the cause of the increased mortality. If this is limited to other digestive system disorders then patients with a negative EMA test but with symptoms should be further investigated for other digestive system disorders. Further investigations into this area are warranted to determine if it is gluten sensitivity that is associated with increased mortality and malignancy or to determine if it is just a marker for other gastrointestinal conditions.

In addition, it is important to determine the risk of malignancy and mortality in patients with a histological confirmation of CD. This study could be extended further by collecting pathology reports on all patients within the study and looking for evidence of villous atrophy. Information on confounding variables such as smoking status could also be collected. However, it is unlikely that patients could be approached because of data confidentiality issues. It is possible that cancer incidence and mortality change depending on the length of time since diagnosis. Therefore, an extension to this study would be to follow-up the patients for a longer period of time. The association with reduced breast cancer incidence is interesting and further studies to investigate this association may identify factors that are associated with reduced breast cancer incidence.

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RAPID COMMUNICATION

Age-related histomorphologic changes in the canine gastrointestinal tract: A histologic and immunohistologic study

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Abstract

AIM: To examine the changes in the histomorphology of the gastric, jejunal and colonic wall of dogs due to physiological aging.

METHODS: Full thickness biopsies were taken from the gastrointestinal tracts of 28 dogs of different ages. The thickness of the different layers of the wall was measured and the numbers of proliferating cells as indicated by immunohistochemical detection of Ki67 were counted.

RESULTS: In the three excision sites, the thickness of all subepithelial layers increased with rising age. The strongest correlation between age and thickness of the intestinal wall was found in the first 10 years of life and in the jejunum (r = 0.6-0.71 for the deep lamina propria mucosa, the muscularis mucosa, and the circular layer of the tunica muscularis). The number of proliferating cells decreased during aging, with the strongest correlation in the lamina propria mucosa and lamina muscularis mucosa of the jejunum and in the colonic submucosa (r = -0.61 to -0.71). Epithelial proliferation was only weakly correlated to the age.

CONCLUSION: The morphology of the deeper layers and the proliferation of mesenchymal cells of the intestinal wall of healthy dogs are correlated with age. Gastrointestinal epithelial proliferation is only weakly age-correlated.

Key words: Age; Canine; Intestine; Ki67; Stomach

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INTRODUCTION

Canine models are well known in the development of gastrointestinal drugs. On the other hand, an increasing number of biopsy samples are taken from the intestines of pet dogs with gastrointestinal symptoms and delivered to pathological diagnostic institutions for evaluation. Lesions, usually found in those specimens, however, are in most cases quantitative aberrations of normal morphology and cellular distribution rather than of a distinct qualitative kind. So a correct diagnosis is largely dependent on the experience of the examiner and the differentiation between physiological and pathological influences on the morphology. Data on the histological changes in the intestinal morphology in dependence on physiological processes, however, are very sparse. Suckling puppies exhibit rapid changes in the mucosal morphology that reach a comparatively stable state by the 42nd d of life[1]. In old dogs, Lafora body-like polyglucosan bodies have been described in the smooth musculature[2] and amyloid in the vessel walls of the large and small intestine without functional or neurological abnormalities[3]. Because of the usage of endoscopic biopsies, however, most examinations are limited to the mucosa[4]. The aim of this study was to characterize morphological alterations during the physiological course of aging including the different layers of the intestinal and gastric wall of dogs.

MATERIALS AND METHODS

Subjects

Tissue samples were obtained from 28 dogs of different age and breed (Table 1), which were patients of the Small Animal Clinic and were euthanatized for diseases unrelated to the gastrointestinal tract. Under general anesthesia, full thickness biopsies of the gastric, jejunal and colonic wall were surgically excised and immediately fixed in 100 mL/L

Table 1 Distribution of age, gender and breed

Age (yr)	Gender	Breed
0.25	M	Collie
0.5	M	Boxer
0.75	F	Rottweiler
0.75	M	West Highland White Terrier
2	F	Newfoundland
3	M	American Canadian White Shepard
3	M	Doberman-Pinscher
5	M	Fox Terrier
5	M	Mongrel
6	M	Pommeranian
6	F	German Shepherd Dog
7	M	Bernese Mountain Dog
7	F	Schnauzer
7	F	Caucasian Shepherd
7	F	Rottweiler
8	M	Mongrel
8	M	German Shorthaired Pointer
8.5	F	Mongrel
9	F	Mongrel
10	M	Mongrel
12	F	English Setter
12	M	Mongrel
12	M	Gordon Setter
13	F	Hovawart
13	F	Mongrel
14	F	Mongrel
14	F	Rottweiler
17	F	Mongrel

M: Male; F: Female.

neutral formalin. After fixation, the samples were routinely embedded in paraffin, cut, and stained with hematoxylin and eosin.

