BIRKHÄUSER

Birkhäuser Advances in Infectious Diseases

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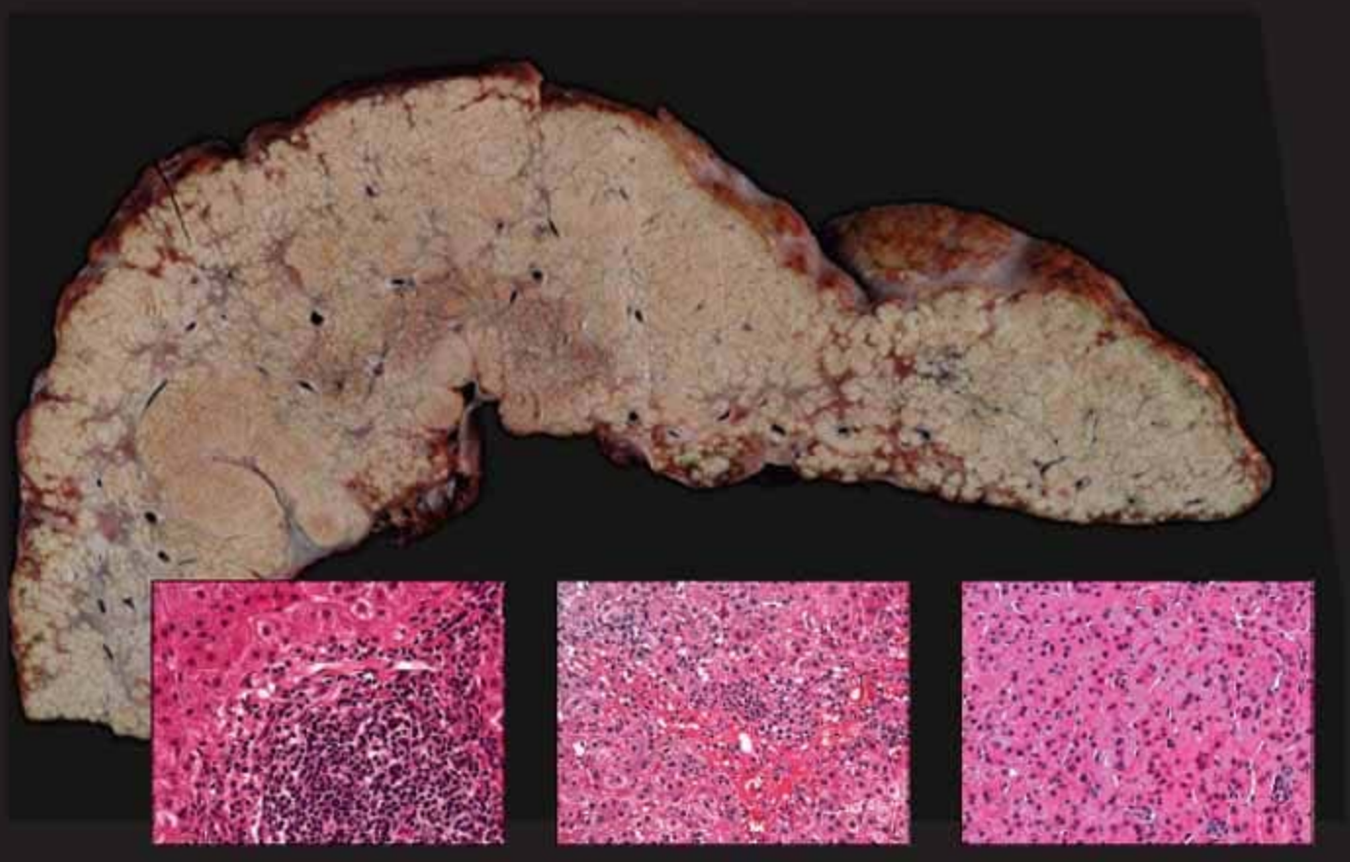
Comparative Hepatitis

Olaf Weber
Ulrike Protzer

Editors



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Comparative Hepatitis

Olaf Weber
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Birkhäuser
Basel · Boston · Berlin

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Cover illustration: Section through a cirrhotic human liver. Small pictures: left, portal lymphoid follicle in HCV infection (H&E, original magnification 200×); middle, zonal pattern with perivenular necrosis in drug-induced hepatitis (H&E, original magnification 100×); right, ground glass hepatocytes in chronic hepatitis B (H&E, original magnification 200×). With kind permission of Thomas Longerich and Peter Schirmacher.
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Preface

Hepatitis is an inflammation of the liver tissue causing hepatocellular injury which may have different aetiologies. In humans, acute and chronic hepatitis and hepatitis-related diseases such as liver failure, liver cirrhosis and hepatocellular carcinoma are among the most important causes for disabilities and death. Whereas the reaction of the liver tissue is relatively uniform, the causes for hepatocellular injury are heterogeneous and include viruses, toxins, radiation, injury and drugs but also bacteria, parasites and autoimmune reactions.

In this volume of *Birkhäuser Advances in Infectious Diseases* we review today's knowledge about hepatitis with emphasis on comparative aspects between hepatitis in humans and animals, but also between different etiological agents.

This book is dedicated to Heinz Schaller who dedicated most of his life as a scientist to the understanding of the interaction between hepatitis B viruses and their hosts.

Heinz is a personality who managed a difficult thing to do: he successfully crossed barriers. He worked as an enthusiastic scientist crossing classical barriers between chemistry, biology and medicine. He guided scientists and students and helped to form new ways of thinking and organizing academic research in Germany. A remarkable number of his fellows made outstanding carriers in different scientific areas.

Furthermore, he has in significant ways enhanced our understanding of the pathogenesis of hepatitis B virus infections, has contributed to the development of the current vaccine, has improved the care of patients infected with the virus, and has trained some of the most distinguished members of the current generation of hepatologists and virologists.

But he also crossed borders by searching for applications of his research. He is one of the few who, in addition to a successful academic career, have set the stage for a commercial success story – Heinz was a co-founder of one of the first companies dedicated to biotechnology: Biogen Idec. Thanks to his major input and intervention, the Center for Molecular Biology Heidelberg (ZMBH) as a biomedical research center with world-wide reputation was founded. Last not least, Heinz has used this success for a sustained support of biomedical research in academics with the "Chica and Heinz Schaller Stiftung".

Heinz's work has been catalytic for our understanding of hepatitis B – one of the most prevalent forms of chronic hepatitis. Crossing borders – are there that many?

Here, we would like to take the opportunity to thank Heinz for his dedication to science and wish him all the best for the years to come.

January 2008

Olaf Weber
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Comparative Hepatitis
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Hepatitis in the clinics – Treatment options

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Abstract

Hepatitis is an inflammation of the liver based on different aetiologies. Clinicians distinguish acute from chronic hepatitis. Pathophysiological changes lead to damage and hepatocellular degeneration. The causes for hepatocellular injury are heterogeneous, such as viruses, toxins, drugs, autoimmunity, cryptogenic. The latest official classification of chronic hepatitis by the International Association the Study of the Liver (IASL) [1] is still valid and is based on:

1. Aetiology
2. Inflammatory activity (Grading)
3. Fibrosis stage (Staging)

This classification has become very relevant since we nowadays not only diagnose the different liver diseases according to their aetiology but also have developed specific treatments that are targeting specific aetiologies of chronic liver disease. Overlap syndromes with primary biliary cirrhosis and primary sclerosing cholangitis and genetic liver diseases add to the clinical spectrum of this syndrome.

In this chapter we will describe the different causes of hepatitis, their treatment options and differential diagnosis.

Introduction

Hepatitis is an inflammation of the liver due to various reasons. Pathophysiological changes lead to hepatocellular damage. Causes of this hepatocellular degeneration are heterogenous such as toxins, drugs, viruses and autoimmunity. The pathologic changes due to different aetiologies share similarities; these include lobular disarray, inflammation involving portal tracts and lobules and hepatocellular degeneration in the form of ballooning and apoptosis. Microorganisms such as bacteria, viruses, fungi or parasites induce the secretion of biologically active molecules by macrophages after penetrating the epithelial surfaces of the body. Activated macrophages secrete cytokines which are defined as proteins released

Table 1. Classification of chronic hepatitis on the basis of pathogenesis [1]

Hepatitis type	HBsAg	HBV-DNA	HDV antibody (HDV-RNA)	HCV antibody (HCV-RNA)	Autoantibodies
B	+	+/–	–	–	–
D	+	–	+	–	~10% anti-LKM-3
C	–	–	–	+	~2% anti-LKM-1
Autoimmune					
type 1	–	–	–	–	ANA
type 2	–	–	–	–	LKM-1
type 3	–	–	–	–	SLA/LP
Drug-induced	–	–	–	–	Some: ANA, LKM; LM
Cryptogenic	–	–	–	–	–

SLA, soluble liver antigen antibody; LP, liver-pancreas antigen antibody; LM, liver cell membrane antibody

by cells that affect their behaviour, or other cells that bear receptors for them. The cytokines and chemokines initiate the process known as inflammation. Later inflammatory responses also involve lymphocytes, which in the meanwhile have been activated by microbial antigens. Infection with microorganisms, physical or chemical injury leads to cell disintegration or necrosis which is associated with the release of proteins and enzymes from the injured cells. These pathophysiologic changes may not only result in an increase of transaminases, but also in liver dysfunction, e.g., impaired bilirubin metabolism or coagulation factor synthesis. Chronic hepatitis is a progressive disease of heterogeneous multifactorial aetiology defined as continuous inflammation of the liver without improvement for six months or longer [1] (Tabs 1 and 2).

Infection

The most common causes for acute and chronic hepatitis are viral infections. Five viruses have been identified that can primarily manifest clinically as acute hepatitis: hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV) and hepatitis E virus (HEV). While HAV and HEV are transmitted by the faecal-oral route and are often associated with acute icteric hepatitis, they do not lead to chronic infection. By contrast, HCV, HBV and HDV are transmitted parenterally and sexually. They are the most common cause of human viral infections leading to chronic hepatitis [2]. HBV, HCV and HDV infections can lead to viral persistence that may lead to chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC).

Table 2. Causes of hepatitis

1. Infection	Hepatitis viruses	Hepatitis A Hepatitis B Hepatitis C Hepatitis D Hepatitis E
	Primary non-hepatotropic virus infections	Epstein-Barr-virus Cytomegalovirus Herpes simplex virus
2. Autoimmune hepatitis		
3. Differential diagnosis	Alcohol induced liver disease Drug-induced liver disease Chemical intoxication Primary biliary cirrhosis Primary sclerosing cholangitis	
	Metabolic liver disease	Non-alcoholic steatohepatitis (NASH)
	Genetic liver diseases	Haemochromatosis Alpha1-antitrypsin deficiency Wilson disease

Hepatitis A

Hepatitis A is considered the most common cause of acute viral hepatitis with seroprevalences from less than 30% in western European countries and up to 90% in developing countries [3, 4]. The major form of transmission is person to person *via* the faecal-oral route. The incubation period is approximately 15–45 days [4]. The onset of symptoms is abrupt with prodromal symptoms such as fatigue, weakness, nausea, abdominal pain and vomiting [5]. Diarrhoea is not typical for adults but is in the case of children with acute hepatitis A [5]. A fulminant course of hepatitis A is a rare event in young patients; however, mortality due to a fulminant course increases with age. Approximately only 4% of fulminant hepatitis cases are due to acute hepatitis A in the Western world [6]. Supportive measures are the only treatment for acute HAV infection. Prevention of HAV infection requires maintenance of high hygienic standards and strategies for active immunisation. Chronic hepatitis has not been reported to develop after hepatitis A infection.

Hepatitis B

Acute hepatitis B varies from an asymptomatic infection to cholestatic hepatitis and acute liver failure. There is no established treatment for acute HBV infection. Severe acute hepatitis B is now being treated with lamivudine within a German national multicentre study (GAHAB) sponsored by BMBF, DFG and Hep-Net, our National Network of excellence on viral

hepatitis. Acute hepatitis B resolves in most symptomatic patients. Rarely, however, the disease takes a fulminant course with a mortality rate of 65% [7, 8]. The best way to avoid a HBV infection is active immunisation with a recombinant hepatitis B vaccine. Usually the acute hepatitis B is self-limiting, but in some cases, particularly in patients with an asymptomatic course of disease, hepatitis B develops a chronic course. This occurs in 5–10% of adults with an intact immune system but in more that 90% of adult patients with immunodeficiency states such as HIV coinfection, post organ transplantation and following antitumour chemotherapy as well as neonates with a still underdeveloped immune system.

Patients with a chronic HBV infection and liver cirrhosis should receive antiviral therapy if there is viremia independent of the level of viral replication. In patients without liver cirrhosis viremia, inflammation, fibrosis score and the level of transaminases should be taken into consideration when considering treatment. The new German guidelines for the diagnosis and treatment of hepatitis B [9] are a joined effort of Hep-Net, the German national excellence network on viral hepatitis, the German societies for Gastroenterology (DGVS), Pathology (DGP), Virology (GfV) and Pediatric Gastroenterology and Nutrition (GPGE). The treatment goal in hepatitis B is to decrease HBV replication in order to decrease liver inflammation, thereby preventing the progression of fibrosis and the development of cirrhosis and its complications, including HCC [10]. Currently, Interferon-alpha, nucleotides and nucleosides are available for antiviral therapy of chronic HBV patients. In contrast to the oral nucleoside analogues, interferon has direct immunomodulatory properties and treatment response is sustained in a significant proportion of patients. Pegylated interferon alpha 2a has been found to be more effective than conventional interferon in the treatment of HBV infection in a Phase II study [11]. A number of factors are predictive in the response to Interferon-alpha, e.g., low serum HBV DNA levels, high serum alanine aminotransferase (ALT) levels and certain HBV-genotypes, in particular genotype A.

Nucleoside and nucleotide analogues have a good safety record and show less adverse effects than interferon. However, they often lead to drug resistance and viral rebound after the termination of treatment is usual. Therefore, knowledge of the pros and cons of the various treatment options nowadays available is important to allow the best choice of treatment for the individual patient based on an individual judgement of the appropriate benefit risk ratio [9] (Tab. 3).

Hepatitis C

The rate of chronicity in patients with acute hepatitis C is 50–90%. Patients with asymptomatic acute hepatitis C are more likely to develop chronic hepatitis. Therefore, the main aim of optimal patient management in acute

Table 3. Efficacy of oral antiviral agents in naïve hepatitis B patients ADDIN [9]

		Nucleoside analogues			Nucleotide analogues
		Lamivudine (Zeffix®)	Telbivudine (Sebivo®)	Entecavir (Baraclude®)	Adefovir (Hepsera®)
Dose (once a day)		100 mg	600 mg	0.5 mg (1.0 mg in patients with Lamivudine resistance)	10 mg
HBeAg-positive patients week 48/52	HBV-DNA <300 cop/ml	36%	60%	67%	21% <400 cop./ml
	HBeAg Seroconversion	18%	23%	21%	12%
	ALT normalisation*	60%	77%	68%	48%
HBeAg-negative patients week 48/52	HBV-DNA <300 cop/ml	72%	88%	90%	51% <400 cop./ml
	ALT normalisation*	71%	74%	78%	72%
Resistence development (virological breakthrough)	Week 48/52	10–32%	3–5%	<0,5%**	0%
	Week 96/104	22–42%	9–22%	<0,5%**	3–20%
	3 years	–53%		<0,5%**	11%
	4 years	–70%			18%
	5 years				29%

*The biochemical response was variable defined in different studies (normalisation of transaminases or ALT decrease <1,25 (Entecavir) or 1,3 (Telbivudine) upper limit of normal
**In lamivudine resistant patients virological breakthrough developed in 7% of patients after 1 year, in 16% after 2 years and 27% after 3 years treated with Entecavir. Treatment was stopped in patients with a viral replication $>7\times10^5$ copies/ml after treatment week 48 (about 5% of patients)

hepatitis C patients lies in avoiding chronicity, and subsequently in preventing cirrhosis associated complications. Fulminant acute hepatitis C is almost unknown in the Western world. Fortunately, it has been shown that the initiation of interferon monotherapy during the acute phase of hepatitis C virus infection significantly reduces the evolution of chronic hepatitis C [12]. Immediate treatment of acute HCV infection within 2–3 months after infection led to 98% of sustained virological response rates between 84–89% using PEG-interferon-alpha-2b for 6 months in subsequent trials [13]. At present a nationwide Hep-Net study compares immediate antiviral treatment of PEG-interferon alpha 2b monotherapy with a delayed start of treatment after 12 weeks with combination of PEG-interferon alpha 2b plus weight based dosing of ribavirin. Chronicity is associated with the risk of developing liver cirrhosis and subsequently to develop hepatic decompensation and hepatocellular carcinoma.

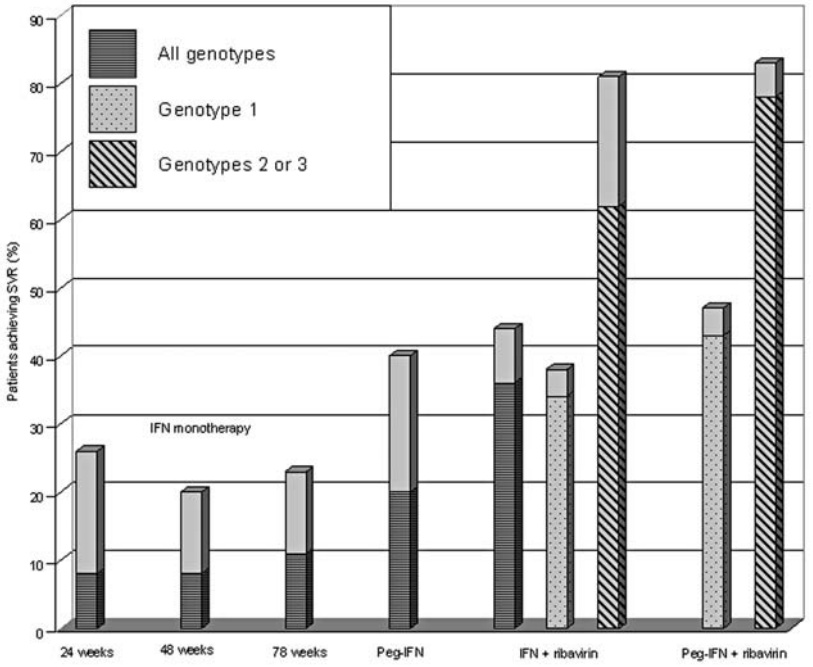

Figure 1. Development of antiviral treatment in chronic hepatitis C in the recent 20 years. Modified according to Manns et al. [18]

Worldwide more than 150 million people are considered to be chronically infected with the hepatitis C (HCV) virus. Combination therapy of chronic hepatitis C with pegylated interferon-alpha plus ribavirin is still the standard of care since 2001 [14]. However, this treatment is only successful in approximately half of the patients chronically infected with hepatitis C, genotype 1. This therapy can be associated with significant side effects and costs [15]. The standard of care for the treatment of chronic hepatitis C patients PEG-interferon alpha plus ribavirin has been optimised over the recent years after 2001, which led to further improvements in response with sustained virological response rates (SVR) up to 60% for the difficult to treat genotypes 1, and more that 80% for the so-called easy to treat genotype 2 and 3 patients [14, 16–18] (Fig. 1). Both interferon and ribavirin induce side effects that have to be considered in the management of patients with chronic hepatitis C. The interferon related side effects can be divided into interferon induced bone marrow depression, flu-like symptoms, neuropsychiatric disorders and autoimmune syndromes. The main problem of ribavirin is haemolytic anaemia. Overall, side effects result in 10–20% premature withdrawals from therapy and an additional 20–30% of patients

require dose modifications [19]. The big unmet need in the treatment of chronic hepatitis C is the therapy of non-responders to previous PEG-interferon and ribavirin treatment. Here only the new small direct antiviral drugs, the so-called STAT-C drugs, will lead to further improvements [18].

Hepatitis D

The hepatitis delta virus (HDV) is an RNA viroid dependent for infection on obligatory helper functions provided by the HBV; it therefore can only infect individuals with simultaneous HBV infection. Acute infection with HDV can be simultaneous with acute HBV infection (coinfection) or can occur in patients chronically infected with HBV as a super-infection. While super-infection usually is associated with chronicity and a higher rate of cirrhosis development compared to monoinfected patients [20–22], acute HBV/HDV coinfection usually leads to spontaneous clearance and chronicity is less than 10%. A recent study has shown that PEG-interferon-alpha-2a displays a significant antiviral efficacy in more than 40% of HDV/HBV coinfected patients alone or in combination with adefovir. Adefovir does not alter HDV-replication, but may be considered for patients with high HBV-levels. Combination therapy of PEG-interferon-alpha-2a plus adefovir has no advantages for HDV-RNA reduction, but is superior in reduction of quantitative HbsAg levels [23]. HBsAg loss was only seen in the group of patients treated with a combination of PEG-interferon alpha 2a plus adefovir.

Hepatitis E

Hepatitis E is transmitted by the faecal-oral route. The symptoms are unspecific and similar to hepatitis A, i.e., flu-like symptoms, including fever, abdominal pain, anorexia, nausea, diarrhoea and vomiting [24, 25]. Like other forms of acute hepatitis, the mainstay of therapy is in monitoring the complications and in treating the symptoms. The HEV infection is usually self-limited. However, fulminant hepatitis may occur. Acute hepatitis E is almost absent in the developed world but is the most prevalent cause of acute hepatic in some parts of the developing world like Kashmir, parts of India, Taschkent and at the horn of Africa like parts of Ethiopia. So far chronic hepatitis has not been reported to develop after hepatitis E infection.