Evaluation of slides

All measurements were performed in a blind fashion, i.e., without knowledge of the animals' age. Distances and structural measurements were performed using a computer sustained manual system (ASM 68k), in which a line was projected into the microscopic ocular, that could be manually adjusted to the outlines of the respective structure to be determined. The resulting length of the line was calculated by the supporting computer system.

The following parameters were determined: in the stomach, depth of foveolae (epithelial length from the surface to the isthmus), length of whole gastric glands (epithelial length from the surface to the base of the gland), estimated percentage of chief cells and parietal cells in the pars principalis, number of fiber layers between the gland base and the lamina muscularis mucosa, thickness of the lamina muscularis mucosa, submucosa, and, because of the inconsistent orientation and borders of the single muscle layers in the stomach, of the tunica muscularis as a whole (Table 2); in the jejunum, villus length (length from the tip following the central lymphatic vessel to the crypt mouth), villus mid and base width (shortest distance from epithelial surface to epithelial surface in the middle of the villus and at the crypt mouth, respectively), crypt depth (length from the crypt mouth to the crypt base following the crypt lumen), thickness of the stratum compactum (distance between crypt base and lamina muscularis mucosa), lamina muscularis mucosa, submucosa, circular and longitudinal layer of the tunica muscularis mucosae (Table 3); in the colon, crypt depth, thickness of the lamina muscularis mucosa, submucosa, circular and longitudinal layer of the tunica muscularis mucosa (Table 4). All measurements were performed at four points of a well oriented section, which were equally distributed over its total length.

Table 2 Absolute values in the stomach

Age (yr)	Depth of foveolae (μm)	Glandular length (μm)	Chief cells (%)	Fibre layers (*n*)	Muscularis mucosa (μm)	Submucosa (μm)	Tunica muscularis (μm)
0.5	210	952	40	6	89.8	60	2076
0.75	226	1073	40	3	99.3	1157	2341
0.75	146	1076	60	4	146.8	365	1746
2	152	1249	70	6	133.0	866	2526
3	155	795	50	11	96.8		
3	228	1052	0	4	212.5	341	1600
5	169	942	70	4	142.3	867	2221
5	198	1013	60	5	96.5	972	1652
7	139	1012	40	3	140.0	869	3140
8	204	927	70	2	192.8	524	1488
8	141	774	30	7	136.8	653	2684
8	219	1074	30	4	154.8	169	968
9	167	986	60	4	249.0	551	2974
10	220	1146	40	6	122.8	1075	2337
12	245	1017	50	9	178.8	3501	2875
12	166	1165	40	5	112.8		
12	230	1059	70	6	121.3	1102	2191
14	189	795	20	12	154.5	1250	1205
17	294	815	20	6	120.5	694	2038

Immunohistochemical assay

In the jejunum and colon, where the most obvious changes in the thickness of wall layers were found, immunohistochemical detection of Ki67 was carried out to determine the proliferative index. The immunohistochemical reaction was performed as follows: Paraffin-embedded sections were dewaxed and treated with 50 mL/L H_2O_2 in ethanol for 30 min to inhibit endogenous peroxidase, followed by rinsing three times in phosphate-buffered saline (PBS). Antigen-retrieval was achieved by microwave treatment (20 min, 0.01 mol/L citrate buffer, pH 6). After demasking, the slides were incubated overnight with the first antibody (MIB-1, DAKOCytomation, Glostrup, Denmark) diluted 1:100 in PBS with 10 g/L bovine serum albumin. Binding of primary antibody was detected with a biotinylated second antibody (goat anti-mouse, diluted 1:200 in PBS) and the ABC-reagent (both Vector Laboratories, Burlingame, USA) according to the manufacturer's instructions using diaminobenzidine as chromogen. Finally, the slides were counterstained with hematoxylin. Negative control was performed with an identical procedure without the first antibody in the PBS/serum incubation step. An internal

Table 3 Absolute values in the jejunum (μm)

Age (yr)	Villus length	Crypt depth	Stratum compactum	Muscularis mucosa	Submucosa	Circular tunica muscularis	Longitudinal tunica muscularis
0.25	572	448	17	58	92	443	254
0.5	622	354	30.75	52	327	573	387
0.75	981	1051	24.25	82	273	729	478
0.75	570	269	35	56	193	519	304
2	536	435	39.75	88	161	542	182
5	894	412	67.25	67	229	764	368
5	882	303	37.75	62	183	658	338
6	867	406	62.75	68	217	585	249
7	589	623	45	73	176	602	346
7	906	466	32.25	99	228	766	454
7	630	381	56.25	106	292	639	418
8	596	333	87.25	162	232	802	285
12	647	314	108.25	150	294	1152	446
12	653	426	46.75	59	161	579	334
13	703	506	73	112	364	1047	217
13	709	344	53.4	146	339	672	290
14	726	475	44.75	89	267	793	370
14	700	514	104.2	136	233	971	535
17	695	403	40.5	75	309	693	304

Table 4 Absolute values in the colon (μm)

Age (yr)	Crypt length	Muscularis mucosa	Submucosa	Circular tunica muscularis	Longitudinal tunica muscularis
0.25	520	41.3	122	387	1235
0.5	530	40.3	165	454	707
0.75	488	60.0	203	338	537
0.75	515	52.8	133	540	530
2	515	56.3	362	541	562
5	609	54.0	388	557	968
5	577	82.3	349	792	682
6	533	48.3	531	728	701
6	363	51.0	375	449	855
7	716	77.7	299	617	1307
7	443	63.0	165	534	586
7	404	38.1	146	519	553
8.5	343	73.0	162	812	787
10	567	44.5	223	734	1034
12	485	40.5	462	510	568
12	460	59.5	372	641	810
13	399	45.5	191	776	1780
13	612	72.5	201	810	981
14	481	37.8	1397	784	575
17	607	48	315	607	359

positive control for the proliferation marker was present in all sections in the epithelial renewal zone of crypts and gastric pits.

Evaluation of Ki67-positive cells

In the jejunum and colon, the number of Ki67-positive cells (i.e. proliferating cells) was examined for the epithelia. Myocytes, myofibrocytes and fibrocytes could not be properly differentiated by morphological means in the immunohistochemically stained slides. For this reason, the number of proliferating cells per area comprises all cells of the lamina propria mucosa, lamina muscularis mucosa, submucosa, circular and longitudinal layer of the tunica muscularis, except round cells, especially leucocytes. All epithelial cells of approximately the lower 40% of the crypts stained positive for Ki67. Hence, comparison was made between the relative and absolute length of this compartment of the crypts. For the other layers, the absolute numbers of positive cells were counted in one whole section and set in relation to the total area of this layer in the respective section (Tables 5 and 6).

Statistical analysis

Values were checked for normal distribution. r is the Pearson product moment correlation coefficient as calculated by OpenOffice.org Calc.

RESULTS

General considerations

In the examined sections, no qualitative changes, such as scars or amyloid deposits, were found irrespective of the age of the dogs.

Stomach: In the fundic mucosa, only minor changes could be noted with increasing age. A tendency towards a relatively increased depth of foveolae could be noted and the lamina muscularis mucosa became thicker (Table 7). The relationship between age and lamina muscularis mucosa thickness was highest in dogs younger than 10 years (n = 14).

Jejunum: The mean thickness of all layers of the jejunum increased in size during aging (data not shown). The most significant changes were detected in the deeper layers of the intestinal wall during the first 10 years of life (n = 12), most obviously in the deepest part of the mucosal lamina propria in the jejunum. The distance between the crypt base and the lamina muscularis mucosa showed a strong correlation with the age of the examined dogs (Table 8). While villus length and crypt depth did not show any clear relationship with the age, the whole mucosal thickness increased with aging, mainly in the first 10 years of life. In the lamina muscularis mucosa, submucosa and circular layer of the tunica muscularis, the thickness also increased with age. In addition, in the lamina muscularis mucosa and circular layer, these changes displayed a stronger relationship in dogs less than 10 years. The longitudinal layer of the tunica muscularis did not show age-related changes.