Primary non-hepatotropic virus infections

Several other viral infections may damage the liver, but are not primarily regarded as hepatotropic. These infections include Ebstein Barr virus, cytomegalovirus and herpes simplex virus (Tab. 1).

Autoimmunity hepatitis

Autoimmune hepatitis (AIH) is another entity of chronic hepatitis. AIH is characterised by a loss of tolerance against hepatic tissue which leads to a chronic, mainly periportal hepatitis and destruction of hepatic parenchyma. AIH may also start as acute hepatitis and may even manifest as fulminant hepatitis. The estimated prevalence in Northern Europe is 170 cases per million [26]. There is no single sensitive and specific diagnostic marker of AIH. Diagnosis is made from a combination of clinical, biochemical, serological and histological features [27]. AIH is most prevalent in females and is often found clustered in families. Serologic detection of autoantibodies is one of the distinguishing features [28] that has lead to the sub classification of AIH in three groups. AIH type 1 is characterised by the presence of antinuclear antibodies (ANA) and/or anti-smooth muscle antibodies (SMA) directed predominantly against smooth muscle actin. AIH type 2 is characterised by anti-liver-kidney microsomal autoantibodies (LKM-1) directed against cytochrome P450 (CYP) 2D6 and with lower frequency against UDP-glucuronosyltransferases (UGT) [29, 30]. AIH type 3 is characterised by autoantibodies against soluble liver antigens (SLA/LP) [31, 32]. Seropositivity in fluorescence tests is considered at titres greater than 1:80 [33].

The clinical presentation ranges from a spectrum of benign asymptomatic disease to fulminant hepatitis [1, 34]. In half of the patients the onset is insidious and half show features of chronic liver disease. AIH is therefore characterised by non-specific features such as right upper abdominal pain, fatigue, arthralgias, myalgia, jaundice and pruritus. About 25% of patients show an acute onset of AIH and rare cases of fulminant progression of AIH leading to acute liver failure have also been reported [34]. Next to the autoantibodies, the diagnosis of AIH is established by the exclusion of other aetiologies of chronic hepatitis.

The liver histology in AIH is characterised by the presence of interface hepatitis in which several mononuclear cells infiltrate the portal tract, invade the adjacent liver parenchyma and surround dying hepatocytes [1]. Studies have shown that untreated AIH had a very poor prognosis with 5–10 year survival rates of 50 and 10% and furthermore demonstrated that immunosuppressive treatment significantly improved survival [35, 36]. The goal of treatment is a complete biochemical and histological resolution of inflammation as well as clinical remission of symptoms. The standard treatment of AIH is either prednisolone monotherapy or combination therapy with prednisolone and azathioprine. Combination therapy is preferred on the whole, because it allows the reduction of prednisolone and thereby the reduction of steroid-associated side effects.

Overlap syndromes of AIH and PBC and of AIH and PSC both appear to be present in about 8% of AIH patients [37, 38]. Overlap syndromes are conditions in which patients have clinical, histological and also immunologi-

cal features of both diseases – AIH and primary biliary cirrhosis (PBC) or AIH and primary sclerosing cholangitis (PSC).

Differential diagnosis

Alcohol induced liver disease

Alcohol induced liver disease may be distinguished into fatty liver (steatosis), alcoholic steatohepatitis (ASH), and alcohol induced liver cirrhosis. Alcoholic steatohepatitis, which is mainly seen in middle-aged women, may present clinically as a severe subacute disease with an unfavourable prognosis, no scientifically proven treatment options, high mortality and disappointing results so far after liver transplantation. In 2001, liver cirrhosis was the 12th leading cause of death in the USA, and 44% of the cases were alcohol-related [39]. It is assumed that a certain threshold of the amount of daily consumed alcohol and the length of time has to be exceeded for development of alcoholic liver disease. This has been proposed as being a daily intake of alcohol over 10–12 years of doses in excess of 40–80 g/day for males and 20–40 g/day for females [40]. Studies have been performed in order to explain the biochemical and histological features of acute alcoholic liver disease. It has been assumed that the metabolism of alcohol may result in the generation of fatty liver, oxidative stress and acetaldehyde, all of which are thought to be important in disease pathogenesis. In some, if not the majority of patients immune mechanisms seem to contribute to pathogenesis. Liver fibrosis and ultimately cirrhosis is the final common pathway of alcoholic liver disease. Cirrhosis itself is a precancerous condition. Chronic alcohol abuse produces a wide range of morphological changes in the liver, the most frequent being fatty liver (steatosis), alcoholic hepatitis and cirrhosis [41].

Patients with cirrhosis may develop ascites, splenomegaly, icterus, pruritus, hepato encephalopathy or even hepatorenal syndrome. Cutaneous manifestations of alcohol liver disease include Dupytren's contracture, alcohol causes flushing, teleangiectases and palmar erythema. Also peripheral neuropathy and cerebellar ataxia can be associated with alcoholic liver disease. Patients may also show signs of feminisation with hypogonadism and gynecomastia. The outcome of patients with cirrhosis is dictated by the development of complications of portal hypertension such as variceal bleeding and ascites, the onset of hepatic encephalopathy and hepatocellular carcinoma.

Pharmacotherapy and transjugular intrahepatic portosystemic shunts (TIPS) are important in the prevention of variceal bleeding. Non-selective beta-blockers reduce portal pressure through a reduction in portal and collateral blood flow. Propanolol is the most common non-selective beta-blocker most widely used in the pharmacological treatment of portal

hypertension. Studies showed that with the use of β-blockers, there was a significant reduction in the risk of bleeding of about 40% compared to untreated controls or patients receiving a placebo [42]. There are several contraindications in the use of β-blockers including bradycardia, hypotension and severe asthma bronchiale.

Patients with high risk varices and contraindications to β-blockers may be considered for oesophageal band ligation. Oesophageal band ligation has replaced sclerotherapy as the preferred form of management of oesophageal varices today.

Treatment options for the management of acute bleeding oesophageal varices are, for example, octreotide/somatostatin, vasopressin/nitrates, terlipressin, endoscopic therapy-sclerotherapy/banding, ballon tamponade, TIPS and surgical procedures like porto-caval shunting [43].

The development of ascites is a major complication of cirrhosis and is associated with a decreased quality of life and an increased risk for infections and renal failure. Among patients with compensated liver cirrhosis nearly 50–60% of patients will develop ascites in the natural course of their disease [44]. Therapies for ascites include sodium restriction, diuretics, large volume paracentesis, the transjugular intrahepatic portosystemic shunt (TIPS) and the peritoneovenous shunt, the latter being only rarely applied these days. Spontaneous bacterial peritonitis is a complication related to ascites with a high mortality rate. Immediate diagnosis and early therapy with antibiotics is essential.

Hepatic encephalopathy can be defined as a spectrum of neuropsychiatric abnormalities occurring in patients with advanced liver disease. Patients often present changes in behaviour including somnolence and confusion. Therapy of hepatic encephalopathy is based on prevention of production and resorption of ammonia.

All the described complications of liver cirrhosis are common to all forms of liver cirrhosis independent of aetiology; therefore they are also consequences of chronic hepatitis virus infections.

Furthermore increased alcohol consumption is often an important cofactor of morbidity in other causes of chronic liver disease, in particular chronic hepatitis C and its consequences.

Drug-induced liver disease

Hepatotoxicity is the most frequent cause of drug-induced mortality. Toxicity may be mainly dose dependent and predictable. Many environmental hepatotoxins, including halogenated molecules and toxic mushrooms can lead to liver failure. Except in the case of acetaminophen (paracetamol), which induces intrinsic hepatotoxcity, most cases of drug-induced liver disease are associated with suicide or incorrect drug administration. Drugs associated with hepatotoxicity are for example anticonvulsants, neurologic

agents, antimicrobial agents, antineoplastic and immunosuppressive agents. The prognosis is generally good if the drug is stopped at the first symptom or sign of liver disease.

As mentioned previously, acetaminophen induces intrinsic hepatotoxicity. The clinical presentation of acute liver failure due to acetaminophen includes a hyperacute progression from jaundice to hepatic encephalopathy [45]. Hepatic transaminases rise within 12–24 h, where as, peak transaminases and prothrombin times usually observed 3 days after investigation. However prognosis of acetaminophen induced fulminant hepatic failure has a more favourable outcome compared to other causes of drug induced liver disease.

In chronic liver disease of unknown aetiology, so-called cryptogenic liver disease, drug induced liver injury has always to be considered as a cause. Diagnosis often relies on the termination of drug administration and subsequent follow up. Only after careful evaluation of the benefit risk ratio a re-exposure with the suspected drug may be used to prove or disprove the diagnosis of a drug induced liver disease. In a minority of cases with chronic liver disease and a drug-induced aetiology the disease is immune mediated. Then, specific autoantibodies may be detectable and may indicate aetiology and pathogenetic mechanisms.

Chemical intoxication

It is difficult to make a definitive statement about hepatotoxicity of specific chemicals and environmental agents because the effects may be extremely diverse. Hepatotoxic chemicals are, for example, halogenated aromatic hydrocarbons, nitroaromatic compounds, chlorinated ethylenes and pesticides. Also metals, like arsenic, beryllium and copper can lead to chronic liver disease. Although chemicals are often accused of causing chronic liver disease it is often very difficult to prove the aetiology in the individual patient.

Primary biliary cirrhosis

Primary biliary cirrhosis (PBC) is a chronic progressive cholestatic disease, characterised by a granulomatous destruction of the intrahepatic bile ducts, which is associated with the development of portal and periportal inflammation, subsequent fibrosis and can lead to cirrhosis. PBC is primarily a disease of middle-aged women, with most cases occurring between ages 40–60 years [46, 47]. Prevalence estimates range from 19 to 240 cases per million of the population [48, 49]. Antimitochondrial antibodies (AMA) are present in 90–95% of cases of PBC, often before clinical signs and symptoms appear and are therefore important in the diagnosis. Among patients with positive

AMA, some patients may not have clinical symptoms and biochemical abnormalities; in such cases a liver biopsy is recommended.

Symptomatic patients describe pain in the right upper abdominal quadrant, nausea and anorexia. Other symptoms attributed to PBC including fatigue, pruritus and keratoconjunctivitis xerostomia. Jaundice is usually a late symptom. The most characteristic biochemically abnormal marker in PBC is an elevated serum alkaline phosphatase, which is usually measured three to four times the upper limit of normal [50]. Serum total bilirubin levels often rise during disease progression but are commonly within normal limits at diagnosis.

As the pathogenesis of PBC is unclear, it is difficult to treat the disease. Only ursodexoxycholic acid (UDCA) has been shown to be effective in patients with PBC (US Food and Drug Administration approval 1998). There is some evidence that other drugs like cyclosporine and azathioprine are effective and prednisolone or budesonide may also have a beneficial effect. There is an increased risk of osteopenia in patients with PBC, associated with female gender and cholestasis, therefore calcium and vitamin D should be given to all patients with PBC.

The natural history of patients with PBC is heterogeneous. However, liver transplantation often remains the only effective therapy for those patients with end-stage liver disease from PBC.

Primary sclerosing cholangitis

Primary sclerosing cholangitis (PSC) also is a chronic cholestactic liver disease. PSC is characterised by concentric obliterative fibrosis of intrahepatic and extrahepatic biliary tree, leading to its destruction and to biliary cirrhosis [51, 52]. The aetiology of PSC is unclear, although growing evidence supports that several immune and none immune mediated mechanism, as well as genetic predisposition, play a significant role in disease aetiopathogenesis. There is a strong association between PSC and inflammatory bowel disease with between 75–80% PSC patients of northern European origin having underlying inflammatory bowel disease [53, 54], especially ulcerative colitis. The true prevalence of PSC is unknown. Almost half of all PSC patients are asymptomatic at the time of diagnosis. The asymptomatic individual has incidental discovery of mildly abnormal liver blood tests. The presence of symptoms does not correlate well with disease severity. Fatigue and pruritus are the most common symptoms. Jaundice, abdominal pain and weight loss are usually present in the later stages of the disease. Fever and chills may be manifestations of ascending bacterial cholangitis.

Endoscopic retrograde cholangiopancreatography remains the gold standard to diagnose large duct PSC. The finding of multifocal strictures and dilatations with beaded appearance and occasional diverticular formation

involving the intrahepatic and/or extrahepatic biliary trees, characterises the disease [51]. The classic laboratory finding in patients with PSC is an increased alkaline phosphatise level. Most patients also have raised aminotransferases.

The clinical management of PSC patients should focus on alleviating symptoms and complications of end-stage liver disease. Several treatment options have been studied for PSC, including pharmacological, endoscopic and surgical modalities. Since PSC patients are at a high risk of developing cholangiocellular carcinoma and other malignant tumours mainly of the GI tract, they should be regularly screened. In most cases, liver transplantation remains the only therapy that improves survival in these patients.

In particular in children an overlap syndrome between PSC and autoimmune hepatitis is common. Biochemical and histological characteristics of both diseases determine diagnosis and therapeutic approach.

Metabolic liver disease: Non-alcoholic steatohepatitis (NASH)

Non-alcoholic steatohepatitis is defined as a constellation of histological abnormalities identified on liver biopsies that are similar to those seen in alcoholic liver disease but these histological abnormalities are found in patients who consume little or even no alcohol. The prevalence of NASH has increased over the last years in parallel with the increasing prevalence of obesity and type 2 diabetes. The most common underlying factor for NASH is the presence of insulin resistance [55–57]. Obesity and a sedentary lifestyle are the two largest risk factors for insulin resistance [58]. NASH is most commonly asymptomatic. Some patients have symptoms like right upper abdominal pain, fatigue and malaise. Liver biopsy is the gold standard for diagnosis. NASH is typically characterised by serum ALT that exceeds the AST by up to two- or three-fold [59].

There is no standard treatment for NASH. General recommendations include improving metabolic risk factors. The goal is weight loss, improving insulin sensitivity and treating hypertriglyceridemia. Antioxidants and ursodeoxycholic acid have only little benefit. The complications that can occur in NASH are liver cirrhosis, acute liver failure and hepatocellular carcinoma. At present, metformin and other antidiabetic drugs are being evaluated.

Genetic liver diseases

Haemochromatosis

Haemochromatosis is a multiorgan disease of excessive iron deposition. The principle organs affected include liver, heart, pancreas and skin.

Haemochromatosis is one of the most common disorders in northern Europe. The highest frequency occurs in Ireland and in the Basque region [60]. The main but not the only gene associated with haemochromatosis was labelled HFE [61]. The HFE gene has two common mutations, C282Y and H63D. About 90% of typical haemochromatosis patients in many regions of the world are homozygous for the C282Y mutation of the HFE gene. Disease manifestation occurs through increased intestinal iron absorption. There is no mechanism for the intestine to increase iron excretion; this leads to a net increase in the iron balance, resulting in progressive accumulation in multiple organs.

In the early stages of the disease, patients usually show no symptoms. With progress of iron accumulation, organ dysfunctions may result. In the later stage of the disease many symptoms of haemochromatosis are secondary to the underlying liver dysfunction, including spider naevi, palmar erythema, fatigue, weight loss and right upper abdominal pain. The most common non liver-related symptoms of haemochromatosis are diabetes, cardiac disease, arthropathy, endocrine abnormalities and pigmentation of the skin. Glucose intolerance has been found in 85% of haemochromatosis patients with liver cirrhosis [62]. Iron accumulation in the pancreas may result in pancreatic endocrine dysfunction and therefore in adult onset diabetes mellitus. Cardiac disease in haemochromatosis includes both cardiomyopathy and arrhythmias. Echocardiography is the preferred initial diagnostic test and abnormalities can be seen in up to 35% of referred cases [62]. The characteristic pigmentation of haemochromatosis is a bronze or grey colour of the skin.

The initial laboratory testing includes serum iron, total iron binding capacity and ferritin. Elevated transferring saturation above 45% and elevated serum ferritin are suggestive of iron overload. Genetic testing of the C282Y Mutation often leads to the exact diagnose of haemochromatosis.

Haemochromatosis can be treated successfully with phlebotomy therapy. The venesection of 400–500 ml is equivalent to approximately 200–250 mg iron. Maintenance venesection after iron depletion of 3–4 venesections per year are done in most patients, although the rate of iron reaccumulation is highly variable [63]. A reasonable target for the transferring saturation is below 25%.

Alpha1-antitrypsin deficiency

Alpha1-antitrypsin is produced primarily by hepatocytes, but also by alveolar macrophages and intestinal cells [64]. The disease is associated with pulmonary emphysema, chronic liver disease and hepatocellular carcinoma; although only about 10% of homozygotes develop a clinically significant liver disease. The pathogenesis of liver disease in patients with alpha1-anti-

trypsin deficiency is less clear. It has been suggested that conformational changes of the unstable alpha1-antitrypsin variants lead to retention of polymers of abnormally folded alpha1-antitrypsin in the hepatocyte endoplasmatic reticulum [65, 66]. Alpha1-antitrypsin deficiency often presents in the neonatal period with jaundice, pale stool, dark urine and elevated liver enzymes. However, the liver disease can remain clinically silent for many years although abnormal biochemical liver enzymes and signs of liver cirrhosis may appear.

There is no specific therapy for alpha1-antitrypsin deficiency-associated liver disease. The only treatment option for patients with progressive liver disease is a liver transplantation. Additionally it is recommended to avoid other risk factors such as passive and active smoking and heavy drinking. Since there is no specific treatment option the goal here is to give these patients the standard medical support and to manage the complications of chronic liver disease.

Wilson disease

Wilson disease is an autosomal recessive disorder that leads to a copper overload in the liver and other organs. The underlying gene has been identified and more than 40 single point mutations of this gene have been identified [43]. Therefore, unlike in haemochromatosis, a single genetic test is not available on a routine basis for Wilson disease. Wilson disease is found worldwide, but most commonly in northern, central and eastern European countries [67, 68]. The clinical presentation can vary. Wilson disease may present as chronic or fulminant liver disease, as a progressive neurological disorder without evident hepatic dysfunction, or as a rather nondescript psychiatric illness or acute episodes of haemolysis. The hallmark of Wilson disease is the Kayser-Fleischer ring, which is presented in 95% of patients with neurological symptoms and somewhat over a half without. Kayser-Fleischer rings are not specific for Wilson disease. They may be found in patients with other types of chronic liver disease.

Medical treatment for Wilson disease involves either chelation or induction of metallothionins. There are two generally accepted oral chelating agents: penicillamine and trientine. Zinc interferes with copper absorption, firstly by competing for a common carrier absorption and secondly inducing metallothionin in enterocytes, allowing copper absorbed into them to be excreted by desquamation [69]. Untreated Wilsons disease is universally fatal, with most patients dying from liver disease and a minority from progressive neurologic disease. However, in most cases liver transplantation often remains the only effective therapy for patients with end-stage liver disease, the genetic defect is cured by liver transplantation.

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Comparative Hepatitis
ed. by Olaf Weber and Ulrike Protzer

Differential diagnosis of human hepatitis

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Abstract

There is a variety of differential diagnoses of acute and chronic human hepatitis regularly seen in clinical praxis. Risk factor assessment for specific entities provides important information and should guide individual diagnostic procedures. Liver screening tests often remain the first indicator for hepatic pathologies and should include quantification of liver enzymes, liver function parameters and cholestatic parameters. Nevertheless, virus serology should always be done during further laboratory evaluation. To estimate the parenchymal liver damage and to exclude biliary obstruction or hepatic lesions an abdominal ultrasound scan is essential. A liver biopsy may complete the diagnostic approach, if either the underlying hepatic pathology remains obscure or parenchymal liver damage needs to be defined.

Introduction

Human hepatitis may present as acute or chronic liver disease, which may cause mild or severe hepatic damage. Thus, the resulting clinical spectrum of human hepatitis ranges from asymptomatic courses to manifest disease. Further diagnostic evaluation is essential, as different hepatic and extrahepatic pathologies may mimic human hepatitis. Beside routine laboratory tests of liver enzymes, which are regularly the first indicator of hepatitis, further diagnostic assessment needs to orchestrate patient history, clinical findings and laboratory investigations. Liver histology and diagnostic imaging are important complementary diagnostic tools to identify the hepatic pathology. Subsequently the differential diagnosis gives first insights into the stage, prognosis and therapeutic options of newly diagnosed hepatitis.

Differentiation of viral hepatitis

Anamnestic findings

During differential diagnosis of human hepatitis a special focus should be set on hepatitis A, B and C, which are the most common causes of viral hepatitis worldwide. In Europe and Northern America patients with identified liver disease show a prevalence of hepatitis B between 0.9% and 1.0%, whereas a prevalence between 7.0% and 18.6% has been reported for hepatitis C. Beside this, a relevant proportion of patients with viral hepatitis also presents with hepatic co-morbidity, which complicates differential diagnosis [1–3].

Viral hepatitis is characterised by risk factors associated with viral infection. Thus, patient history may guide to the viral pathogen. Due to the faecal-oral transmission route of hepatitis A virus (HAV) individuals accommodated in collective housings are at higher risk of acquiring HAV infection. Further risk factors have been identified for clinical employees and sewage workers as well as for homosexual men and individuals from lower social background. Due to global migration and tourism, increasing numbers of patients from HAV endemic regions have to be considered [4]. Only a very few cases of faecal-oral infections with hepatitis E virus (HEV) have been reported in Europe and were usually connected with a previous stay in HEV-endemic areas including India, eastern Asia and Africa. An HEV seroprevalence of 20% in some remote areas of the United States also suggests autochthonous cases of hepatitis E in the northern hemisphere [5].