Colon: The circular layer of the tunica muscularis displayed an age-related increase (Table 9). In contrast to the findings in the jejunum, only weak correlations between age and the thickness of the other mucosal or submucosal layers could be found.

Immunohistochemistry

Jejunum: In all layers of the intestinal wall, the numbers of proliferating cells were highest in young dogs, particularly those less than 3 years (data not shown). A

Table 5 Proliferation in the jejunum

Age (yr)	Proliferative length epithelium		Proliferating cells (*n*)					
	Absolute (μm)	Relative (%)	Villous lamina propria (/villus)	Intercrypt lamina propria (/intercrypt space)	Lamina propria below crypts	Muscularis mucosa (/mm length)	Submucosa (/mm length)	Tunica muscularis (/mm length)
0.25	296.4	69.3	4.57	0.77	22.62	8.25	6.25	1.28
0.5	179.2	75.2	25.21	2.16	35.50	10.50	1.34	0.37
0.75	278.4	61.5	3.79	1.22	14.39	5.19	2.99	0.58
0.75	1006.4	77.0	1.23	0.43	31.07	0.00	0.00	0.06
2	151.2	47.6	1.11	0.29	2.89	0.00	0.00	0.00
5	277.6	70.3	7.48	1.16	5.56	4.17	1.64	0.33
5	314.4	73.1	9.53	0.68	17.84	1.66	1.14	0.21
6	243.8	76.8	3.24	0.00	1.06	0.98	0.00	0.24
7	378.6	71.6	2.25	0.24	8.82	0.96	0.00	0.00
7	388.0	73.2	1.61	0.47	4.66	1.49	0.18	0.00
7	412.4	70.3	7.81	0.00	4.04	0.00	1.04	0.20
8	219.6	57.7	2.89	0.00	2.86	0.39	0.00	0.00
12	139.0	60.8	4.43	0.31	13.20	0.00	0.00	0.00
12	358.0	77.6	6.78	1.33	15.14	0.00	1.65	0.20
13	231.6	77.4	16.18	1.03	19.14	0.91	0.26	0.93
13	291.4	66.8	1.38	0.00	0.00	0.00	0.00	0.00
14	335.2	73.0	2.42	0.29	1.67	0.64	1.12	0.31
14	369.4	77.1	15.55	0.75	15.04	2.70	0.18	0.09
17	248.0	65.8	0.81	0.23	2.65	0.00	0.00	0.00

Table 6 Proliferation in the colon

Age (yr)	Proliferative length epithelium		Proliferating cells (*n*)				
	Absolute (μm)	Relative (%)	Intercrypt lamina propria (/intercrypt space)	Lamina propria below crypts	Muscularis mucosa (/mm length)	Submucosa (/mm length)	Tunica muscularis (/mm length)
0.25	147.4	28.8	2.117	1.59	32.07	5.21	1.33
0.5	179.0	32.2	0.943	1.67	9.20	2.63	0.12
0.75	90.2	20.3	0.388	0.36	0.98	0.78	0.04
0.75	217.8	39.4	2.048	0.79	1.64	1.21	0.09
2	129.8	27.8	0.194	0.28	0.00	0.39	0.00
5	192.0	32.9	2.251	0.89	3.02	1.14	0.17
5	219.5	36.9	0.657	0.71	2.63	0.37	0.11
6	189.2	56.4	0.276	0.11	1.05	0.43	0.59
6	194.4	37.4	0.563	0.33	1.35	0.62	0.10
7	118.4	29.8	0.248	0.00	0.00	0.40	0.00
7	158.0	31.8	0.226	0.18	0.00	1.12	0.05
7	215.8	33.3	0.838	0.63	2.01	0.52	0.00
8.5	150.0	37.1	0.583	0.24	1.12	0.00	0.05
10	132.8	26.1	1.059	1.60	3.18	0.21	0.04
12	151.8	32.8	1.031	1.49	6.13	0.27	0.16
12	173.4	33.9	0.6519	0.55	1.84	0.29	0.00
13	132.8	26.1	0.000	0.33	1.79	0.85	0.20
13	163.6	25.5	0.817	0.29	0.66	0.24	0.02
14	138.2	30.9	0.831	0.37	3.27	0.00	0.00
17	234.0	39.4	0.329	0.40	0.00	0.18	0.00

marked correlation between age and number of Ki67-positive cells could be found in dogs younger than 10 years (n = 12) for the lamina propria between crypts and between lamina muscularis mucosa and crypt base, for the lamina muscularis mucosa, for the submucosa and for the tunica muscularis (Table 9). Including the older dogs (> 10 years), correlation between age and thickness of the lamina muscularis mucosa remained strong. In contrast to the mesenchymal tissues, no clear correlation could be detected between age and epithelial proliferation.

Colon: As in the jejunum, the number of proliferating cells decreased with age. The strongest correlations were found, again, until the age of 10 (n = 13). In particular, the thickness of the lamina propria between crypt base and lamina muscularis mucosa, of the lamina muscularis mucosa and of the submucosa revealed a strong age-

Table 7 Strength of correlation between age and diameter of different layers in the stomach wall of dogs

	All ages	< 10 yr
Absolute foveolar depth	0.44	0.05
Relative foveolar depth	0.54	0.11
Lamina muscularis mucosa	0.17	0.58
Submucosa	0.35	0.17
Tunica muscularis	-0.04	0.01

Table 8 Strength of correlation between age and diameter of different layers in the jejunal wall of dogs

	All ages	< 10 yr
Villus length	-0.01	0.17
Crypt depth	-0.15	-0.2
Whole mucosa	0.5	0.1
Lamina propria	0.54	0.72
Lamina muscularis mucosa	0.51	0.61
Submucosa	0.46	0.13
Circular muscle layer	0.59	0.6
Longitudinal muscle layer	0.12	0.12

Table 9 Strength of correlation between age and diameter of different layers in the colonic wall of dogs

	All ages	< 10 yr
Crypt depth	0.01	-0.21
Lamina muscularis mucosa	-0.05	0.42
Submucosa	0.38	0.27
Circular muscle layer	0.61	0.63
Longitudinal muscle layer	0.2	0.17

Table 10 Strength of correlation between age and number of proliferating cells in the different wall layers of the jejunum and colon

	Jejunum		Colon	
	All ages	< 10 yr	All ages	< 10 yr
Absolute length of proliferating epithelium	-0.21	-0.16	0.07	0.17
Relative length of proliferating epithelium	0.15	0.07	0.02	0.37
Lamina propria of villi	-0.07	-0.28		
Lamina propria between crypts	-0.32	-0.62	-0.36	-0.43
Lamina propria below crypts	-0.46	-0.74	-0.24	-0.65
Lamina muscularis mucosa	-0.56	-0.61	-0.36	-0.5
Submucosa	-0.45	-0.54	-0.57	-0.61
Tunica muscularis	-0.29	-0.56	-0.36	-0.34

dependency in the number of proliferating cells (Table 10). In the submucosa, the correlation remained strong throughout the lifetime. The tunica muscularis and lamina propria between crypts showed only moderately age-related proliferation (r = -0.34 and -0.43, respectively).

DISCUSSION

The layers of the gastrointestinal wall beneath the mucosa comprise the major part of intestinal tissue. However, apart from pathological conditions, these tissues are thought to remain unchanged throughout lifetime. This study revealed that in dogs without gastrointestinal diseases, a continuous thickening of the deeper intestinal layers occurs, mainly during the first 10 years of life. In dogs, this period is regarded as young to middle-aged. Hence senile change in nutritional behavior and metabolism is unlikely to be the primary driving force in this development.

The strongest correlation between age and morphology could be detected in the jejunal lamina propria mucosa between crypt base and lamina muscularis mucosa. In textbooks of veterinary histology, this site is often referred to as stratum compactum[5], when it is easily discernable from the overlying lamina propria, as in the proximal small intestine. In the examined sections, no morphologically distinct stratum compactum could be detected, even in those dogs that presented with a very large distance between the crypt base and the lamina muscularis mucosa. This might be due to the more distally located excision site. This layer is thought to prevent perforation of the intestinal wall by food containing bones[4]. Similarly, the observed proliferation of this zone during lifetime in this study might have been stimulated by mucosal distension and alteration by sharp bone fragments. However, detailed anamnestic data about the diet fed to these dogs during their whole lifetime were not available.