Hepatitis B virus (HBV) and hepatitis C virus (HCV) show parenteral transmission routes and are the predominant pathogens of viral hepatitis. Therefore clinical history should identify patients who have received blood transfusions or surgical interventions. Patients suffering from haemophilia or receiving chronic haemodialysis were identified as risk groups. These risk factors are of special relevance if they originate from the era without efficient hepatitis screening [6, 7]. Since viral screening of blood donors and blood products has been implemented for HBV and HCV, the estimated risk for transfusion-associated HCV and HBV infection has declined to 1/4,400,000 and 1/620,000 per transfused unit in Europe, respectively [8]. Remaining groups at risk for HBV and HCV are employees in the health care setting and intravenous drug addicts. Particularly drug addicts demonstrate a very high HCV prevalence of 48–90% [7, 9].

Unsafe sexual practices are the most important infectious route which is responsible for 30% to 40% of new HBV infections, excluding other risk factors [10]. Sexual transmission rate for HCV is much lower and has been suggested to range from 0% to 7% during long-term sexual partnerships, if no other risk behaviour has been documented [9]. In areas of high HBV prevalence perinatal transmission from HBV-infected mothers to newborns contributes to a large proportion of infections. Vertical HBV transmission

Table 1. Clinical characteristics of viral hepatitis

	Hepatitis A	Hepatitis B	Hepatitis C	Hepatitis D	Hepatitis E
Transmission	faecal-oral (percutaneous)	percutaneous sexual perinatal	percutaneous (sexual) (perinatal)	percutaneous (sexual)	faecal-oral (perinatal)
Incubation phase (days)	15–49	60-–180	14–160	21–45	15–60
Clinical illness	70–80% adults 5% infants	10–15%	5–10%	10% higher with super-infection	70–80% adults
Jaundice	30% adults <5% infants	10–15%	5–10%	unknown	common
Fulminant	<1%	<1%	rare	2–7.5%	<1%, up to 30% in pregnant women
Case fatality rate	0.1–2.7%	1–3%	1–2%	<1% coinfection >5% super-infection	0.5–4% 1.5–21% in pregnant women
Chronic hepatitis	none	5–10% adults 90% infants	80–90%	<5% coinfection 80% super-infection	none

from HBeAg-positive mothers to newborns occurs in 70–90%. The rate of vertical infection declines to 10–40% if the mother is HBeAg negative and reduces further to 5–10% following active and passive HBV immunisation of the newborn [11]. Like with HAV, infections with HBV and/or HCV have to be considered in patients originating from endemic areas. HDV infections only affect HBV-positive patients and may cause severe hepatitis during superinfection whereas co-infection with HDV does not relevantly alter the acute natural course of hepatitis B. Particularly in areas with high HBV prevalence HDV infections have to be considered.

Non-hepatotropic viral infections can cause atypical hepatitis. The predominant viral pathogens, responsible for atypical hepatitis originate from the *herpesviridae* family. Cytomegalovirus (CMV), Epstein-Barr virus (EBV), varicella zoster virus (VZV) and herpes simplex virus (HSV) are the most important pathogens. The course of atypical hepatitis depends on the host immune competency and ranges from mild to fulminant manifestations. Chronic active courses comparable to hepatitis B and C are not observed, but recurrent exacerbations of latent infections are possible and common for CMV. The seroprevalence of *herpesviridae* is high in the general population and reaches about 82% for CMV, 75% for EBV and 58/14% for HSV-1/2 respectively [12–14]. Thus *de novo* infection usually does not cause hepatitis in the adult population and reactivation of latent

infections is the most important pathomechanism. Specifically, reactivated CMV infections have to be considered in patients who are immunosuppressed, receiving high dose chemotherapy or suffering from other causes of immunodeficiency. Exceptional *de novo* infections which may manifest with hepatitis are congenital HSV infections or EBV infections in late childhood or early adolescence [15]. Exotic viral hepatitis is predominantly based on infections with *arboviridae*. Thus the virus is usually transmitted by insect vectors, which are endemic in tropical or subtropical regions. The most common viral entities are Yellow fever and Dengue fever [16, 17]. Also viral zoonoses, which are endemic in tropical and subtropical regions, contribute to hemorrhagic fever with hepatic involvement. Particularly patients with a recent history of foreign travel are at risk of developing exotic hepatitis and should be identified [15, 18].

Clinical findings

Acute manifestations

The clinical findings of acute human hepatitis originate from hepatocyte damage and impaired liver function, which could lead to vegetative symptoms, jaundice and encephalopathy. Conclusively, the clinical findings during human hepatitis are usually non-specific and only the chronological sequence of symptoms might indicate the responsible cause. Viral hepatitis is characterised by three different periods of acute infection. Before onset of symptoms patients undergo an incubation period, which lasts from weeks to months. The duration of the incubation period is highly variable, because it depends on the causative viral agent and on the immune competency of the infected person. Elevated liver enzymes are often the only pathological finding, which usually makes viral hepatitis an incidental diagnosis during its incubation period. The prodromal phase subsequently presents with clinical features of drowsiness, fatigue, flu-like symptoms and epigastric pain. These symptoms usually persist until onset of jaundice, which marks the beginning of the icteric phase. Jaundice usually resolves without residual hepatic damage, if the immune system is able to eliminate the viral pathogen. As jaundice does not develop in a relevant proportion of patients, manifestations of acute viral hepatitis are often misinterpreted as unspecific flu-like symptoms. Sometimes prolonged jaundice is observed without developing chronic infection. Some patients develop extrahepatic manifestations of viral hepatitis, which are based on immune-mediated mechanisms.

The rate of fulminant hepatitis depends on the natural course of the causative viral pathogen and is observed in less than 1% of hepatitis A, in 0.1% of hepatitis B and in rare cases of acute hepatitis C. Patients at increased risk of developing fulminant hepatitis are immunocompromised patients and patients with underlying liver disease or liver cirrhosis. Increased rates of ful-

minant hepatitis have also been observed in pregnant women with hepatitis E and during hepatitis D superinfections. The clinical picture of fulminant hepatitis is characterised by pronounced jaundice and may lead to liver failure and subsequent multiorgan failure. Hepatic encephalopathy, serum electrolyte derangement, cerebral oedema, hemorrhagic coagulopathy and instable glucose homeostasis may develop as severe complications. The resulting case fatality rate has been estimated to be below 0.1% for acute hepatitis A, about 0.1% for acute hepatitis C and 0.4% for acute hepatitis B [19].

Chronic manifestations of viral hepatitis

Symptoms of chronic hepatitis are non-specific and seen infrequently. They consist of fatigue, abdominal discomfort and depressive symptoms. Thus, the differential diagnose of chronic hepatitis may not be as reliant on clinical findings at initial stages. Liver-specific signs of chronic hepatitis are finally determined by progression to end stage liver disease resulting in impaired liver function and cirrhosis. Thus progressive impairment of liver function may gradually cause coagulopathy, jaundice and encephalopathy. Liver cirrhosis and resulting portal hypertension may eventually provoke ascites, peripheral oedema and gastrointestinal haemorrhage. Among these symptoms ascites is considered the landmark sign of decompensated cirrhosis and occurs with a frequency of 5–7% per year [20]. Signs of cirrhosis are often missing unless complications like spontaneous bacterial peritonitis, portal vein thrombosis and hepatocellular carcinoma have induced decompensation. As a result cirrhotic complications may present as acute or subacute hepatic pathology, as long as the underlying cirrhosis has not been recognised before. Sometimes the chronic course of viral hepatitis is complicated by acute exacerbations, which may lead to acute liver failure. Particularly chronic hepatitis B shows inflammatory flares with an associated mortality of 0.7% [21].

Extrahepatic manifestation of viral hepatitis

Differential diagnosis of human hepatitis can be based on extrahepatic manifestations, which regularly occur during acute and chronic liver damage. Extrahepatic manifestations depend on the causative pathology and affect different organ systems. Frequent extrahepatic manifestations during acute viral hepatitis are cutaneous rash, erythema and arthralgia. These extrahepatic symptoms occur in 11–14% during acute HAV infection and in 25% during acute HBV infection and in a less defined rate of acute HCV infections. Extrahepatic manifestations are even more common during chronic viral hepatitis and may specify the underlying viral pathogen. Hepatitis B causes immune complex-mediated vasculitis which may lead to neuropathy,

Table 2. Differential diagnoses of human hepatitis

	Natural course	Characteristics	Diagnostic tests
Metabolic liver disease			
Fatty liver disease	Chronic	Incidence increasing with age, m > w, BMI > 30 kg/m^2, increased waist circumference	Medical history, diabetes and alcohol screening, serum lipids, exclusion of other liver disease
Haemochromatosis	Chronic progressive	Pts. > 40y, m > w, hyperpigmentation, diabetes mellitus	Family history, ferritin > 500, transferrin staturation > 50%, HFE screening
α1 Antitrypsin deficiency	Chronic progressive	Pulmonary emphysema	Family history, α1-antitrypsin serum activity, genetic screening
Wilson disease	Chronic progressive, fulminant hepatic deterioration	Pts. < 40y, coombs negative haemolysis, Kaiser-Fleischer ring, neurological symptoms	Family history, serum copper, coeruloplasmin level and renal copper excretion
Cholestatic liver disease			
Malignant obstruction	Subacute progressive	Pts. > 50y, incidence increasing with age, weight loss, fever and night sweats	Diagnostic imaging, endoscopic screening if applicable and biopsy of identified lesions
Biliary concrements	Acute, recurrent	Incidence increasing with age, m < w, colic right upper quadrant pain	Diagnostic imaging and retrograde endoscopic cholangiography
Autoimmune liver disease			
Autoimmune hepatitis	Chronic, undulation of remission and increased activity	Pts. 10–20y, 50–60y, m < w, extrahepatic autoimmune disease	Medical history, exclusion of other liver disease, autoimmune serology (particularly ANA, SMA, LKM, SLA)
Primary sclerosing cholangitis	Chronic, recurrent flares of increased activity	Pts. 40–50y, m > w, recurrent cholangitis, inflammatory bowel disease, complicating cholangiocarcinoma	Diagnostic imaging and retrograde endoscopic cholangiography, autoimmune serology (particularly pANCA and ANA)
Primary biliary cirrhosis	Chronic, slowly progressive	Pts. > 25y, m < w extrahepatic autoimmune disease	Medical history, histology, exclusion of other liver disease, autoimmune serology (particularly AMA)
Toxic hepatitis			
Alcohol toxic	Chronic and acute depending on alcohol intake	Incidence increasing with age, m > w, alcohol intake outside meal time and > 30–50 g/day	Medical history, alcohol screening, exclusion of other liver disease
Other toxic agents	Chronic, acute and fulminant, depending on causative agent	New or chronic medication, intentional or accidental intoxication	Drug history, drug screening, exclusion of other liver disease

Table (continued)

	Natural course	Characteristics	Diagnostic tests
Ischaemic hepatitis			
	Chronic, acute and fulminant, depending on causative insult	Underlying disease with deterioration of hepatic or systemic circulation	Medical history, identification of cardio-vascular pathology, sepsis, extensive burns, trauma or hepatic malperfusion
Parasitic and bacterial hepatitis			
	Acute and chronic depending on underlying infection	Systemic manifestations depending on underlying pathogen	Travel anamnesis, immune status and specific microbial, histological and serological diagnostics

Pts., patients; y, years; m, male; w, female; BMI, body mass index; ANA, anti nuclear antibody; AMA, anti-mitochondrial antibody; pANCA, anti-perinuclear neutrophile cytoplasmatic antibody; SMA, smooth muscle antibody; LKM, liver kidney microsomal antibody; LKM, soluble liver antigen antibody

nephropathy or Raynaud-Syndrome. Specifically membranous glomerulonephritis and panarteritis nodosa often involving splanchnic arteries are highly suggestive for hepatitis B. In contrast, hepatitis C is clearly linked to mixed essential cryoglobulinemia which may also cause Raynaud-Syndrome, renal impairment and arthropathy. The association of chronic hepatitis C with lymphoproliferative diseases has not been defined as well but should be considered [22]. Moreover, viral hepatitis could provoke other manifestations, including neuritis, myelitis, Guillain-Barré syndrome [23], Sjogren's syndrome [24], cardiomyopathy [25] and lichen planus [26].

Differentiation of other causes of liver damage

Despite viral hepatitis being one of the leading causes of human hepatitis worldwide, other hepatic pathologies should be identified by clinical history. Differential diagnosis ranges from common toxic or ischaemic events, hereditary entities to autoimmune diseases, which manifest as acute or chronic liver disease. Prior to accurate diagnosis of a specific hepatic pathology, other defined disease entities must first be excluded.

Non-alcoholic steatohepatitis

Particularly hepatic steatosis and/or non-alcoholic steatohepatitis (NASH) have become an increasing medical burden in industrialised countries,

requiring diagnostic attention. Depending on the analysed cohort between 3% and 24% of the general population are affected by hepatic steatosis [27]. Hence, population-based surveys have also stated that hepatic steatosis or NASH might be responsible for 63–69% of patients with elevated liver enzymes [1, 2]. This group of patients is characterised by risk factors which could be identified by clinical history. Specifically obese patients (BMI >30 kg/m^2) have a high prevalence (75.8%) of steatosis hepatitis compared to non-obese individuals (BMI <25 kg/m^2), who show a prevalence of only 16.4% [3]. Enlarged waist circumference, ethnic background and diabetes mellitus are further risk factors of hepatic steatosis [28]. Different studies suggest that patients with steatosis may develop NASH in 10–25% or even end up with cirrhosis in 2–7% of all cases [29]. Differentiation from viral hepatitis is hampered by the fact that chronic viral inflammation induces hepatic steatosis and that steatosis promotes disease progression of chronic viral hepatitis.

Toxic hepatitis

Toxic hepatitis is a further important differential diagnosis, which is often linked to chronic alcohol consumption or hepatotoxic medication. The overall prevalence of alcohol-related liver disease is higher in male patients and increases with age. Alcoholic liver disease should be strongly suspected in patients with an alcohol consumption more than 40–80 g/day, which equals a daily indulgence of about 2.5 l beer, 1 l wine or 200 ml distilled spirits [30]. Specific drinking habits, like alcohol consumption outside mealtimes, should also be recognised as risk factors for alcoholic liver disease [31]. Compared to men who show a higher prevalence of alcoholic liver disease, female individuals consuming alcohol on a regular basis are at higher risk of developing alcohol-related liver disease [30]. Clear aetiological differentiation of liver damage during chronic alcohol consumption is hindered by a relevant proportion of patients with additional viral hepatitis and/or obesity [2]. To identify other causes of toxic hepatitis, clinical history should focus on medications which were started within 3–4 months prior to onset of liver damage. New medications are often responsible for hepatotoxicity. Even medications which were administered for more than 12 months have been identified as causative hepatotoxic agents. In western countries acetaminophen is the number one cause of acute liver failure leading to liver transplantation. As life-threatening amounts of hepatotoxic agents are regularly ingested during suicidal attempts, anamnesis should also focus on life crisis and depressive symptoms. Furthermore, drugs like amphetamines and cocaine have been associated with acute liver damage and should be screened. In Europe accidental ingestion of fungal toxin (death cap mushroom, *Amanita phalloides*) has to be considered during assessment of liver failure.

Ischaemic hepatitis

A further focus should be set on ischaemic hepatitis caused by severe hypotension, liver congestion and thrombo-embolic events. As ischaemic hepatitis usually affects critically ill patients, clinical history should recognise chronic heart failure, myocardial infarction, arrhythmias, extensive trauma and sepsis [32]. Chronic hepatic congestion due to cardiac insufficiency might even cause fibrotic remodelling and end-stage liver disease [33]. Finally, liver-specific vascular pathologies of the liver like Budd-Chiari syndrome and veno-occlusive disease contribute to acute and chronic hepatic damage as well [34, 35]. Ischaemic hepatitis usually does not present with prolonged sub-clinical periods and mimics the clinical picture of acute hepatitis. The interval between the initial ischaemic event and the clinical manifestation of hepatic damage only takes hours. Depending on the extent of ischaemic liver damage, liver failure and its complications may ensue. The symptoms of acute liver damage are essentially the same as for viral hepatitis stated before, though extrahepatic manifestations resemble the underlying pathology.

Hereditary liver disease

Hereditary liver diseases may also present with hepatitis type liver damage. Haemochromatosis is the most common hereditary disease of the caucasian population. Haemochromatosis should be suspected if male patients, usually older than 40 years, present with signs of liver damage. Iron overload also affects the endocrine pancreas and diabetes mellitus might be associated with haemochromatosis. Further indicators for haemochromatosis are atypical cardiac disease, unexplained arthropathy or male sexual dysfunction. For first degree relatives of patients suffering from haemochromatosis further diagnostic workup is also recommended [36]. Wilson's disease may mimic diverse hepatic entities, ranging from acute hepatic failure to cirrhosis. Although it is a rare entity, Wilson's disease should therefore be included in differential diagnosis of acute and chronic hepatitis. Particularly patients between 5 and 40 years of age should be screened for Wilson's disease. Patients highly suspicious for Wilson's disease are those with neurological findings in addition to hepatic pathology. The spectrum of neurological signs includes tremor, ataxia and seizures. Psychiatric symptoms including depression, neurosis or even frank psychosis have been observed. Finally other organ systems including the heart and kidney might be affected by Wilson's disease [37]. Alpha-1-antitrypsin (AT) deficiency, which affects about 1 in 2000–4000 newborns, should also be included in differential diagnosis. The clinical picture of AT deficiency is usually restricted to signs of hepatic and pulmonary disease. Hepatic manifestations might range from elevated liver enzymes and/or jaundice to liver cirrhosis. As the lifetime risk of liver

cirrhosis is about 25–50% in patients showing AT deficiency, patients with unexplained liver damage should undergo diagnostic screening for this entity [38].

Autoimmune disease

Autoimmune hepatitis (AIH) is the predominant autoimmune disease affecting the liver. In Europe AIH shows an incidence of 1.9 in 10,000 persons per year [39]. AIH shows a bipolar incidence during lifetime with a juvenile and an adult peak. Recent observations suggest that AIH may also be common in the elderly population. AIH may present as acute hepatic deterioration or as chronic liver damage and cirrhosis. An inconsistent course with hepatic flare-ups and periods of remission is regularly observed during AIH. The diagnosis of AIH is based on a defined clinical and serological scoring system that requires the strict exclusion of other hepatic pathologies, such as viral, toxic or cholestatic liver disease [40]. Particularly AIH should be excluded in every patient who also presents with other autoimmune diseases. As AIH is associated with genetic factors, like HLA DR3 and DR4, a positive family history might lead to AIH [41]. Chronic autoimmune pathologies affecting the bile ducts, like primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) also mimic chronic human hepatitis and regularly lead to liver cirrhosis and end stage liver disease. Whereas PBC is characterised by a gradual progress of cholestatic manifestations and liver damage, PSC is often complicated by recurring cholestatic episodes. Deterioration of cholestatic symptoms during PSC are usually caused by recurrent cholangitis. They may follow biliary interventions or are based on obstruction by *de novo* cholangiocarcinoma. PSC usually affects male patients and manifests itself between the 4th and 5th decade of life. About 75% of all PSC patients also suffer from inflammatory bowel disease, predominantly ulcerative colitis [42]. PBC primarily affects women and usually does not manifest in patients younger than 25 years. Before liver damage has caused visual sign of jaundice and cirrhosis, patients often report about chronic pruritus. Furthermore, autoimmune disease like Sjogren's syndrome and Sclerodermia are associated with PBC. Beside distinct PSC, PBC and AIH overlap syndromes between these three pathologies are possible [43].

Cholestasis and pathologies of bilirubin homeostasis

Beside parenchymal liver damage cholestatic diseases have to be considered in the differential diagnosis of human hepatitis. Mechanic biliary tract obstruction by gallstones and malignancies are the most common pathologies affecting the biliary tree. Both are associated with typical presentation,

examination findings and clinical history might already allow for differentiation. Thus malignancies should be strongly considered in patients complaining about anorexia, loss of appetite, night sweats and fever. In contrast, gallstones usually present with postprandial colic pain of the right upper abdomen. Particularly female, fertile and obese patients are at higher risk to develop gallstones.

If bile duct obstruction has been excluded, haemolysis should be included into diagnostic workup. Several hereditary, infectious and haematologic entities are possible causes and laboratory testing for haemolysis should be included as an initial step towards differential diagnose. Mild hyperbilirubinaemia resulting from hereditary deficiency of bilirubin metabolism should complete differentiation of jaundice.

Bacterial and parasitic hepatitis

Several bacterial or parasitic infections affect the liver either due to direct hepatic involvement or due to effects of systemic inflammation. Bacterial liver abscess used to be a frequent infectious complication but the incidence has declined since potent antibiotics have been available. Bacterial liver abscesses are usually polymicrobial, including aerobic Gram-negative or Gram-positive species as well as anaerobic bacteria [44]. The bacteria reach the liver either by the biliary or the haematological route. As a consequence the biliary and splanchnic system should be intensively screened for bacterial foci. Amebic abscesses are particularly observed in patients originating from endemic areas or with positive travel anamnesis. Clinical history differentiates preceding bloody diarrhoea which characterise amebiasis in only 10% of all cases [45]. Beside common commensal bacteria, many other bacterial pathogens, including typhoid fever and brucellosis, might cause hepatic deterioration. Leptospirosis, a very common zoonosis, has to be considered if acute flu-like symptoms are accompanied by (haemolytic) jaundice and renal impairment. Also secondary syphilis shows liver involvement with hepatitis in up to 50% of all cases. Beside non-specific general symptoms, lymphadenopathy and characteristic maculopapular rash of the palm and soles may be observed.