Observations of alterations in the size of the lamina muscularis mucosa are restricted to experimental conditions, e.g. an increase in thickness after partial jejunectomy[6] in rats. The observed increase in the thickness of the muscular layer during lifetime is paralleled by observations in the jejuna of rats[7,8]. A possible explanation might be an increased workload for the musculature caused by a decrease in neuronal coordination as a result of the neuronal cell loss during aging, which has been described in humans[9] and rats[10]. If the increased muscular thickness is the result of a hypertrophy of muscle fibers or of the proliferation of connective tissue between fibers needs further, preferentially electron microscopic investigations.

In rats, epithelial cell proliferation increased in the first postnatal week and decreased or remained unaltered in the following months[7,11,12]. Differentiation of the brush border enzymes follows a similar course with a maximal development in young adulthood[13]. Examination of the mechanisms involved pointed to involvement of proliferation, apoptosis and crypt fission in the development of the intestinal mucosal epithelium[14,15], but delivered contradictory results in particular with regard to alteration of the apoptotic rate in aging rats[14,16-18]. In contrast to these findings, the epithelial proliferation in the canine tissues in this study and a study of the jejunal mucosal morphology in dogs of different weights and ages[19] remained stable throughout lifetime. This observation was also made in mice[20]. The discrepancy in the proliferative rate might be explained by different methods applied. While bromodeoxyuridine[16] and Ki-67

(this investigation) cover a small time span of the cycling process, proliferating cell nuclear antigen is expressed during a long period exceeding mitosis. The increased number of proliferative cells in aging rats found in the study using the latter method[18] might indicate rather decreased degradation of the detected enzyme than increased number of cycling cells. The deeper layers of the present study, however, contained a decreasing number of proliferating cells with rising age. This reduced speed of cellular turn-over might lead to a lower adaptability towards a changed digestibility of diet and a slower healing after damage in older dogs.

In conclusion, all layers of the canine jejunal and colonic wall, except for the epithelial monolayer, increase in thickness during aging, while the number of proliferating cells decreases. The underlying mechanisms and possible functional consequences need further investigation.

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AUTHOR'S FEEDBACK

Congratulation on *World Journal of Gastroenterology*

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Dear Professor Lian-Sheng Ma,
Professor Michael Hobsley and I have greatly enjoyed reading the account of all you have done towards the development of "The *World Journal of Gastroenterology*", as described in the article by Zhen-Xi Li in the November 21st. issue of the Journal. It is an amazing achievement! Yours is the only international gastroenterological journal to come out weekly! The standard of the articles is extremely high and the Journal is equal in size to "Gut" (Leaderette: 2005 Impact Factor-Gastroenterology & Hepatology list:1 GASTROENTEROLOGY 12.386; 2 HEPATOLOGY 9.792; GUT 7.692), which comes out only monthly. You have achieved all this in a matter of only 10 years-truly a wonderful achievement. We do congratulate you.

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PHOTO NEWS

President Lian-Sheng Ma met with Dr. Parimal Chowdhury, Professor of University of Arkansas for Medical Sciences

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Parimal Chowdhury (middle of front seat), Professor of University of Arkansas for Medical Sciences, and his wife (left of front seat) met with Lian-Sheng Ma (right of front seat), President of *WJG*, in Beijing, China, on November 29, 2006. The other in turn (from right of rear seat) are Jing Wang, Ye Liu, Yan Jiang, Jing-Yun Ma, Gai-Ping Wang, who are editors of *WJG*. (*WJG* Photo).

President Lian-Sheng Ma, Editor-in-Chief of *World Journal of Gastroenterology* (*WJG*), met with Dr. Parimal Chowdhury, from the University of Arkansas for Medical Sciences, member of *WJG* Editorial Board, and his wife in Beijing on November 29, 2006. The two sides held cordial and friendly talks.

President Ma extended his warm welcome to Mrs. Chowdhury. He firstly introduced to the guests the development of the journal. Currently, the editorial board of the *WJG* consists of 903 members from 52 countries, there are 5 science editors, 27 language editors located in 16 countries, 22 copy editors from 8 countries and 5 electronic editors. Totally 1223 articles were published in *WJG* from issue No.1 to issue No.42 in 2006, with authors distributed in 58 countries and regions, articles coming from outside China accounted for 77.35%, and the rejection rate was 46.44%. *WJG* is the only weekly journal in the fields of gastroenterology and liver diseases among SCI journals in the world. *WJG* was hit 13090137 times and downloaded 1313719 times online from April 15th, 2003 to November 1st, 2006. WJG was cited 2595 times in 2005 by 765 SCI journals, including 412 (15.87%) self-citations. *WJG* is an international academic journal of its kind published in English in China. Based on rapid development of basic research both in basic and clinical gastroenterology as well as growth of international exchange in science and technology, *WJG* converted to a weekly journal from 2005. PubMed and *WJG* electronic version of the text became available beginning from 1998 (ASP, PDF), and readers have free access to read the full texts of *WJG*. In this point, authors can make a timely and broad dissemination of their work to the world, and this has greatly expanded the international influence of the journal and attracted more high-quality manuscripts from different parts of the world.

Dr. Chowdhury was grateful for the hospitality given by President Ma and his staff and expressed his appreciation for the achievements and development of *WJG*. He mentioned that *WJG* is an open journal wide-ranging in contents and timely publication. Furthermore, it has the potential capacity of competing with similar international journals. Dr. Chowdhury also mentioned that Dr. Mark Donowitz, President of the American Gastroenterological Association (AGA), visited Hong Kong recently, is also very concerned about the development of *WJG*. He had a great admiration for the rich content, high quality of editing and printing and fast publishing frequency of *WJG*. It was his pleasure to work with *WJG*, said Dr. Chowdhury, adding that as one of the editorial board members of *WJG*, he will continue to contribute to *WJG* development.

Finally, President Ma expressed his gratitude to Dr. Chowdhury for his support and for his comments on *WJG*, for writing reviews, and organizing a series of articles as topic highlights in the past. Ma also expressed that *WJG* will continue to follow international standards and improve the academic quality in all aspects including peer review, copy editing, printing, *etc.* *WJG* will target to be the most outstanding international journals with respect to academic level be quality of editing from a scientific perspective and to be one of the top academic international journals in the next 3 to 5 years with the strong support of our country, the authors, readers, all editorial members and the international community.

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Meetings

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Digestive Disease Week
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Washington Convention Center,
Washington DC

American College of
Gastroenterology Annual Scientific
Meeting
12-17 October 2007
Pennsylvania Convention Center
Philadelphia, PA

15th United European
Gastroenterology Week, UEGW
27-31 October 2007
Le Palais des Congrès de Paris,
Paris, France

NEXT 6 MONTHS

Meeting ILTS 13th Annual
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Rio De Janeiro
www.ilts.org

Meeting Canadian Digestive Diseases
Week (CDDW)
16-20 February 2007
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cagoffice@cag-acg.org
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htm

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of the Liver
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Meeting ESGAR 2007 18th Annual
Meeting and Postgraduate Course
12-15 June 2007
Lisbon
fca@netvisao.pt

Meeting Falk Symposium 160:
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15-16 June 2007
Portoroz
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Meeting Falk Symposium 158:
Intestinal Inflammation and
Colorectal Cancer
23-24 March 2007
Sevilla
symposia@falkfoundation.de

Meeting 42nd Annual Meeting of
the European Association for
the Study of the Liver
11-15 April 2007
Barcelona
easl2007@easl.ch
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Meeting European Society for
Paediatric Gastroenterology,
Hepatology and Nutrition
Congress 2007
9-12 May 2007
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Meeting 9th World Congress
on Gastrointestinal Cancer
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Meeting Falk Symposium 159: IBD
2007-Achievements in Research
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4-5 May 2007
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Meeting BSG Annual Meeting
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Meeting Gastrointestinal Endoscopy
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Meeting Falk Workshop: Mechanisms
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Meeting Falk Symposium 161: Future
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11-12 October 2007
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Meeting Falk Symposium 162: Liver
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Meeting 15th International Congress
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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

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Author(s) and editor(s)

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Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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