For patients with a positive travel history schistosomiasis and malaria are important differential diagnosis of human hepatitis. Particularly schistosomiasis contributes to a large number of patients with liver fibrosis worldwide and has to be considered in patients originating from endemic areas in Eastern Asia and Northern Africa. As recurrent schistosomiasis may aggravate progression of chronic viral hepatitis, both entities require diagnostic attention and treatment. Particularly in Egypt where efforts to eradicate schistosomiasis with intravenous medications have contributed to a high HCV prevalence by contaminated syringes, co-infections with both entities have become a serious medical burden [46]. Malaria transiently

affects the liver throughout its lifecycle and causes haemolytic jaundice during erythrocytic stages. Nevertheless, hepatocyte damage is observed predominantly during *Plasmodium falciparum* infections and particularly in patients with underlying viral hepatitis acute liver failure has been documented [47].

Diagnostic tests

Liver enzymes

In up to 80% of all cases, acute hepatitis does not present with clinical symptoms, so laboratory findings are often the only indicators for hepatitis and help to differentiate the causes of liver damage. A population-based survey in Europe for example has identified elevated liver enzymes (defined as >1.5 times the upper reference limit, URL) in up to 17% of the general population.

Elevation of alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST) ten times beyond the URL are reliable markers to discriminate acute hepatic damage from unspecific or chronic causes of ALT and AST elevations. Cut off values to identify acute hepatic injury are 200 U/l for AST (sensitivity: 91%, specificity: 95%) and 300 U/l for ALT (sensitivity, 96%; specificity, 94%) [48].

A proportion of up to 18% of mild liver enzyme elevations (>1.5 x URL) were associated with viral hepatitis in industrialised countries and the numbers are declining due to vaccination and screening efforts. Elevated ALT (>1.5 × URL) occurs in 63% of HCV- positive individuals and in 31% during HBV infection [49]. In contrast, only about 4% of pronounced AST elevations >400 U/l are caused by viral hepatitis in the patients attending clinic [50]. Kinetics of AST and ALT elevations during acute viral hepatitis are characterised by a gradual slope, which reaches its maximum after a variable period of three or more weeks from onset of first enzyme elevation. During further time-course the AST and ALT levels decline gradually. AST/ALT Ratio <1 are regularly observed during viral hepatitis in about 90% of the infected patients [51]. Though viral hepatitis is not the main cause, which is responsible for severe liver damage, laboratory findings occur in other settings.

In a significant proportion of patients (50%) with incidentally diagnosed AST elevations (>400 U/l), it was attributed to ischaemic hepatic events. Particularly during very high AST and ALT elevations >3,000 U/l ischaemic hepatitis should be considered. This laboratory findings further limits the spectrum of potential differential diagnosis to ischaemic, toxic or acute viral hepatitis [52]. Clinical observations of ischaemic hepatitis have shown that the median peak ALT level reaches 1,500 U/l during congestive heart failure and 2,100 U/l during hypoxemia [53]. At time of clinical admission patients

Table 3. Characteristic laboratory patterns of human hepatitis

Disease	peak ALT (×URL)	AST/ALT ratio	peak Bili (mg/dl)	PT (seconds)
Viral hepatitis	10–40	<1	<15	<3
Alcoholic hepatitis	2–8	>2	<15	1–3
Toxic hepatitis	>40	>1 early	<5	>5 (transient)
Ischemic hepatitis	>40	>1 early	<5	>5 (transient)

×URL, times upper reference limit; PT, prothrombin time prolongation

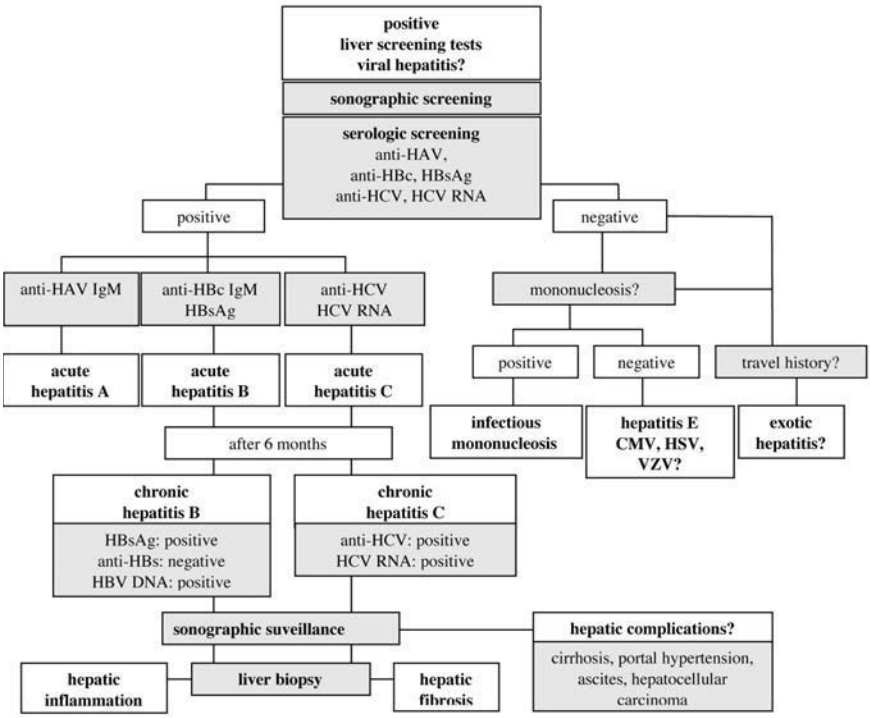

Figure 1. Diagnostic algorithm of viral hepatitis

with ischaemic hepatitis often have already reached maximum peak ALT values, suggesting that the ischaemic event causes a fast and steep incline of liver enzymes prior to clinical admission. Therefore the ischaemic event itself is regularly not witnessed by the clinician. During further follow up

a fast decline of liver enzymes is typically observed during ischaemic liver damage. Ischaemic hepatitis is also characterised by a lactate dehydrogenase (LDH) elevation, which reaches median peak level >2,500 U/l [53]. The mean ALT/LDH ratio is significantly lower during ischaemic hepatitis compared to viral hepatitis and a ALT/LDH ratio >1.5 is useful to differentiate ischaemic hepatitis from viral hepatitis with a sensitivity of 94% and a specificity of 84% [54].

Toxic hepatitis shares several laboratory features of ischaemic hepatitis. Beside fast kinetics and high peak levels of liver enzyme alterations the ALT/LDH ratio usually does not exceed a cut-off value of 1.5 [54]. Despite the different characteristics of toxic hepatitis the diagnosis is often missed. The leading factor towards the right diagnosis is a careful history of toxin or drug exposition. Uncomplicated alcoholic hepatitis is the most common toxic hepatitis and usually presents with mild laboratory alterations. During uncomplicated alcoholic hepatitis aminotransferases often do not exceed >10 times the upper reference limit. It has been shown that AST/ALT ratio >2 is reasonably specific to identify alcoholic hepatitis from other causes of acute liver damage and the majority of patients (80–90%) with alcoholic hepatitis show an AST/ALT ratio >2 [51].

In the general population presenting with AST elevations >400 U/l almost 24% of all cases were contributed to cholestatic disease [50]. Liver enzyme elevation during mechanical biliary obstruction exceeds 2,000 U/l in 1–2%. Characteristically the elevated liver enzymes resolve within a week despite persisting cholestasis [55]. Serum alkaline phosphatase (AP) derived from the cholangial epithelial is an important laboratory indicator for cholestasis. Mild AP elevations below three times the reference limit are considered non-specific and may occur during other hepatic pathologies. In contrast, striking AP elevations strongly suggest bile duct obstruction, if extrahepatic (skeletal) sources of AP have been excluded. Gamma-glutamyl-transpeptidase (GGT) is considered to be more specific for hepatic pathologies, because no relevant quantities of GGT are found in bone tissue. As GGT is mainly located in the microsomal cellular compartment it may be induced by alcohol and toxic drugs. If GGT induction is suspected the AP/GGT ratio >2.5 offers a helpful parameter to identify cholestatic disease.

During chronic hepatitis elevation of liver enzymes shows variable profiles and is even missing in a relevant proportion of patients. Therefore, the diagnostic value to differentiate the causes of chronic liver damage or chronic hepatitis is relatively low. Nevertheless, the AST/ALT ratio helps to identify liver cirrhosis in patients with chronic liver disease of different aetiology if alcoholic liver disease has been excluded [56–58]. As mentioned before combinations of laboratory parameters like AST/ALT- and ALT/LDH-ratio or additional testing of cholestatic parameters might help to increase discriminatory power of liver tests regarding chronic entities.

Parameters of liver function

Liver function tests are mainly based on analysis of serum bilirubin, prothrombine time and albumin. Time course and quantity of impaired liver function provides additional specifications of different pathologies and their prognosis. Hyperbilirubinaemia may indicate hepatitis and usually shows delayed kinetics compared to aminotransferase levels. Total serum bilirubin consists of a major conjugated (direct) and a minor unconjugated (indirect) fraction. If hepatocellular malfunction and hepatobiliary obstruction is responsible for hyperbilirubinaemia the proportion of direct bilirubin usually does not fall below 50%. Hence, lower concentrations of direct bilirubin suggest another cause for jaundice such as haemolysis [59]. Despite the fact that hyperbilirubinaemia is associated with liver damage, jaundice is an inconsistent finding during acute viral hepatitis. Particularly children with acute viral hepatitis often do not develop jaundice or show mild bilirubin elevations. A clinical observation described peak bilirubin >171 mmol/l (10 mg/dl) in only 1% of children with acute hepatitis [60]. In contrast adults may develop jaundice in 70% of cases of acute hepatitis A [61], 33–50% of cases of acute hepatitis B [62, 63] and about 33% of cases of acute hepatitis C [64].

Bilirubin, prothrombine time (PT) and serum albumin help to estimate prognosis of acute and chronic hepatic disease as the deterioration of liver function might be associated with worse outcome. For acute hepatic damage a PT cut-off >20 seconds (international normalised ratio >6.5), a total serum-bilirubin >15 g/dl (257 mmol/l) and albumin <25 g/l indicates severe liver damage or predicts likelihood of death [65]. Assessment of chronic hepatic damage relies on similar variables, but other cut-off values have been defined to estimate the outcome. In conclusion, some of these laboratory parameters have been included into standardised assessment of acute and chronic liver failure and are important to define the indication for liver transplantation for different aetiologies [66–68].

Specific serological and molecular tests

Since the introduction of specific serological and molecular tests for different types of viral hepatitis, they are the most important diagnostic tool to confirm or rule out different entities of hepatitis. In addition autoimmune serology is able to narrow the panel of differential diagnosis of autoimmune-mediated liver diseases.

Immunoglobulin M antibodies against HAV (anti-HAV IgM) are typically present at onset of symptoms, and remain detectable for an average of 3–6 months after infection. Some serological studies suggest that positive HAV IgM for more than 4 months present in only about 13% of all cases. In contrast total anti-HAV IgG titres are very durable and persist for long

periods after infection. Therefore detection of HAV IgM is considered as routine test to identify acute HAV infection and HAV IgG resembles past exposure and immunity against HAV [69].

Enzyme immunoabsorbent assays (EIA) are standard diagnostic tests to identify acute or chronic hepatitis B virus infection. The standard screening tests are able to identify HBV infection as early as 2–6 weeks prior to the onset of symptoms of acute hepatitis B. The recommended standard screening test for HBV is based on the detection of hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) specific antibodies. In a small proportion of patients HBsAg targeted assays fail to detect HBV infection. These false negative HBsAg tests result from HBsAg titres below the detection limit during chronic HBV infection and from HBsAg escape mutations, which are not detected by antibodies employed for routine screening. Tests against HBeAg and HBV nucleic acids are recommended complementary tests to identify active HBV replication. In contrast, neutralising anti-HBsAg antibodies (>10 IU/ml) indicate an eliminated HBV infection or immunisation against HBV [70]. In patients with positive HBsAg titres and a high risk of HDV infection assays of total anti-HDV antibodies are considered as sufficient test to identify hepatitis D co- or super-infection.

First generation EIAs to detect anti-HCV specific immunglobulins have been introduced by 1990 as screening for HCV infection [71]. Nowadays third generation EIAs are implemented in clinical practice, which detect a panel of specific immunglobulins against epitopes of different structural and non-structural HCV proteins. These third generation EIAs show a specificity of about 97% and have reduced the diagnostic window after HCV infection to about 16 weeks [72]. Patients with HCV infection have detectable antibodies in 50–70% at clinical presentation, but after three months about 90% will have developed detectable antibody titres [73]. IgM antibodies are no reliable markers to discriminate acute from chronic HCV infection as 50–70 % of chronically HCV infected patients still show positive anti-HCV IgM titres [73, 74]. Patients with signs and symptoms of acute hepatitis and negative HCV serology should undergo HCV nucleic acid tests. HCV PCR assays show a high sensitivity and are able to detect a minimal HCV RNA threshold of 5–50 IU/ml in the serum. The high sensitivity detects active HCV replication already 1–3 weeks after HCV infection. Quantitative HCV RNA tests and HCV genotyping are complementary tests designed to identify prognosis of HCV infection and to monitor response to HCV therapy [73].

Autoimmune-mediated liver damage is associated with different types of auto-antibodies. Indicators of AIH are elevated antibody titres directed against liver/kidney microsomes (LKM), soluble liver antigen (SLA) and liver pancreas antigens. An elevation of total serum gammaglobulin is also indicative for AIH. AIH is also associated with high titres (>1/80) of anti-nuclear antibody (ANA) and/or anti-smooth muscle antibody (SMA).

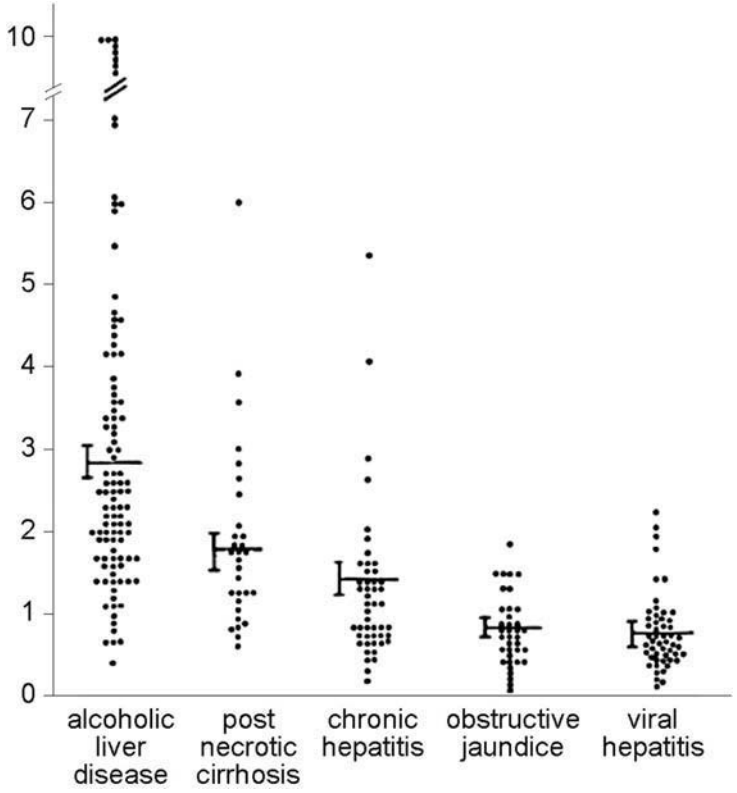

Figure 2. Differentiation of liver disease by AST/ALT ratio
Serum specimens were obtained from patients with histologically proven liver disease. Ratio was calculated from the highest AST and ALT levels during follow up [51]. With kind permission from Springer Science and Business Media.

The diagnostic value of auto-antibodies is limited by the fact that they are missing in a significant number of patients with AIH. Therefore, a clinical scoring system introduced by an international panel does not regard auto-antibodies as an absolute prerequisite for diagnosis of AIH [40].

Another marker of autoimmune disease, anti-mitochondrial antibody (AMA), is found in almost all patients (90–95%) with PBC [43]. The identified AMA during PBC are specifically directed against the mitochondrial pyruvate dehydrogenase complex (M2 type of AMA), which helps to discriminate other entities which may be associated with positive AMA titres. M2 type of AMA shows a specificity of 96% and a sensitivity rate of 98% for the diagnosis of PBC [75]. Other auto-antibodies found in patients with PBC are SMA, ANA, rheumatoid factor and antithyroid antibodies. About 5–10% of patients have features of both PBC and AIH which is considered as overlap syndrome [76].

Finally, PSC which affects hepatic bile ducts is associated with high titres of perinuclear anti-neutrophile cytoplasmatic antibodies (pANCA) in 80% of all cases and with anti-nuclear antibodies (ANA) titres in up to 50% of all cases [77]. Patients with PSC might share characteristics of AIH. These cases are named autoimmune cholangitis or are classified as AIH overlap [42].

Diagnostic imaging

Diagnostic imaging is important to differentiate acute from chronic liver disease and to recognise bile duct obstruction or hepatic lesions. For this purpose ultrasound is a very important cost effective screening tool and should be routinely included in differential diagnosis of human hepatitis. Acute liver damage for example correlates with changes of hepatic morphology like an enlarged liver diameter, rounded liver margins and a parenchyma of reduced echogenicity. Moreover, acute hepatic deterioration might be based on hepatic malperfusion. In this setting, dilated hepatic veins are indicators for hepatic congestion and duplex ultrasound might identify thrombotic occlusions or reduced blood flow of arterial, portal or venous vessels.

The sonographic characteristics to identify pronounced liver fibrosis or cirrhosis are quite well defined but detection of mild hepatic fibrosis is still challenging. Predominantly, an irregular liver surface and interrupted sonographic signals of the hepatic capsule are associated with liver fibrosis or cirrhosis. Sonographic assessment of the liver surface reaches a sensitivity between 54% and 57% and a specificity between 88% and 95% for the detection of liver fibrosis or cirrhosis [78–80]. Relative hypertrophy of the caudal lobe, changes of the liver diameters and irregular patterns of the intrahepatic vessels are additional signs of chronic liver damage [78, 81]. Further assessment of the venous blood flow, arterial resistance and portal velocity might increase the sensitivity for the detection of liver cirrhosis or portal hypertension [78, 82, 83]. Splenomegaly is an extrahepatic indicator for portal hypertension and could identify haemolytic disease, especially if sonographic signs of liver cirrhosis are absent. Finally, enlarged lymph nodes localised in the liver hilum may indicate viral hepatitis [84]. Unfortunately, differentiation of hepatic fibrosis from hepatic steatosis is hampered by the fact that characteristics of hepatic steatosis, like increased parenchymal echogenicity and ultrasound absorption, often lead to underestimation of coexisting mild fibrosis [79, 85].

Cholestatic disease is a crucial differential diagnosis of human hepatitis. Cholestasis is routinely identified by ultrasound, depending on the anatomic site of biliary obstruction. Beside gallstones and cholecystitis, where the sensitivity reaches more than 95%, ultrasound is reasonably sensitive (~75%) for detection of intrahepatic cholestasis, whereas sensitivity for detection of choledocholithiasis without dilated bile ducts drops to 50%. Computed tomography reaches similar levels of sensitivity for detection for biliary

obstruction, e.g., about 75% [86]. Further improvement of biliary imaging has been proposed by magnetic resonance cholangiography but is not yet available for routine use. Up to now, endoscopic retrograde cholangioscopy (ERC) is the most sensitive diagnostic tool to identify biliary pathologies and should be considered if biliary obstruction is suspected for clinical reasons. ERC also detects minimal bile duct irregularities and therefore is considered the golden standard for diagnosis of PSC and offers treatment options for dominant strictures or biliary concrements [42].

Beside ultrasound which is a valuable tool to screen and monitor focal hepatic lesions, contrast enhanced computed tomography (CT) is the standard procedure to confirm hepatic metastasis or HCC. Especially in context of liver cirrhosis early arterial phase enhancement of HCC nodules allows detection of HCC. Compared to ultrasound, which reaches a sensitivity of 71% enhanced CT shows a sensitivity of 82% or more for the detection of HCC. Therefore CT is standard for the baseline assessment and monitoring of cirrhotic livers in context of HCC treatment [87, 88]. Contrast enhanced ultrasound appears to be a very promising alternative and might be equivalent for the diagnosis of HCC and other focal hepatic lesions.

Histology

Histo-morphological assessment of the liver is a hallmark of differential diagnosis. Liver histology defines the inflammatory activity of hepatitis and is able to quantify the extent of acute and chronic liver damage caused by different entities. Hence, liver histology may predict the prognosis of the underlying liver disease.

Viral hepatitis is characterised by necro-inflammatory changes and chronic fibrotic alterations of liver histology. The heterogenic manifestation of hepatic necrosis during viral hepatitis may show focal lobular necrosis restricted to hepatic lobules, piecemeal necrosis of the periportal plate or extensive bridging necrosis, which is not restricted to the hepatic lobule. The typical inflammatory infiltrates during viral hepatitis consist of mononuclear cells [89]. Additional cytopathic effects of viral hepatitis have been suggested, such as bile duct lesions and hepatic steatosis, which are more common features during hepatitis C compared to hepatitis B [90–92]. To quantify the inflammatory activity (grading) and the fibrotic damage (staging) during viral hepatitis standardised scores have been introduced. The extent of cellular infiltrates and hepatic necrosis are included in the assessment of inflammatory activity. Histological grading quantifies extent of periportal fibrosis, the formation of septal fibrosis up to the destruction of portal architecture in case of complete cirrhosis [89, 93].

The histological signature during hepatitis might discriminate other entities from viral hepatitis. AIH often causes a more pronounced picture of hepatic damage at the time of diagnosis. Hence, hepatocellular necrosis,

multinucleated hepatocytes and broad areas of parenchymal collapse are typically observed in AIH. Whereas bile duct damage or loss and hepatic steatosis are uncommon during AIH [94]. Primary biliary cirrhosis (PBC) is characterised by a chronic non-suppurative destructive cholangitis of the small bile ducts. Granuloma formation, progressive loss of bile ducts and cellular infiltrates with an non-homogenic distribution throughout the liver are typical findings [43]. Histology of PSC may also show bile duct destruction, signs of cholangitis and cholestasis. Nevertheless, a precise cholangiographic assessment is the most important diagnostic tool, as PSC usually affects the median and large bile ducts [42, 95]. Also granulomatous hepatitis is a common histological finding, which is found in 2–10% of liver specimens. Beside PBC mentioned above, the causes of granulomatous hepatitis include autoimmunological, infectious and toxic entities. Sarcoidosis and tuberculosis remain the most important differential diagnosis and are responsible for 50–60% of all cases with granulomatous hepatitis. Furthermore, the differential diagnosis of granulomatous hepatitis should be extended in patients with acquired immunodeficiency syndrome to atypical mycobacteria, histoplasmosis and cryptococcosis. If no specific pathology could be identified toxic medications should be considered as alternative cause.

Conclusions

Differential diagnosis of human hepatitis should always consider the most common causes of liver damage, which includes steatohepatitis, alcohol consumption, viral hepatitis and biliary obstruction. The most frequent causes of acute hepatic deterioration with particularly high liver enzyme elevations are viral hepatitis, hepatic ischaemia and toxic events. Differential diagnosis of chronic human hepatitis needs to be extended to hereditary metabolic defects and autoimmune diseases if other common hepatic pathologies are unlikely. Patient groups with risk factors for rare entities of human hepatitis require a specific diagnostic approach.

In laboratory assessment elevated liver enzymes are usually the first indicator for human hepatitis. Additional parameters of liver function are essential to estimate prognosis and extent of underlying liver damage. Viral serology should always be included into the diagnostic algorithm as it is highly reliable to detect or exclude viral hepatitis. Further special diagnostic tests, for example auto-immune serology, may be applied depending on specific patient history and clinical findings.

Finally, ultrasound of the liver parenchyma is a cost effective method to screen for biliary obstruction, hepatic masses and pronounced liver fibrosis or cirrhosis; it is therefore recommended during differential diagnosis of human hepatitis. Liver biopsy may complete the diagnostic approach, if either the underlying hepatic pathology remains obscure or a definite quantification of liver fibrosis and hepatic inflammation is mandatory.

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Comparative Hepatitis
ed. by Olaf Weber and Ulrike Protzer

Comparative pathology

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Abstract

Hepatitis is defined as a necroinflammation of the liver displaying liver cell damage, inflammatory cell infiltrates, and regeneration to a variable extent. In contrast to acute hepatitis, which is characterised by a self-limiting damage of the acinar parenchyma, chronic hepatitis is pathomorphologically defined by the persistence of necroinflammation, portal predominance of mononuclear inflammatory infiltrates, and the development of fibrosis. Whereas HAV and HEV do not cause chronic hepatitis in humans, HBV (alone or in combination with HDV) and HCV infection may result in chronic liver disease in humans, which may progress to liver cirrhosis and HCC. Initial animal models for the study of viral hepatitis belonged to the group of nonhuman primates, especially chimpanzees, which can be infected by human hepatitis viruses. These models develop a milder acute hepatitis compared to humans and although chronic hepatitis may also develop, progressive liver disease and liver cirrhosis are generally absent. In the case of HBV, several mammalian and avian HBV-related viruses are known. The most intensively characterised model is the Eastern woodchuck infected with WHV. This example is the only model in which chronic liver disease and HCC frequently develop. Recently, the tree shrew (*Tupaia belangeri*) has become an interesting model since it can be infected with human hepatitis viruses, it can be handled more easily compared to chimpanzees, and it can be used in transplantation models. Additionally, several transgenic mice and human mouse chimera have been developed, which allow for the studying of viral replication and novel therapeutic approaches *in vivo*.

Introduction

Comparative pathology of hepatitis may address several topics: the differences between acute and chronic hepatitis, differences in histological features due to causative agents as well as typical and atypical forms of hepatitis (e.g., following liver transplantation). Additionally, the features of hepatitis can be compared between different species, e.g., humans and rodents. Finally, certain areas within human hepatitis can be studied in animal models.

Hepatitis represents a necroinflammation of the liver, which is histologically characterised by a variable extent of liver cell damage, inflammatory cell infiltrates, and regeneration. In contrast to acute hepatitis, which is characterised by a predominance of damage in the acinar parenchyma, chronic hepatitis is pathomorphologically defined by the persistence of necroinflammation, the predominance of portal mononuclear inflammatory infiltrates, and variable necroinflammatory activity at the portal-parenchymal interface, as well as the development of fibrosis.

The morphological pattern of hepatitis

Acute hepatitis

General aspects

The severity of acute hepatitis may vary from asymptomatic to fulminant hepatic failure. Accordingly, the histological changes seen in acute hepatitis may vary. Mild acute hepatitis typically shows a variable picture including hepatocellular swelling, single cell necrosis, apoptosis (acidophilic bodies), cholestasis (canalicular and hepatocellular), acinar inflammation, Kupffer cell activation (prominence, ceroid phagocytosis), and liver cell regeneration. The combination of hepatocellular cell death ('drop-out') and small regenerating hepatocytes may lead to a disarray of liver cell plates, which is an important diagnostic criterion of acute hepatitis. Of note, a mild portal inflammation (mostly lymphoplasmacytic), sometimes even including some mild bile duct damage, may be a minor component of acute hepatitis. In contrast to chronic hepatitis, portal inflammation does not predominate and the outline of the portal tract remains well defined towards the acinar parenchyma. In more severe disease, there may be confluent necroses, frequently pronounced around perivenular areas, or even bridging necroses connecting central-portal or central-central areas. Consequently, a collapse of the reticulin fibre framework may be seen in appropriate stains (e.g., modified Gomori's), which may be replaced by fibrous scarring after the hepatitis has resolved. In case of extensive confluent necroses, a ductular reaction, which consists of ductular proliferations, associated fibrosis and neutrophilic infiltration occurs at the portal interface. This feature may mimic extra-acinar cholestasis (e.g., bile duct obstruction) and has thus to be differentiated. In case of fulminant acute hepatitis, panacinar and multiacinar necrosis are observed, which are associated with approximation of individual portal tracts, large collaptic areas and extensive futile ductular reaction.

Acute hepatitis may be caused by viral infection (e.g., hepatotropic viruses, but also Epstein-Barr virus (EBV), cytomegalovirus (CMV), Herpes simplex virus (HSV), Varicella zoster virus (VZV), adenovirus, enterovirus,

etc.), drugs, toxins, alcohol exposure, bacterial infection as well as autoimmunity, and Wilson's disease.

Since there are only a few pathognomonic features that allow a precise, histologically based definition of the etiology, it is rather the challenge for the clinician than for the pathologist to determine the underlying etiology. Nevertheless, a specific and sometimes overlapping pattern may point to the underlying etiology.

Specific features in acute hepatitis

The histological spectrum by which the liver may react to certain agents or damage is limited. Nevertheless, an evaluation of the morphological injury pattern including the recognition of subtle features (e.g., acidophilic bodies, eosinophils) may allow for the identification of the causative agent. Additionally, immunohistological and molecular pathological analyses may allow diagnosis in a subset of cases. The most important diagnostic patterns include virus-type hepatitis, steatohepatitis, zonal restricted parenchymal damage, mononucleosis-like hepatitis, granulomatous hepatitis, and focal necrotising hepatitis.

Virus-type hepatitis

The typical lesion in acute virus hepatitis consists of spotty cytolytic necrosis resulting in a 'drop-out' of individual hepatocytes within the hepatocellular plates and apoptosis resulting in acidophilic bodies (Fig. 1A). The remnants of these cells are either removed by blood flow or they are phagocytosed leading to ceroid-laden macrophages and prominence of Kupffer cells.

In hepatitis A virus (HAV) and hepatitis E virus (HEV) infection, there may be two common, sometimes overlapping pictures pointing to the causative agent: one includes a periportal accentuated inflammatory pattern, rich in plasma cells, with no or little perivenular necrosis [1, 2]. The second feature is so-called cholestatic hepatitis with prominent perivenular cholestasis and only minimal inflammatory infiltration. Rarely, HAV may present as acute hepatic failure [3].

Steatohepatitis

Steatohepatitis represents the progressive form of fatty liver disease and is defined by three histological features: steatosis, ballooning and necroinflammatory lesions [4, 5]. The histological changes are usually pronounced in the perivenular region. Eventually lipogranulomas, Mallory-Denk bodies and glycogenated nuclei may be seen. Steatohepatitis is usually caused by chronic alcohol abuse or non-alcoholic conditions including adipositas, type II diabetes mellitus, metabolic syndrome and drugs (e.g., amiodaron, nifedipine, steroids).

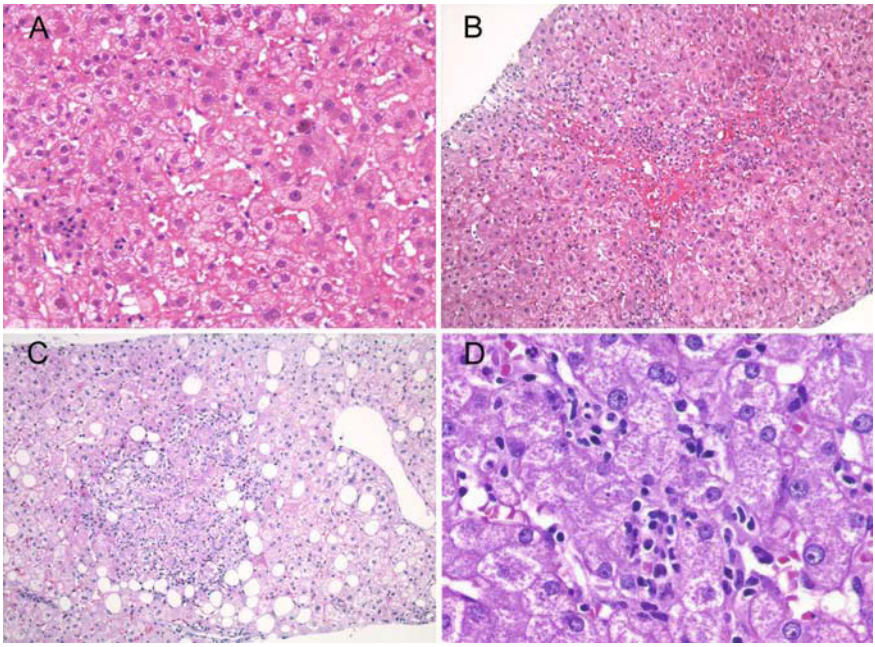

Figure 1. Hepatitis pattern: (A) Viral-type hepatitis pattern showing focal cytolytic liver cell necrosis and acidophilic bodies. (B) Zonal pattern: There is perivenular necrosis with leucocytic demarcation and haemorrhage in this case of drug-induced hepatitis. (C) Hepatic sarcoidosis with epithelioid cell granuloma with multinucleated giant cells. (D) EBV-hepatitis with single-file rowing of leucocytes showing some nuclear atypia.

Zonal pattern

The zonal pattern shows necrosis restricted to a certain area of the liver acinus, in particular of the acinar zone 3, as seen in ischaemic liver cell damage (Fig. 1B). This pattern may sometimes be observed in severe acute viral hepatitis, but particularly when associated with prominent eosinophilic or neutrophilic infiltration, cholestastis, or peribiliary basophilia may point to drug- and toxin-induced liver disease (e.g., phenprocoumon) [6]. Notably, drug- or toxin-induced hepatitis, which can produce all forms of acute, chronic, vascular and neoplastic liver diseases, has to be considered generally in hepatitis of uncertain etiology [7].

Mononucleosis-like hepatitis

The mononucleosis-like pattern shows only mild or absent liver cell damage and prominent lymphocytes are seen within the hepatic sinusoids, typically with single file-like rowing, and portal tracts (Fig. 1D). This pattern is typical for acute liver involvement in EBV infection. In some cases of acute hepatitis C virus (HCV) infection, there may be a prominent intrasinusoi-

dal lymphocyte aggregation without severe liver cell damage, reminiscent of EBV hepatitis [8]. An additional differential diagnosis for this pattern includes malignant lymphoma (e.g., hepatosplenic T-cell lymphoma) [9]. Immunohistological and molecular pathological analyses (detection of viral nucleid acids, clonality of T-cell receptor rearrangement) may allow differentiation in most cases.

Granulomatous pattern

Hepatic granulomas consist of histiocytic, even epithelioid cells, a varied number of mononuclear cells and (sometimes) multinucleated giant cells. Histologically caseating (e.g., tuberculosis) granulomas, non-caseating granulomas, lipogranulomas, and fibrin ring granulomas can be differentiated. In most cases, hepatic granulomas develop in response to infections (especially bacterial or parasitic), drug hypersensitivity (e.g., acetylsalicylic acid, amoxicillin) or systemic diseases (e.g., sarcoidosis, Fig. 1C). Histochemical stains like PAS, Gram, Giemsa, Grockott, Warthin-Starry, or Hale may be helpful for determining the cause of a given granuloma.

Caseating granulomas in contrast to non-caseating granulomas show a central necrosis surrounded by epithelioid cells, multinucleated giant cells and histiocytes. They are typically seen in tuberculosis (Fig. 2D). Non-caseating granulomas in portal tracts near damaged bile ducts are almost certainly due to primary biliary cirrhosis. Lipogranulomas are frequently seen in steatohepatitis and consist of a fat vacuole surrounded by lymphocytes. Occasionally, fibrin ring granulomas composed of a fat vacuole surrounded by a ring of fibrin, epithelioid cells, giant cells and neutrophils may be seen. They may be observed in infectious diseases (e.g., Q fever), systemic diseases (Hodgkin's disease, systemic lupus erythematodes) or drug-induced liver damage (allopurinol) [10–13].

Focal necrotising pattern

The focal necrotising pattern of acute hepatitis shows non-zonal restricted group or bridging necrosis with haemorrhage, which may be demarcated by polymorphic neutrophils. This type of acute hepatitis is mainly seen in atypical forms of hepatitis (including HSV- (Fig. 2A), VZV-, adenovirus (Fig. 2B), enterovirus etc.) and most frequently occurs in the setting of immunosuppression. Atypical hepatitis differs from classical hepatitis in that it frequently allows diagnosis due to additional typical morphological features. Adenoviral infection, which rarely occurs in liver allografts, may mimic CMV hepatitis (microabscess formation, Fig. 2C) in its initial stages, but may proceed to confluent haemorrhagic necroses resulting in usually fatal infection [14]. Beside haemorrhagic necroses, the presence of 'smudge cells' is a diagnostic feature in adenoviral hepatitis. Another frequently fatal viral infection is acute necrotising Herpes simplex or Varizella zoster hepatitis, which show characteristic eosinophilic or basophilic nuclear inclusions (Cowdry bodies) in viable hepatocytes adjacent to necroses [15].

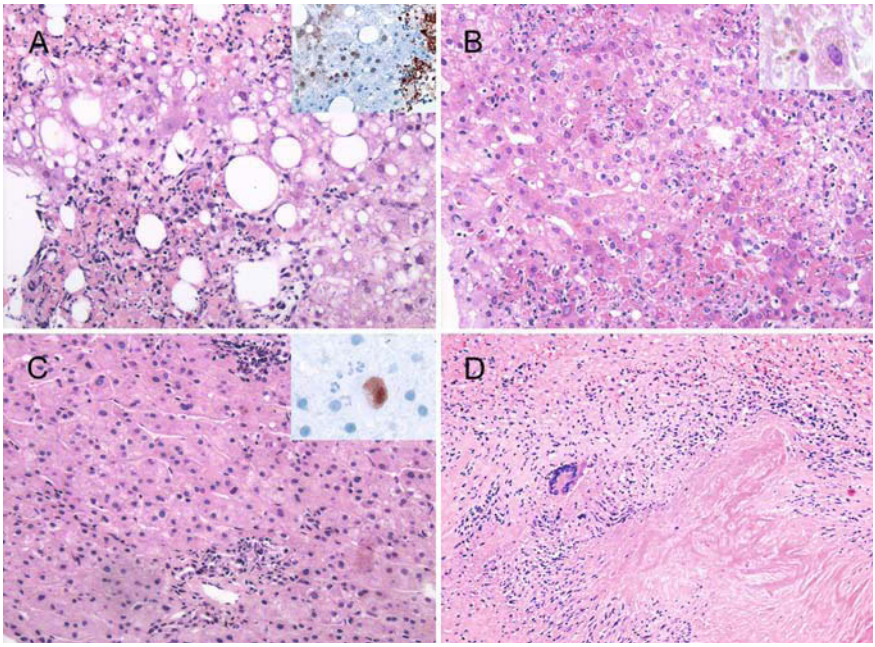

Figure 2 Atypical hepatitis: (A) Focal necrotising Herpes simplex hepatitis. Note the eosinophilic cowdry-type nuclear inclusions in the vital hepatocytes adjacent to the necrosis (insert: HSV-immunohistochemistry). (B) Focal necrotising adenovirus hepatitis with numerous smudge cells (insert). (C) Microabscess formation in CMV-hepatitis (insert: CMV-immunohistochemistry). (D) Hepatic tuberculosis showing caseating necrosis surrounded by epithelioid granuloma with langhans type multinucleated giant cell.

Chronic hepatitis

General aspects

In adults, chronic hepatitis is primarily grouped according to the underlying etiology [16]. Thus, infections, drugs/toxins, autoimmunity, metabolic, inherited diseases and non-defined 'cryptogenic' mechanisms are differentiated.

Classical infectious agents causing chronic hepatitis include hepatitis B virus (HBV) infection (alone or in combination with hepatitis D virus (HDV)) and HCV infection. Infrequently, EBV infection may induce chronic hepatitis [17]. Whether a few bacteria such as the *Helicobacter* species may cause chronic hepatitis or accelerate disease progression is rather questionable [18]. Beside pure autoimmune hepatitis, so-called autoimmune overlap syndromes with primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are diagnosed very infrequently. The list of medications which may induce chronic hepatitis is still growing due to the increasing number of approved drugs. Classical agents include antibiotics

(e.g., nitrofurantoine) and antiepileptics (e.g., phenytoin). Importantly, even herbs such as the Chinese medicinal herb Jin Bu Huan have been identified to induce chronic hepatitis. Metabolic diseases mostly do not belong to the classical etiological spectrum of chronic hepatitis, although especially Wilson's disease may show hepatitic features.

Pathomorphology and special features of chronic hepatitis

By definition, chronic hepatitis shows a predominance of portal inflammation. Interface hepatitis (in older terminology: piecemeal necrosis), acinar inflammation, and portal or septal fibrosis are facultative features. Disease progression is driven by the extent of interface hepatitis and is morphologically characterised by cytoplasmic degeneration and apoptosis of periportal hepatocytes in close contact with infiltrating lymphocytes. The features of cytoplasmic degeneration include hepatocellular ballooning, granular aggregation of cell organelles and partial dissolution of nuclear and cellular membranes [19].

The morphology-based definition of the underlying etiology is not possible in most cases and it requires the clinical-pathological context to prevent non-essential additional diagnostic procedures. Thus, clinical and serological information are important for the assessment of a liver biopsy specimen. Nevertheless, there are several characteristic features indicating the causative agent. In case of HBV, these include ground-glass hepatocytes (Fig. 3A) or so-called 'sanded nuclei' (frequently in immunosuppressed patients). Ground-glass hepatocytes are the morphological correlate of HBs antigen overload of the endoplasmic reticulum. 'Sanded nuclei' are characterised by eosinophilic, homogenous, fine-grained nuclear inclusions giving the impression of sand-like granulation. They are less frequently seen compared to ground-glass hepatocytes and are caused by nuclear HBc antigen overload [20]. Beside chronic HBV infection, they are observed more frequently in the case of an HBV-/HDV coinfection. In these cases, they are immunohistologically positive for the delta antigen [21]. Generally, the liver histology in HBV-/HDV coinfection shows more severe inflammatory activity compared to HBV monoinfection. Cytoplasmic or membranous HBc antigen staining as well as membranous HBs antigen staining have diagnostic importance since they mark viral replicating cells [22]. Molecular biological approaches for PCR-based detection of viral nucleic acids have been established, but they are only used in selected cases in which diagnosis is difficult (e.g., unclear morphology).

HCV infection is generally milder and more frequently shows (periportal, macrovesicular) steatosis, sinusoidal inflammatory aggregates with pronounced Kupffer cell activation, and portal lymph follicle formation (Fig. 3B) compared to HBV [23]. Especially the formation of portal lymph follicles is considered typical for HCV infection, although it is not pathog-

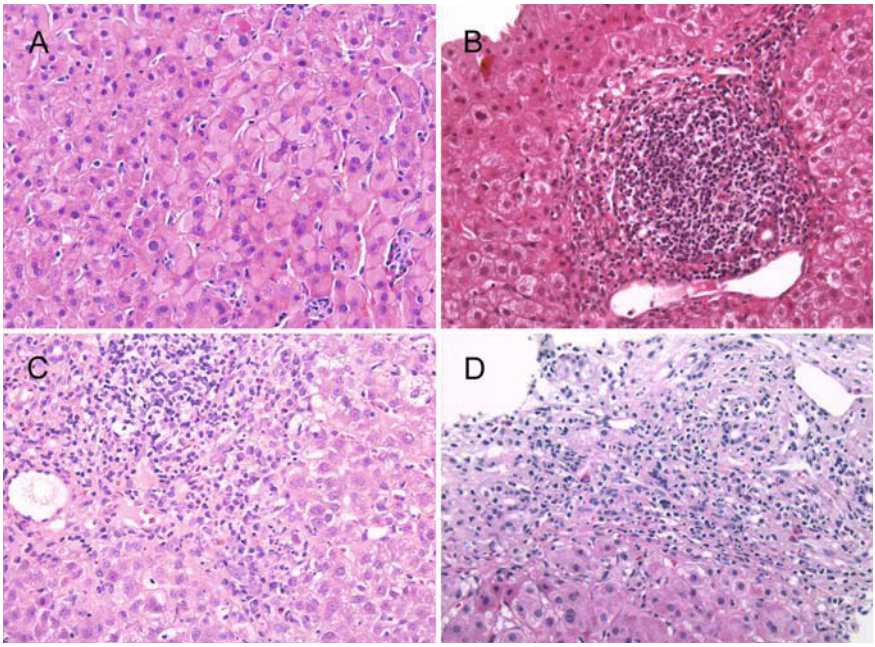

Figure 3. Special features of chronic hepatitis: (A) Chronic hepatitis B with numerous ground-glass hepatocytes. (B) Portal lymphoid follicle in HCV-infection. (C) Autoimmune hepatitis with marked interface hepatitis, numerous plasma cells, and pseudogland formation. (D) Drug-induced hepatitis with sparse portal mononuclear inflammatory infiltrates and intermingled eosinophils.

nomonic in any case. Immunohistological detection of HCV proteins is possible on snap-frozen tissue, but results obtained from formalin-fixed paraffin-embedded tissue are unreliable in most cases [24, 25].

Histological characteristics of untreated autoimmune hepatitis (Fig. 3C) are severe inflammatory activity and almost always include the features of chronicity at time of diagnosis. There is typically severe interface hepatitis, rosette formation of periportal hepatocytes, plasma cell-rich portal inflammatory infiltrates, and, by definition, no or only minimal bile duct damage [26].

Inflammatory activity and fibrosis in chronic viral hepatitis

The degree of inflammation and hepatocellular damage (necroinflammation) may vary considerably interindividually as well as during the course of a chronic hepatitis in a given patient. Thus, the inflammatory activity seen in a liver biopsy specimen (grading) only reflects a snapshot of the disease process [27]. Nevertheless, inflammatory activity, especially the

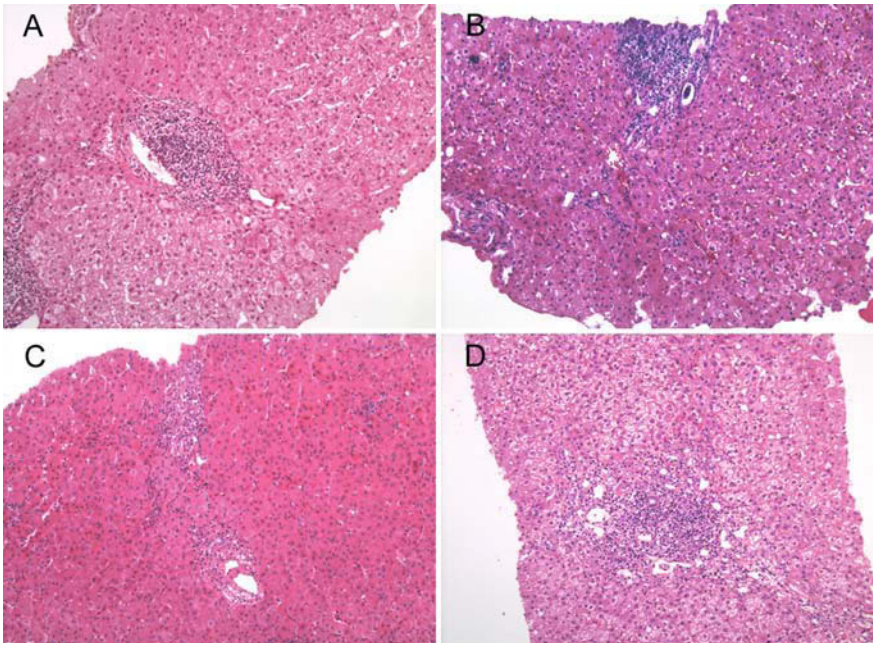

Figure 4. Grading of chronic hepatitis: (A) Minimal chronic hepatitis (grade 1) showing moderate portal inflammation and an intact limiting plate. (B) Mild chronic hepatitis (grade 2) additionally showing focal piecemeal necrosis and some acinar single cell necrosis. (C) Confluent interface hepatitis and numerous parenchymal cytolytic necrosis in this case of moderate chronic hepatitis (grade 3). (D) Severe chronic hepatitis (grade 4) with marked interface hepatitis and hepatocellular group necrosis (top).

degree of interface hepatitis, has a high predictive value with respect to disease progression [28]. During the last decades, several attempts have been made to assess inflammatory activity more objectively by introducing grading systems that score the various parameters of inflammatory activity. Several scoring systems have been broadly accepted, but until now, no single worldwide consensus for the use of a certain system has been achieved. Systems proposed include the scores according to Desmet et al. [16], Batts and Ludwig [29], Ishak's modified histological activity index (mHAI) [30] (originating from Knodell's score [31]), and the METAVIR score for assessment of chronic HCV infection [32]. Desmet's score (Tab. 1) is descriptive in terms of inflammatory activity and distinguishes four grades (minimal, mild, moderate, severe = grade 1 to 4, Fig. 4A–D). This grading system has several advantages: compared to other scores it has a good inter- and intraobserver reproducibility [33, 34]; it also has a broad applicability and, importantly, it has a good correlation to the more complex mHAI score (Tab. 2).

The degree of fibrosis and tissue remodelling (architectural distortion, staging) is the most important integrative measure for progression,

Table 1. Grading of chronic viral hepatitis according to Desmet & Scheuer [16]

grade	verbal	mHAI-Score	histological features
1	minimal	1–3	mild portal inflammation, none or minimal acinar inflammation/ few single cell necrosis, no interface hepatitis
2	mild	4–8	mild to moderate portal inflammation, focal interface hepatitis, some single cell necrosis, no confluent necrosis
3	moderate	9–12	moderate to severe portal inflammation, confluent interface hepatitis, multiple single cell necrosis, some confluent necrosis, no bridging or panacinar necrosis
4	severe	13–18	severe portal inflammation and interface hepatitis, severe acinar inflammation including confluent, bridging and panacinar necrosis

Table 2. Ishak's modified histological activity index (mHAI) [30]

interface hepatitis	0	none
	1	focal, few portal tracts
	2	focal, majority of portal tracts
	3	continuous <50% of portal tracts
	4	continuous <50% of portal tracts
confluent necroses	0	none
	1	focal
	2	few zone 3-necrosis
	3	multiple zone 3-necrosis
	4	zone 3- and few portocentral bridging necrosis
	5	zone 3- and multiple portocentral bridging necrosis
	6	panacinar/multiacinar necrosis
single cell necroses	0	none
	1	1 focus/MPF (objective 10×)
	2	2 to 4 foci/MPF
	3	5 to10 foci/MPF
	4	>10 foci/MPF
portal inflammation	0	none
	1	mild, few or all portal tracts
	2	moderate, few or all portal tracts
	3	moderate, all portal tracts
	4	severe, all portal tracts

reversibility, and functional hepatic reserve in chronic liver disease. Thus, the staging of chronic hepatitis is an important histological criterion to evaluate prognosis and therapeutic options/indication, which include both initial and time-course biopsies. During the last years, several non-invasive tests have been introduced to assess liver fibrosis [35], but so far all have

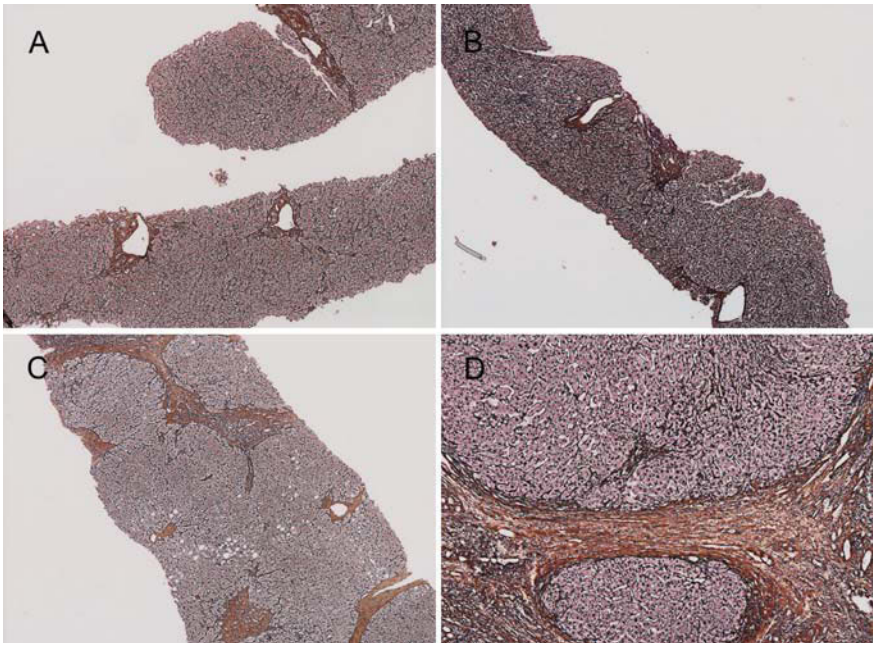

Figure 5. Staging of chronic hepatitis: (A) Fibrous extension of the portal tracts (stage 1). (B) Portal and septal fibrosis (stage 2). (C) Portal and septal fibrosis with formation of incomplete pseudolobuli and architectural distortion (stage 3). (D) Complete cirrhotic remodelling (stage 4).

failed in identifying clinically relevant stages of early progression, in the evaluation of architectural distortion, and in the measuring of fibrosis in fatty liver disease adequately. Since those patients have a high need for treatment, liver biopsy remains the gold standard in routine diagnostics [36]. Analogous to the grading systems, several scoring systems have been proposed for the staging of chronic hepatitis [16, 29, 30]. The score according to Desmet et al. (Tab. 3), which was developed from Scheuer's score [37], descriptively separates five stages (none, mild, moderate, severe fibrosis, and cirrhosis, Fig. 5A–D). The advantages of this system include simple applicability, clear definition and comparably small inter- and interobserver variability [34]. Of note, Desmet's score does not differentiate the extent of cirrhosis, the final stage of fibrosis, which is a limitation of all qualitative scoring systems introduced so far. Since it is now well-known that effective antiviral therapy may lead to the regression of liver fibrosis [38], in therapeutic studies the use of the semiquantitative fibrosis scores, such as the one established by Chevalier et al. (Tab. 4), is recommended [39].

Table 3. Staging of chronic viral hepatitis according to Desmet & Scheurer [16]

score	verbal	histological features
0	no fibrosis	no portal fibrosis
1	mild fibrosis	enlarged portal tracts without septa
2	moderate fibrosis	incomplete portoportal septa, preserved architecture
3	severe fibrosis	portal fibrosis with septa and architectural distortion (differences in central vein – portal tract distances, no evidence of complete cirrhotic remodelling)
4	cirrhosis	probable or definite cirrhotic remodelling

Table 4. Semiquantitative Severity Score [39]

criterium	score	verbal
central vein	0	normal or absence of vein (cirrhosis)
	1	moderately thickened (stellate aspect of vein wall)
	2	Markedly thickened wall (annular aspect of vein wall with numerous fibrous extensions between hepatocytes)
perisinusoidal fibrosis	0	normal
	1	localised fibrosis
	2	diffuse fibrosis
portal tract	0	normal
	1	enlarged without septa
	2	enlarged with septa
	3	cirrhosis
number of septa	0	none
	1	≤6 septa/10mm
	2	>6 septa/10mm
	3	nodular organisation
width of septa	0	thin and/or incomplete
	1	thick and loose connective matrix
	2	very thick and dense connective matrix
	3	>2/3 biopsy area

Determining factors of hepatitis caused by hepatotropic virus

The general mechanisms of viral hepatitis include hepatocellular damage, inflammatory cell infiltrates, and regeneration. They are common to all hepatitis virus infections. The development of chronic viral hepatitis is a result of the insufficiency of the host immune system to clear the agent. Several viruses have developed mechanisms that facilitate their escape from the host defensive mechanisms. Thus, chronicity and its course represents a complex interplay between viral and host factors determining the morphology and final outcome of a hepatitis virus infection.

Causative agent

In contrast to HBV infection, HCV infection becomes chronic in most cases, which is the result of several factors. The degree of viral replication in acute HCV infection is comparably high, usually surpassing the capacity of the immune system. Although a type I interferon response is initiated by the innate immune system, it often fails to induce viral clearance *in vivo* due to interactions of HCV proteins with mediators of the interferon response system [40]. Some HCV proteins have been shown to inhibit the activity on NK and dendritic cells [41, 42]. During HCV infection numerous quasispecies are produced due to the poor proofreading potential of the viral DNA polymerase, overwhelming the capacity of the adaptive immune system [43]. Despite these viral mechanisms, about 30% of HCV infections are cleared during the acute phase. Importantly, the addition of antiviral therapy leads to an almost 100% cure of acute HCV infection. In contrast, chronic HBV infection persists even following seroconversion, which may be due to HBeAg secretion producing T-cell tolerance and downregulation of HBV replication [43]. Thus, there is a balance between minimised viral replication and control of the infection by the cellular and humoral immune system resulting in life-long protective immunity.

Host factors

Whether an acute hepatitis resolves or becomes chronic is ultimately determined by the host's defense mechanisms in clearing the infection. The T-cell response seems to play a major role in the resolution of a hepatotropic viral infection and patients suffering from chronic infection only show a narrowed and transient T-cell response compared to those who cleared the virus [40, 43]. One of the best examples in this regard is the common chronicity rate in infants vertically infected by HBV in contrast to infected adults, which is mainly due to the immaturity of the immune system. Additionally, reactivation of an HBV infection may occur following immunosuppression due to organ transplantation [44, 45]. Importantly, HBV as well as HCV infection may show an atypical re-infection pattern after liver transplantation known as fibrosing cholestatic hepatitis (FCH), which typically develops within the first month after transplantation and rapidly leads to graft failure. Histologically, FCH is characterised by hepatocellular ballooning, cholestasis with prominent ductular proliferation and intense perisinusoidal fibrosis, but there is a lack of significant inflammatory cell infiltration. In the case of HBV, ballooned hepatocytes show intense cytoplasmic and nuclear staining for HBcAg, suggesting maximal viral replication [46, 47].

The presence of concurrent liver diseases may negatively influence the progression of chronic viral hepatitis. Considering recent epidemiologic data, fatty change either in alcoholic or non-alcholic fatty liver disease (e.g.,

obesity, insulin resistance, metabolic syndrome) probably represents the most important issues in this context, since it has been shown that fatty change causes single cell necrosis and apoptosis, accelerating fibrosis progression [48, 49]. Whereas periportal (or midzonal) macrovesicular steatosis may belong to the spectrum of HCV infection (especially genotype 3) [50], perivenular accentuation is highly indicative of an additional alcoholic or non-alcoholic liver cell damage, especially when seen in combination of non-necrosis associated perisinusoidal fibrosis and/or Mallory-Denk bodies.

Although endothelial iron deposits have been observed in chronic viral hepatitis, hepatocellular siderosis should raise the question of concurrent genetic haemochromatosis (e.g., HFE mutation). Concurrent liver disease may be observed in up to 20% of biopsies obtained from HCV-infected individuals.

Animal models

Animal models and natural occurring variants of human hepatitis viruses

Hepatitis A virus

Several nonhuman primate animal models have been used for study of HAV infection, but most of the data come from inoculation experiments in two animal species: chimpanzees and tamarins (*Saguinus mystax*). Additionally, some information is derived from the infection of owl monkeys [51–53]. Histological changes in the liver of these animals closely resemble HAV infection in man including focal necrosis, ballooning degeneration, apoptosis, cholestasis, and Kupffer cell proliferation. The inflammatory infiltrate is mainly composed of lymphocytes, but neutrophils, eosinophils, and plasma cells are also observed. The severity of hepatitis ranges from mild with focal necrosis to moderate with bridging necrosis and all animals recover completely. In contrast to humans, animals are almost never symptomatic during experimental HAV infection [54].

Hepatitis E virus

Macaque species, chimpanzees, and owl monkeys are the most frequent animal models used for study of HEV, but additionally, pigs and rats may be useful for some studies. Histological changes in the liver of nonhuman primates and humans are similar. There is focal cytolytic necrosis with minimal inflammatory infiltration in both species. Inflammatory infiltrates, if present, consist mainly of neutrophils. Cholestatic hepatitis (ballooning, hepatocellular cholestasis) is common and pseudogland formation has

been reported [54, 55]. The mortality of hepatitis E in the third trimester of pregnancy in humans is reported to be approximately 20%, which is much higher than in pregnant rhesus monkeys [56, 57]. Additionally, in contrast to humans, in whom immunity develops, recurrent hepatitis E has been reported in tamarins [54].

Hepatitis B virus

Natural animal model systems for the studying of HBV infection can be differentiated in those being permissive for human HBV and those infected by HBV-related viruses.

Chimpanzees can be infected with HBV and this model contributed much to our current understanding of the molecular virology of chronic hepatitis B. The advantages of this model include the fact that it is the only model for immunological studies of the natural course of the infection, the possibility to use defined inocula and study the early phases of infection. The disadvantages of this model are ethical considerations limiting biomedical research on primates, comparably high costs and limited availability of chimpanzees (listed as an endangered species since 1988). The clinical course of the infection is only very mild and the humoral immune response is weak and more restricted compared to humans. Additionally, vertical transmission occurs only rarely (in contrast to HBV in humans). In chimpanzees, there are two histologically distinctive types of infection: the first type is a self-limiting, acute hepatitis. The second type results in chronic hepatitis with portal lymphocytic infiltration, formation of lymphoid follicles (usually not seen in humans) and variable, usually mild liver cell necrosis. Interface hepatitis is generally not seen in HBV-infected chimpanzees. Therefore, chimpanzees do not develop progressive fibrosis and liver cirrhosis [58, 59]. It has been shown that infection with HBV-related viruses naturally may take place in chimpanzees, gibbons, orang-utans, and rhesus monkeys, but these animals do not seem to develop morphological equivalents of hepatitis [60–63]. Another transiently HBV-infectible animal model represents the tree shrew species *Tupaia belangeri* [64], which develops short-term replication followed by seroconversion, but does not show histological changes of hepatitis.

HBV-related viruses can be grossly divided in mammalian hepatitis viruses (orthohepadnaviruses) and avian hepatitis viruses (avihepadnaviruses). The first HBV-related virus described was the woodchuck hepatitis virus (WHV), which causes chronic hepatitis with some interface activity comparable to that in humans with chronic HBV infection, although inflammatory activity is generally milder. Additionally, WHV-infected woodchucks develop hepatocellular carcinoma (HCC) with higher frequency, but in contrast to humans, they do not develop liver cirrhosis [65, 66]. Other mammalian HBV-related viruses include the ground squirrel hepatitis virus

(GSHV) [67, 68], the arctic squirrel hepatitis virus (ASHV) and the woolly monkey hepatitis B virus (WMHV). In general, all of these animals show only mild histological liver abnormalities despite sometimes high serum titres and HCC development is seen infrequently [69, 70]. Woolly monkey hepatitis B virus (WMHBV) usually causes chronic hepatitis with no or minor symptoms, but fulminant WMHBV hepatitis may occur. The development of liver cirrhosis has not been reported [71]. About one-third of aged arctic squirrels develop focal liver lesions, which include liver cell adenomas, HCCs, and large nodules showing microvesicular steatosis [72]. Importantly, these lesions are also observed in animals not infected with the arctic squirrel hepatitis virus (ASHV). Thus, this model differs significantly from WHV infection, in which most infections result in HCC development within 2–3 years [73].

The group of avihepadnaviruses include the prototypic duck hepatitis B virus (DHBV) and several related family members: heron hepatitis B virus (HHBV), snow goose hepatitis virus (SGHV), ross goose hepatitis B virus (RGHBV), white stork hepatitis B virus (WSHBV), and crane hepatitis B virus [74–78]. DHBV infection causes an acute hepatitis and its severity correlates with the degree of viremia, but in contrast to mammalian hepatitis viruses, only very mild or even no histological changes occur during DHBV persistence. HCC development has been observed in DHBV-infected Pekin ducks, but only in a few cases such that its relation to DHBV is questionable [74, 77–79].

Hepatitis D virus

Since HDV represents an incomplete virus, which has an absolute requirement for HBV for replication and transmission, animal models are restricted to those permissive for HBV infection [80]. Both in humans and in animal models, the replication of HBV is diminished during acute HDV replication. HDV infection may either occur as a co-infection with HBV or as a superinfection in HBV carriers. In the chimpanzee model, co-infection results in a moderately active hepatitis, which may show two different courses. Whereas in the unimodal course of disease there is parallel expression of HBc and delta antigen in the nuclei of infected hepatocytes, the bimodal infection is characterised by the expression of one or the other antigen during the second phase [81]. In contrast, a superinfection of HDV results in a more severe hepatitis, which in more than 50% of cases results in viral persistence [82]. Additionally, the HDV genotype seems to influence the severity of liver disease [83]. Although HDV infection results in a more severe hepatitis in chimpanzees compared to other hepatitis viruses, they do not develop a progressive disease compared to the one seen in humans.

Although there is no indication that HDV infection may take place naturally in WHV infection, it has been shown that HDV can be transmitted

to WHV-carrying Eastern woodchucks, which results in acute and chronic hepatitis in a high proportion of animals [84].

Hepatitis C virus

The situation with HCV is even more complex since the only true infection model is the chimpanzee. In contrast, other mammals have not been infected successfully with HCV [85, 86]. Acute infection of chimpanzees usually shows a variable, but mild degree of focal inflammation and hepatocellular apoptosis, whereas portal inflammatory infiltration is not prominent. In contrast, chronically HCV-infected chimpanzees show a variable degree of portal inflammatory infiltrates and formation of lymphoid aggregates. Sometimes mild interface hepatitis may be seen, which is not associated with significant fibrosis even after more than ten years of chronic infection. Acinar inflammation includes sinusoidal lymphocytic infiltration, parenchymal inflammatory foci and apoptosis [87]. Although HCV infection in chimpanzees results in acute hepatitis, about 2/3 of chimpanzees convalesce, whereas about 1/3 will become persistently viremic; a rate which is much lower compared to humans [88].

Recently, transient acute HCV infection has been reported in tree shrews (*Tupaia belangeri chinensis*) resulting in anti-HCV antibody production, although the efficiency (approximately 30%) is much lower compared to HBV infection [89]. Since the animals used in this study had been caught in the wild, it is not clear whether the minimal histological alterations observed in this study had been really due to HCV infection.

GB viruses

GB viruses A and B (GBV-A and GBV-B) are members of the Flaviviridae family and were isolated from tamarins infected with serum from a human hepatitis patient [90, 91]. A third member of this group represents a related human virus, GB virus C, or alternatively, hepatitis G virus (GBV-C/HGV) [92, 93].

GBV-A has been shown to be a common New World primate virus causing chronic infection without apparent liver disease [94]. GBV-B, which is phylogenetically related to human HCV [95], is capable of causing acute hepatitis in tamarins [96]. Since indigenous isolates of GBV-B have not been isolated from wild tamarins, it seems questionable whether tamarins are the natural host. GBV-C/HGV seems to be a lymphotropic virus rather than a hepatotropic virus. Phylogenetically, it is more related to GBV-A than GBV-B.

GBV-B, a pleiotropic virus, has been proposed as a potential model for the study of human HCV infection, since it is phylogenetically most closely

related to HCV and causes an acute, self-resolving hepatitis in tamarins, resulting in an increase in alanine aminotransferase and mild hepatitic changes in liver histology. Importantly, GBV-B does not cause chronic hepatitis in tamarins, which is different from HCV in humans. Additionally GBV-B infection causes protective immunity after resolution of the infection, which is not the case in resolved human hepatitis C [96].

Transgenic mouse models

Although transgenic and other mouse models have elucidated some important aspects of the pathogenesis of viral hepatitis, they have several limitations: murine hepatocytes are not permissive for human hepatitis virus infections. Therefore, studies of the complete natural life cycle of hepatitis viruses are not possible using these systems. Therefore, most of these mice do not exhibit a liver-specific phenotype and studies on virus-related, immune-mediated mechanisms of liver injury are restricted. Inducible systems (e.g., tTA, *Cre/loxP*) may overcome this problem in the near future. The description of the entire spectrum of transgenic models of viral hepatitis is beyond the scope of this chapter. Therefore, only general remarks are given and some interesting facts deriving from these models are presented.

Several transgenic mouse strains expressing partial or complete copies of the HBV genome have been developed [97–99]. HBV transgenic mice inoculated with HDV develop viral replication and assemble new HDV particles [100]. Recently, a transgenic mouse model for chronic hepatitis was developed [101]. In this model, chronic liver disease resulted from the adoptive transfer of unprimed, syngeneic splenocytes in HBV-transgenic immunodeficient SCID mice. As a result, the HBs antigen and viral DNA were cleared within a few weeks, which were often accompanied by HBs antibody seroconversion, but it did not result in complete viral elimination as indicated by the reappearance of HBV DNA at a later time. Histologically, the livers of these mice showed portal inflammation, acinar inflammation, hepatocellular degeneration, and regeneration as seen in human chronic viral hepatitis.

Chimeric mouse models

There are basically two different chimeric mouse models: the first represents the Trimera mouse model, which is developed *via* preconditioning of normal mice by lethal body irradiation and radioprotection by SCID mouse bone marrow transplantation. Subsequently *ex vivo* infected human liver tissue fragments are implanted under the kidney capsule or in the ear pinna of Trimera mice [102]. A modification of this model is the non-obese

diabetic/severe combined immunodeficiency (NOD/SCID) mouse transplanted with human liver tissue [103].

The second mouse model, of which several modifications exist, is based on the albumin-urokinasetype plasminogen activator (uPA) transgenic mouse. This mouse develops a liver-auto-toxic phenotype with progressive depletion of transgene-bearing hepatocytes and reconstitution of the liver parenchyma by transgene-deficient hepatocytes *via* transgene rearrangement by 2 months of age [104]. When these mice are cross-bred with RAG-2-deficient mice lacking mature T and B lymphocytes [105], hepatocytes may xenograft the livers of the compound transgenics (e.g., woodchuck, human, tree shrew) [106–108]. Recently a derivative model, the uPA/SCID mouse was characterised, which shows occupation of up to nearly 90% of the liver parenchyma by expanded engrafted human hepatocytes [109, 110]. The advantage of these chimeric systems is the ability to study viral replication and viral spread *in vivo* in an animal system, which allows for therapeutic and vaccination studies in a small animal system. Nevertheless, chronic viral hepatitis cannot be evaluated in this model due to the immunodeficiency background of RAG-2- respectively SCID-mice.

Clinically relevant hepatitis in domestic animals

Chronic liver disease in dogs is similar to chronic hepatitis in humans in that there are many different etiopathological factors such as the canine adenovirus type 1, *Leptospiroa interrogans* var. *grippotyphosa*, canine acidophil cell hepatitis virus, copper accumulation, α1-antitrypsin deficiency, drug toxicity, and autoimmunity, although in many cases the etiological cause remains obscure [111–118]. Histologically, canine chronic hepatitis is comparable to human hepatitis and shows portal inflammation with interface hepatitis as well as an acinar inflammatory component and progression to liver cirrhosis [119, 120].

Atypical frequently fatal hepatitis has been reported in lambs by ovine adenovirus type 7 [121]. Simian Varicella Virus may cause fulminant hepatitis in Old World monkeys, which histologically shows similarities to Varicella zoster virus hepatitis in humans (nuclear inclusions) [122].

Concluding remarks

Although liver biopsy may be a valuable tool in evaluating acute hepatitis in humans, especially in transplants, in which specific histological features may facilitate expeditious diagnosis and adequate treatment, liver biopsy represents the gold standard in the assessment of chronic hepatitis since it provides clues for the diagnosis, prognosis (grading, staging) and therapeutic options. Many small and large animal models (including transgenics)

have been developed for studies on chronic viral hepatitis, which have elucidated important aspects of the molecular biology and pathogenesis of viral hepatitis. Nevertheless, the histological disease course in animals differs from that in humans in that the degree of inflammatory activity is generally milder and the development of liver fibrosis or cirrhosis is rarely seen. Future developments (e.g., inducible transgenic models) may allow closer simulation of the human situation and help to improve our understanding of the pathogenesis and prognosis of chronic hepatitis.

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Comparative Hepatitis
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Hepatitis B virus: Lessons learned from the virus life cycle

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Abstract

The human hepatitis B virus (HBV) is the prototype member of the family of hepadnaviridae, small enveloped viruses which replicate their compact and highly organized DNA genome via reverse transcription. In humans, HBV may cause inflammatory liver disease, hepatitis B. With more than 350 million chronically infected people at high risk to develop liver cirrhosis or hepatocellular carcinoma, HBV is one of the most important human pathogens. In recent years, the viral life cycle has been characterised in considerable detail, our understanding of immunology and pathogenesis of hepatitis B has largely improved, and nucleos(t)ide analogues have been established as antivirals. However, current treatment options are still limited because they only rarely eliminate the virus, and thus long-term treatment is required. Following a general introduction, we therefore discuss which steps in the viral life cycle may serve as targets for novel therapeutic strategies.

The human hepatitis B virus

The human hepatitis B virus (HBV) is the prototype member of a family of small enveloped DNA viruses. All members of this family are characterised by a pronounced liver tropism and they are therefore subsumedly named hepadnaviruses (for *hepa*totropic *DNA viruses*). HBV is a non-cytopathic virus since its assembly in hepatocytes proceeds without cell disruption. Thus HBV-producing cells continuously release progeny viral particles. This has been demonstrated in stably transfected hepatoma cell lines [1] as well as in HBV-transgenic mice [2] and explains why HBV infection *per se* does not cause liver damage. It also explains why HBV elicits little or no innate immune responses [3]. When the adaptive immune system becomes activated, however, inflammatory liver disease called hepatitis B becomes evident and the infection may be cleared. This usually happens if adults become infected, but often is missing during neonatal infection.

The viral genome (3.2 kb) consisting of a partially double stranded, relaxed circular (rc) DNA shows an extremely compact organisation with overlapping open reading frames of the viral polymerase and structural proteins encoding the viral capsid and the three envelope proteins. In addition, regulatory elements like promoters, enhancers and the polyadenylation signal overlap with coding sequences [4]. Like the animal hepadnaviruses (e.g., duck hepatitis B virus (DHBV), woodchuck hepatitis B virus (WHV), HBV multiplies its genome by reverse transcription of an RNA pregenome (Fig. 1) [5]. Following fusion of the viral and the cellular membrane the nucleocapsid is transported to the nuclear pore complex where the rc DNA genome is released into the hepatocyte nucleus. Inside the nucleus the rcDNA is converted to a covalently closed circular DNA (ccc DNA) by cellular enzymes. cccDNA encodes four unidirectional, overlapping open reading frames, and constitutes the transcription template for the pregenomic and the subgenomic RNAs.

The pregenomic RNA serves as mRNA for the viral core and polymerase proteins. In addition, it contains a hairpin-loop at the 5'-end, which triggers its encapsidation together with the viral polymerase into an icosahedral nucleocapsid. The nucleocapsid in the virion consists of 240 subunits of the HBV core protein which organise to some extent the surrounding envelope proteins [6]. The viral envelope is built of a lipid bilayer and is densely packed with the large (L), middle (M) and – predominantly – small (S) surface proteins. These proteins are translated from three subgenomic mRNAs. In addition, HBV and other mammalian hepadnaviruses encode a regulatory protein called X, which is required to establish infection *in vivo* in woodchucks [7, 8] and displays pleiotropic effects when studied in cell culture-based assays. Infected cells secrete – in high excess to complete virions – subviral particles, which are empty envelopes of spherical (22 nm particles) and filamentous shape. Spheres contain mostly the S protein and can be detected in the serum of infected individuals as hepatitis B surface antigen (HBsAg). The HBV precore protein, whose coding sequence encompasses that of the core protein, is not required for HBV replication or infection, but it is processed and secreted from infected cells as HBeAg [4, 9].

Hepatitis B virus infection and related disease

HBV infects livers of humans, humanoid primates like chimpanzees [10] and gorillas, and surprisingly *Tupaia belangeri*, a small mammal belonging to the order scandentia [11, 12]. Depending on the host immune response, the virus establishes either transient or persistent infection. HBV is transmitted by perinatal, percutaneous, and sexual transmission routes, as well as by close interindividual contact during early infancy. The latter occurs presumably by open cuts or sores [9]. Vertical transmission from mothers to

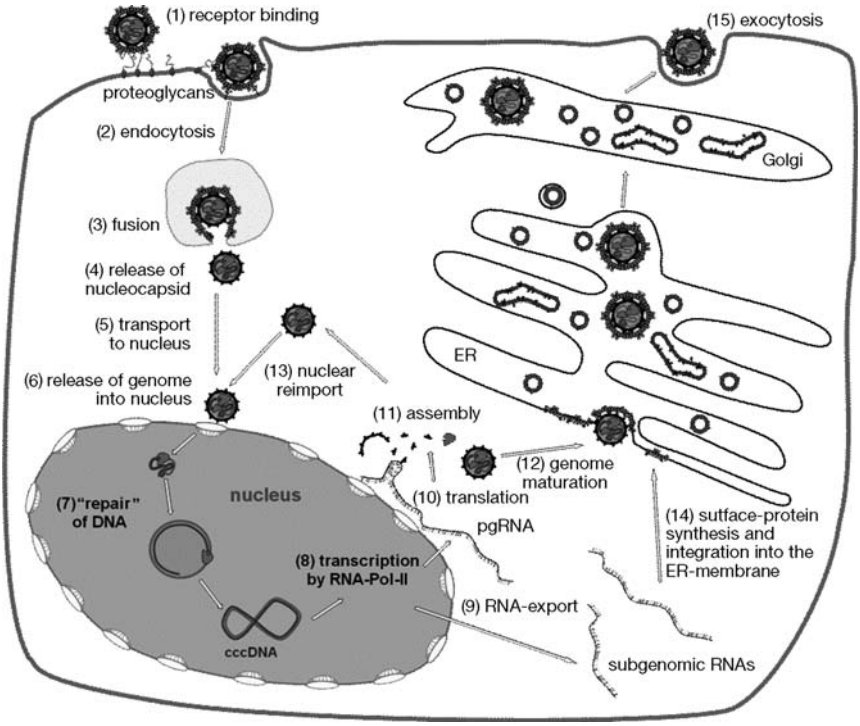

Figure 1. (1) HBV attaches *via* the preS-domain of the L-protein to heparansulfate-proteoglycans of the hepatocyte surface. (2) After endocytosis by an unknown mechanism, fusion of the viral and the vesicular membranes occurs (3) and the DNA-containing nucleocapsid is released (4). After transport to the nucleus (probably along the cytoskeleton of the hepatocyte) (5) and release of the capsid into the nuclear pore complex (6), the partially double-stranded DNA enters the nucleus and is converted to the covalently closed circular (ccc) DNA by cellular enzymes (7). After Pol-II-mediated transcription (8) and export (9) of the pregenomic RNA (pgRNA) into the cytoplasm translation of core protein and the HBV polymerase occurs (10). After self assembly of the complex of pgRNA, polymerase and core protein into an RNA-containing nucleocapsid (11) reverse transcription of the pgRNA to a mature genome takes place (12). This DNA-containing nucleocapsid can either be re-imported into the nucleus (13) or enveloped by the HBV surface proteins at the ER membrane (14). These surface proteins have been synthesised from independently transcribed and exported subgenomic RNAs. Exocytosis of autonomously assembled subviral particles and progeny virions proceeds via the secretory pathway by a still ill understood route (15).

their neonates, or infection during the first year of life, results in >90% in persistent, often life-long infection. In contrast, infection during adulthood is cleared in >90% of cases, and results in life-long protective immunity [13].

In humans, HBV infection may cause inflammatory liver disease – hepatitis B. Worldwide, more than 350 million persons are persistently infected. Chronic HBV infection may be symptomatic or asymptomatic. Persistently

infected individuals without significant, ongoing necroinflammatory disease are termed HBsAg carriers. Necroinflammatory disease is defined by the presence of inflammatory activity and/or fibrosis in liver tissue biopsies and often detected by an elevated serum transaminase activity. Individuals in whom the virus persists for more than six months and in whom HBV causes chronic necroinflammatory disease are classified as having chronic hepatitis B.

The ongoing inflammation in chronic hepatitis B results in liver cirrhosis and hepatocellular carcinoma in more than 25% of patients [9]. Of chronic hepatitis B patients, 15–40% will develop serious sequelae during their lifetime, accounting for 0.6 to 1 million deaths per year worldwide [14].

Immune response to HBV

Viral clearance during self-limited HBV infection is characterised by a vigorous, polyclonal and multispecific $CD4^+$ and $CD8^+$ T-cell response to viral proteins [15, 16]. In contrast, a weak and oligoclonal T-cell response was described in persistently infected individuals [17, 18]. Thus, in patients who develop chronic infection either no sufficient T-cell response is induced or an initially vigorous T-cell response is diminished.

In self-limited experimental HBV infections in chimpanzees a two-phasic T-cell response has been observed. In the initial phase T-cells control viral replication and antigen expression in a non-cytotoxic fashion by the secretion of cytokines with interferon (IFN)-γ being the main player. Hereby, the number of antigen-positive hepatocytes as a potential target of T-cell killing is reduced. In a second phase, the cytotoxic activity of T-cells is predominant and remaining infected cells are eliminated [19, 20].

Translation of viral proteins during replication results in the processing of virus-derived peptides. These peptides are presented by major histocompatibility antigens class I (MHC I) molecules on the surface of infected hepatocytes. Cytotoxic T-cells, which recognise these antigens in the context of the respective MHC I molecule, may be activated to kill the infected cell either by secretion of perforin and granzyme or by induction of apoptosis. Since HBV infected cells continuously express viral proteins, they are potential targets of T-cell killing, if activated T-cells gain access to the infected cells.

The failure to induce or maintain a vigorous T-cell response is astonishing since huge amounts of HBsAg and HBeAg circulate in the blood of infected patients. The majority of T-cell epitopes are shared among all viral envelope proteins. Thus, HBsAg-derived peptide antigens should be sufficient to elicit cellular as well as humoral immune responses targeting the viral envelope. HBeAg is small enough to cross the placenta and is discussed to induce neonatal tolerance [21, 22]. Since HBeAg shares most of its coding sequence with HBV core proteins, both proteins share the T-

cell epitopes. In adult, T-cell receptor-transgenic mice, HBeAg suppressed the T-cell response to HBV core protein expressing cells, indicating that HBeAg/core cross-reactive T-cells either are deleted or become anergised [23].

Several possible reasons for a diminished T-cell response during chronic viral hepatitis are discussed: T-cell modulation due to or local induction of tolerance in the liver [24, 25], systemic immunomodulation by the induction of regulatory T-cells and/or the exhaustion of T-cells due to antigen overload [13, 26].

Taken together, induction and maintenance of a strong and vigorous T-cell response seems to be crucial to achieve virus elimination. However, it is important to notice that a cytotoxic T-cell response is thought to be responsible for both, viral clearance and liver injury during HBV infection [13, 27].

Antivirally active cyokines

Cytokines are released by cells of the immune system, but also by parenchymal cells. Their main role is to mediate immune responses, but some have been proven to affect HBV replication in hepatocytes: type I and II IFN, tumour necrosis factor (TNF)-α and IL-6. Whereas secretion of IFN-α/β, TNF-α and IL-6 may be induced by recognition of molecular patterns by membrane bound or viral nucleic acids by endosomal or cytoplasmic pattern recognition receptors in a broad variety of cell types, IFN-γ is released by immune cells, e.g., T-cells, NK-cells or macrophages.

IFN-α is long known to elicit antiviral activity in HBV infection, and has been the first approved antiviral treatment for hepatitis B. After binding to specific receptors on the surface of infected cells, IFN-α/β have the potential to trigger the activation of multiple noncytolytic intracellular antiviral pathways that, for example, interfere with translation of viral RNAs or induce their degradation.

Adoptively transferred virus-specific T-cells can abolish HBV gene expression and replication in the liver without killing the hepatocytes. This antiviral function was mediated by IFN-γ and TNF-α secreted by the transferred T-cells or by antigen-nonspecific macrophages or NK cells activated following the T-cell transfer [28]. This showed that IFN-γ and TNF-α are central players in the non-cytopathic control of HBV infection.

In a similar manner, application of IL-12 to HBV-transgenic mice either prevented the assembly or triggered the degradation of the nucleocapsid particles within which HBV replication occurs [29]. IL-6 influences HBV gene expression [30]. Antiviral cytokines may thus activate independent virocidal pathways influencing viral gene expression or eliminating HBV nucleocapsid particles and their cargo of replicating viral genomes.

Current therapeutic options

Currently approved treatment options for chronic hepatitis B are related to the direct or immunmodulatory action of interferon-α and the inhibition of the HBV reverse transcriptase activity by nucleoside analogues. Presently there are six approved medications: IFN-α, PEG-IFN-α, Lamivudine (3TC), Adefovir, Telbivudine and Entecavir. Although these drugs to a different level reduce viral loads down to several logs, elimination of the virus is hardly achieved [31–33]. In addition the selection of resistant mutants during therapy arises. Nevertheless, it has clearly been demonstrated that high viremia is an important risk factor associated with progression of HBV-related liver disease and development of hepatocellular carcinoma [34]. Therefore, current therapeutic goals are sustained suppression of HBV replication and remission of liver disease [35, 36].

IFN-α has direct antiviral effects and is immunomodulatory and thus shall stimulate the host immune response to eliminate the virus. Pegylated versions improve its bioavailability and thus its therapeutic efficacy [37]. However, its side effects (e.g., induction of hepatitis flares, fever, myalgias, thrombocytopenia, and depression) make it a difficult treatment for many applications and exclude therapy of advanced or decompensated liver disease. In addition, therapeutic effects are limited: only about 20–30% HBeAg-positive patients profit from IFN-α treatment, seroconvert to anti-HBe and lose serum HBV-DNA. Loss of HBsAg (indicating virus clearance) is achieved in <8% of patients. Although HBeAg-negative patients have a better response rate, response is not durable in most cases [33].

Nucleos(t)ide analogues inhibit the viral reverse transcriptase and are usually well tolerated. Nucleos(t)ide analogues control HBV replication but rarely eliminate the virus. 30–50% of treated, HBeAg-positive patients seroconvert after long-term treatment to an anti-HBeAg state and control HBV replication (summary in [36]). However, this is close to the numbers that spontaneously seroconvert, and HBeAg-seroconversion alone does not protect from the development of complications [38]. HBV cccDNA persists in the host cell nucleus, continues to produce HBsAg, and may cause a viral rebound and recurrent disease if the treatment is stopped. Continuous treatment is therefore required, which often selects resistant viral variants which are characterised by specific amino acid exchanges in the HBV polymerase [39]. Emergence of antiviral-resistant mutations can lead to negation of the initial response, and in some cases hepatitis flares and hepatic decompensation [33]. Because of the overlapping reading frames of the polymerase with HBV envelope proteins, mutations which mediate resistance may alter the latter or lead to premature translational termination [40–42]. This may affect virion secretion or result in the generation of surface proteins with a higher oncogenic potential [43].

Taken together, true cure of infection (loss of HBsAg and sustained disappearance of viremia, as measured by stringent PCR assays) is achieved

only infrequently (<1%) with the current regimens [33]. Without an efficient immune response, long-time treatment is required, which raises concerns about drug efficacy and safety, but also high treatment costs [44]. An increasing number of antiviral drugs targeting reverse transcription but also other steps in the viral life cycle are currently developed, which might improve treatment outcome in the future [45].

Novel and established therapeutic approaches targeting the HBV life cycle

Receptor binding and entry

Due to the lack of an infectable cell line and the dependence on primary human hepatocytes the mechanism of HBV entry was for a long time not understood. The recent development of a cell culture-based HBV infection system [46] and the possibility to produce sufficient amounts of recombinant HBV [47, 48] or HDV [49, 50] particles with mutations in envelope proteins facilitated the mapping of important determinants of HBV entry. Having these tools now at hand, it again became a topic of intensive research which cellular proteins are functionally involved in entry and at which specific stages of entry the subsequently described viral determinants play specific roles.

One general theme of hepadnaviral infectivity is the requirement of N-terminal myristoylation of the respective L-proteins for infection. Knocking out [51–53] or displacement of the myristoylation site of the L-protein to the N-terminus of the M- or S-protein [54] results in a loss of viral infectivity. Through deletion analysis it became furthermore clear that the N-terminal 77/75 amino acids of the preS1-domain of the L-protein are essential for infection [48, 55] and do not interfere with assembly. In contrast, the preS2-domain is dispensable [55–58] although the presence of an amphipathic motif which supports cell permeation of foreign proteins [59] initially suggested [60] a critical role. In addition to the N-terminal 2/3 of the preS1-domain of the L-protein, the HBV S-domain plays (a) crucial role(s) for the infectivity of the virus. Introduction of mutations into the cytosolic loop [61] as well as replacement of Cys-residues in the antigenic loop [62, 63] renders hepatitis delta virus, which carries an HBV envelope, non-infectious. Since accumulating evidence suggests that HDV and HBV use identical entry pathways, HBV most likely also requires the S-domain for infection [64].

The observation that both, myristoylation of the HBV L-protein as well as the integrity of the N-terminal 77 preS1 amino acids are essential for the infectivity of HBV initiated investigations on the potential of synthetic and recombinant myristoylated or otherwise acylated HBV-preS-derived peptides and fusion proteins to inhibit HBV and HDV infection *in vitro* [46, 47, 65–67] and *in vivo* [68]. The outcome of these experiments revealed

that HBV-preS-derived lipopeptides are very potent entry inhibitors of HBV and HDV infection with *in vitro* IC_{50} in the low nonomolar or even picomolar range [67]. Their activity essentially depends on a highly conserved sequence motif 9-NPLGFF-15 which is substantially increased by the subsequent 30 amino acids. The activity also increases with the length of the N-terminal acyl moiety (e.g., IC_{50} of C_5-HBVpreS/4-48: ca. 1 μM, IC_{50} of C_{18}-HBVpreS/2-48: ca. 250 pM) [67]. The observation that five subcutaneous administrations of less than 0.2 mg/kg of a myristoylated HBVpreS/2-48 lipopeptide completely prevented HBV infection in the liver of transplanted uPA/RAG-2 mice makes this novel class of HBV entry inhibitors interesting for further clinical applications, especially to treat chronic HBV and HDV infections, possibly in combination with other therapies.

Uncoating and nuclear import

Although all viruses need to uncoat their genomes and many of them need to import it into the nucleus, they usually abuse cellular pathways for this. Therefore, targeting these steps in the viral life has not succeeded yet. Once the HBV capsid has reached the cytoplasm, we have presently no tool in hand to prevent the virus establishing its genome in the nucleus.

Viral gene expression and RNA export

Natural helioxanthin compounds have been described to elicit antiviral activity against a variety of viruses and potently inhibit HBV replication. Helioxanthin analogue 8-1 has been characterised to inhibit HBV promoter activity by reducing the activity of the essential transcription factor [69].

Antiviral cytokines may affect HBV replication by altering gene expression at transcriptional and posttranscriptional steps. Similar to 8-1, IL-6 affects the activity of promoters in the HBV genome directly by affecting transcription factors. It is, however, discussed controversially whether it thereby activates [30] or inhibits (Hösel et al., unpublished results) HBV replication.

IFN-α/β reduced HBV RNA specifically possibly by affecting the balance of transcription factors [70]. In addition, interferon-induced RNA-binding proteins have been described which may stabilize and/or destabilise the pregenomic HBV RNA by binding an RNAse-sensitive stem-loop structure [71]. However, the main activity of type I IFN is on cytoplasmic HBV capsids (see below).

The possibility to use RNA interference to selectively prevent translation and induce degradation of mRNAs promoted investigation about its antiviral potency. Because of the largely overlapping open reading frames in the HBV genome and due to the fact that all HBV RNAs use the same

polyadenylation site, HBV is a very interesting target for RNA interference. Therefore, several studies used transfection of small interfering RNAs (siRNA) or expression of short hairpin RNAs (shRNAs) to address the question whether HBV-specific shRNAs may abrogate HBV gene expression in cell culture and *in vivo* in HBV replicating transgenic mice [72–75]. Since these molecules are easier to handle and show a higher efficacy, they replaced the use of antisense oligonucleotides or ribozymes.

Adenoviral transduction of hepatocytes from HBV-transgenic mice with shRNA targeting genomic and subgenomic RNAs resulted in a drastic decrease of HBV secretion and a significant reduction of HBV core expression [75]. Adenoviral vectors efficiently target the liver, but a high dose application, which would be needed to target all HBV-infected hepatocytes, may result in unwanted side effects. Therefore extensive efforts are undertaken to allow a direct administration of siRNAs as therapeutics and selectively target them to the liver. Major problems are the serum stability and a possible activation of pattern recognition receptors by single- or double-stranded RNA molecules, which may cause side effects [76]. The latter, however, may also be used as an antiviral strategy (Protzer and Hartmann, unpublished results), but requires a liver-specific recognition to minimise side effects.

Through lipid encapsidation of chemically modified and thereby stabilised siRNAs, Morrissey et al. [77] demonstrated a more than 1 log reduction of HBV serum DNA-levels in treated HBV transgenic animals. This proves that siRNAs have a therapeutic potential for the treatment of HBV infection. However huge efforts are necessary to optimise the application form and specificity and efficacy of liver targeting of siRNA-based therapies in the future.

Encapsidation of pregenomic RNA and capsid assembly

Another promising new therapeutic strategy to specifically interfere with a yet unaddressed step in the HBV life cycle is the prevention of nucleocapsid assembly followed by an accelerated degradation of the partially assembled capsid protein oligomer. This therapeutic option is based upon the observation that a group of synthetic substances called dihydroarylpyrimidines are able to prevent secretion of HBV particles in the constitutively HBV-expressing cell line HepG2.2.15 [78]. Two prototype members of dihydroarylpyrimidines, Bay 39-5493 and Bay 41-4109, have been characterised in some detail with regard to their activity and their mechanism of action. They display an IC_{50} of 30 nM and 50 nM, respectively, and differ in their potential to bind and misdirect capsid assembly. Interestingly, Bay 41-4109 not only prevented nucleocapsid formation but also induced core protein degradation *via* a proteasome-dependend pathway. Its activity in HBV-transgenic mice after oral application [79] indicated that this substance

targeted the liver, was taken up into hepatocytes and induced a reduction of HBV secretion *in vivo*.

More recent studies aimed at deciphering the mechanism of action of dihydroarylpyrimidines. They indicate that Bay 41-4109 acts in a concentration-dependent manner in a dual mode by accelerating and misdirecting capsid assembly [80, 81]. Recently, a whole set of dihydroarylpyrimidine derivatives with increased activities have been developed and are presently characterised with regard to their bioavailability, genotype specificity of action, toxicity and *in vivo* activity (Urban, unpublished results). Presently we lack detailed structural information on the exact binding site(s) of Bay 41-4109 or its derivatives to the HBV core protein. Structural information would allow estimation of the probability of viral escape mutants, which may evolve under selection pressure. Furthermore, *in vivo* application requires induction of core protein degradation in order to prevent its intracellular accumulation. Irrespective of this lack of knowledge, dihydroarylpyrimidines represent a highly interesting group of new molecules for further clinical developments.

Interestingly, the antiviral activity of heme oxygenase 1 (HO-1) targets the same step in the viral cycle – capsid assembly. HO-1 is a metabolic enzyme catalysing the initial and rate-limiting step in the oxidative degradation of heme. In addition, HO-1 has immunomodulatory properties and, e.g., prevents apoptotic liver damage in immune-mediated hepatitis in mice [82]. It has recently been shown to ameliorate HBV-associated liver damage in mouse models of acute and chronic hepatitis B [83]. Furthermore, it showed a profound antiviral activity repressing HBV replication at a posttranscriptional step. Interestingly, induction of HO-1 in hepatocytes reduced the stability of the HBV core protein leading to a reduction of capsids containing viral replicative intermediates, and – as a consequence – blocked the refill of nuclear cccDNA [83]. Thus, induction of HO-1 might be an interesting novel therapeutic option for inflammatory flares of hepatitis B.

Cytoplasmic HBV capsids as target of intracellular defence pathways

IFN-α/β as well as IFN-γ, upon binding to their receptors on hepatocytes, trigger intracellular events that either inhibit the assembly of pgRNA-containing capsids or accelerate the degradation of capsid subunits or complete capsids [19, 84, 85]. Whereas IFN-α may also downregulate HBV RNA at the transcriptional level [70, 86], the activity of IFN-γ remains posttranscriptionally [70, 87]. Wieland et al. [19] proposed that IFNs activate hepatocellular mechanism(s) preventing the formation of replication-competent HBV capsids and, thereby, inhibit HBV replication. TNF-α destabilises HBV capsids independent of IFN-signaling pathways *via* activation of NF-κB [88, 89]. Thus, cytoplasmic HBV-RNA containing capsids seem to be a common

target of intracellular defense mechanisms activated by cytokines which are antivirally active against HBV.

Reverse transcription

The fact that HBV, although carrying a DNA genome in the virion, replicates *via* reverse transcription (RT) of an RNA intermediate (pgRNA) and thus encodes a polymerase with RT- and RNAseH-activities constitutes the basis for blocking this RT step. Selectively active nucleos(t)ide-analogues efficiently block RT in presently used therapeutic regimens. However, RT inhibitors alone do not eliminate the virus, since they act at a late step in the viral life cycle and do not directly affect established cccDNA in the nucleus,

Lamivudine (LMV, Zeffix®), a cytidine-analogue (3TC) was initially developed for the therapy of HIV patients and was approved for chronic hepatitis B in 1998 [90–92]. However LMV monotherapy results in a fast development of resistant HBV mutants (56% resistance within 3 years of therapy), most frequently in an M204V/I exchange within the YMDD-motif of the polymerase [93]. This mutation is often followed by compensatory mutations that increase the replication defect caused by the YMDD mutation [94, 95].

Resistance results from genetic adaptation of the virus to the selection pressure caused by drug treatment. The development of resistance is promoted by (i) the high error rate of the viral polymerase (no proof-reading activity), (ii) the strength of the selection pressure of the drug (iii) the number of mutations needed to gain resistance combined with 'fitness' of the resistant HBV variant (iv) the replication space in the liver and (v) the virus titre in the patient. The fast development of LMV resistance might be caused by an only partial repression of replication during therapy (virus titres in patients are reduced by 'only' 4 logs in patients treated with 100 mg LMV daily). Moreover, the fact that a single point mutation can already mediate resistance and the observation that compensatory mutants can reverse the initial drop of replication competence also abet therapy failure during long treatment periods.

Three other oral nucleos(t)ide analogues are currently marketed and approved as a first line of therapy for the treatment of chronic hepatitis B: adefovir dipivoxil (ADV, Hepsera®), an AMP analogue; entecavir (Baracude®), a guanosine analogue, and telbivudine (Sebivo®), a thymidine analogue. In addition, tenofovir, which is approved for HIV treatment, proved more active than adefovir in HIV/HBV coinfected patients [96], and is currently tested in HBV monoinfection. All therapies lower the viral load, reduce serum transaminase activity and improve liver histology.

Several studies have shown that development of resistance in adefovir-treated patients is less frequent than in LMV-treated patients (18% after 3

years of therapy) [97]. In addition to this slower rate of resistance development, Adefovir is effective against lamivudine resistant mutants [98–101] and can therefore be used in 'add on'-therapies in patients with viral breakthrough under LMV treatment.

For Entecavir a much lower dose is needed for an efficient reduction of viral loads in the serum of chronically infected patients (0.5–1.0 mg/patient/day). As expected, monotherapy with Entecavir results in a drastically reduced rate of arising resistant mutants [102]. However, LMV-resistant mutants are to a large extent cross-resistant to entecavir, and thus need alternative regimens. Telbivudine also proved more effective as a first line therapy and selected fewer drug-resistant viral variants than LMV [103, 104].

Since the main hurdle of RT-inhibitor therapy is the selection of drug resistant mutants, combination therapy is often discussed, but has not proven to be superior over monotherapy so far.

Nuclear reimport of viral genomes and accumulation of a cccDNA pool

Currently, no means are available to prevent import of viral genomes into the nucleus –whether this is the incoming viral genome (see above) or these are newly synthesised viral genomes. In the nucleus, the incomplete DNA genomes are completed by host enzymes to cccDNA molecules serving as the viral transcription template and as the viral persistence form. Newly formed capsids may be targeted from the cytoplasm to the nucleus and thus may increase the number of nuclear viral replication templates, i.e., cccDNA molecules. When reverse transcription of the HBV genome or maturation of viral capsids is efficiently blocked, accumulation of a pool of HBV cccDNA in the nucleus of infected cells is hindered, and HBV cccDNA may be lost by cell division. However, direct targeting of the nuclear cccDNA is hard and may only be achieved by targeted nucleases.

Envelopment and release of progeny virions

So far, no direct inhibitors of the envelopment of HBV capsids and maturation or release of virions are available. However, since at least trace amounts of HBV envelope proteins are displayed on the plasma membrane of infected hepatocytes, these may serve as a target for T-cell-based treatment strategies. T-cells redirected against HBV surface proteins by artificial T-cell receptors, which recognise native S or L protein on the cell surface using a single-chain-antibody fragment as an antigen binding domain, selectively kill infected hepatocytes in cell culture [105]. However, efficacy and safety of such an approach remains to be shown in *in vivo* models.

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Comparative Hepatitis
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Chronic hepatitis C: Portrait of a silent epidemic and the etiologic agent

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Abstract

Chronic liver disease caused by infection with the hepatitis C virus (HCV) is an important medical problem. Although primary infection usually is mild, and often asymptomatic, in the vast majority of cases HCV establishes persistence that in the course of one or several decades may result in chronic liver diseases including liver cirrhosis and hepatocellular carcinoma. Therapeutic options are limited and a vaccine to prevent HCV infection is not in sight. Diagnostic tests to detect HCV-containing blood products have become available after the first molecular cloning of the viral genome. This achievement also opened the field for studies of the viral replication cycle as well as the development of selective antiviral drugs. HCV is a hepatotropic flavivirus which has several remarkable properties. Among these are the potent inhibition of the induction phase of the innate antiviral response, the high genomic plasticity and the unusual strategy of virus particle assembly that occurs in close association with lipid droplets and the very-low-density lipoprotein production pathway. This chapter will briefly summarise some old and new aspects of chronic hepatitis C and of the pathogen responsible for this disease.

Introduction

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) worldwide. End-stage liver disease due to chronic hepatitis C is now the leading indication for liver transplantation. A protective vaccine is not available yet and therapeutic options are still limited. As a consequence, the number of patients presenting with long-term sequelae of chronic hepatitis C, including HCC, is expected to further increase for the next two decades even if the incidence of new cases has diminished since the introduction of anti-HCV screening of blood and blood products [1, 2]. Given this scenario, there is an urgent need to develop more effective and better tolerated therapies for chronic hepatitis C.

Epidemiology

It is estimated that 120–180 million people worldwide are infected with HCV [3]. The virus is parenterally transmitted. With the introduction of anti-HCV screening of blood and blood products in 1990, new cases of posttransfusion hepatitis C have virtually disappeared. Indeed, over the last 20 years the risk of posttransfusion hepatitis C could be reduced from about 1 per 100 blood units transfused to 1 per 2,000,000–10,000,000 [4]. Unfortunately, the lack of systematic screening of blood donors continues to result in HCV transmission in countries with developing or transitional economies. In these countries, large-scale immunisation and parenteral therapy programs (e.g., for the treatment of schistosomiasis in Egypt or leishmaniasis in India) as well as surgical and dental procedures with inadequately sterilised equipment have also been important routes of transmission [5, 6]. In the Western world, intravenous drug use is now the major identifiable mode of HCV transmission [3]. In addition, HCV transmission has been described in the nosocomial setting [7–9]. Occupational needlestick injuries from anti-HCV positive sources result in seroconversion in on average 3% of recipients. Intranasal cocaine use has been identified as a possible mode of transmission ('straw sharing') [10]. Sexual transmission is rare and correlates with high-risk sexual practices. The risk of perinatal transmission is probably less than 5% unless the mother is co-infected with HIV [11]. Intriguingly, in clinical practice no epidemiologic risk factor can be identified in up to one third of patients with hepatitis C ('sporadic hepatitis C').

Natural history

After an incubation period of 3–12 weeks HCV infection is usually followed by a clinically inapparent hepatitis [12]. Only about 25% of patients are symptomatic. Fulminant hepatitis C is very uncommon. One of the most important clinical features of hepatitis C is its progression to chronicity in 50–80% (Fig. 1). Typically, patients with chronic hepatitis C have few, if any symptoms and these are usually nonspecific, intermittent, and mild. The most common symptom is fatigue.

The natural history of chronic hepatitis C has been analysed in several retro- and prospective studies [13, 14]. While no increased mortality was found in the retrospective Veterans Administration study [15], other studies indicated that chronic hepatitis C frequently progresses to cirrhosis and HCC [16]. Studies with a follow-up of up to 25 years performed in cohorts of women infected at a young age *via* contaminated anti-D immunoglobulin have shown a benign course [17, 18]. By contrast, a recent report describing a cohort of male plasma donors infected in the 1970s revealed a poor outcome, with one third of patients having advanced liver disease and 15% having developed HCC or end-stage liver disease after a follow-up of more

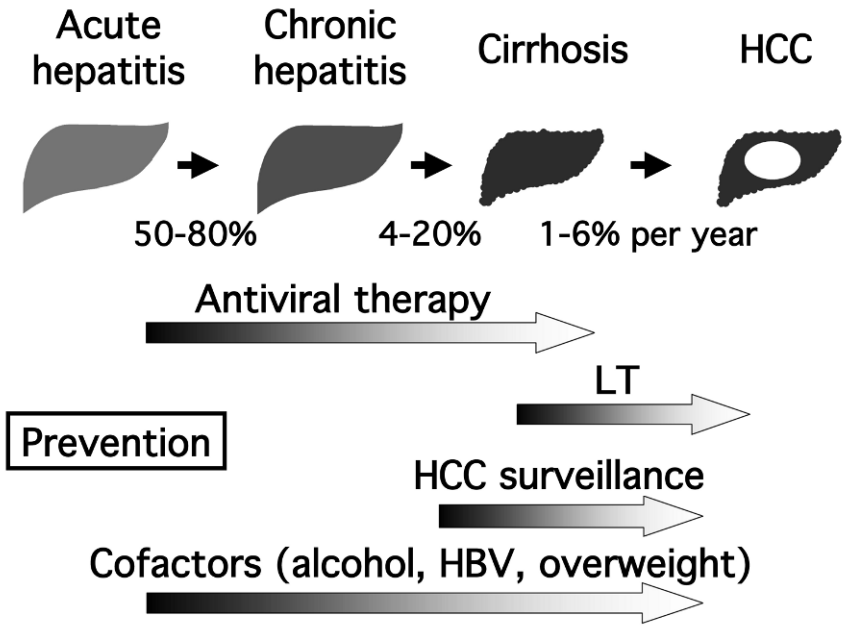

Figure 1. Natural history and management of hepatitis C. Comprehensive management of hepatitis C involves not only antiviral therapy but also preventive measures, liver transplantation (LT) for patients with end-stage liver disease, surveillance for hepatocellular carcinoma (HCC) in patients with cirrhosis, and the control of cofactors of disease progression, including, among others, alcohol, hepatitis B virus (HBV) or other coinfections, and overweight.

than 30 years [19]. Factors associated with more frequent and rapid progression to cirrhosis are higher age at the time of infection, male sex, alcohol consumption, coinfections with HBV or HIV, nonalcoholic fatty liver disease (NAFLD), hepatic iron overload, smoking and immunosuppression. Comprehensive management of chronic hepatitis C takes these factors into consideration and aims at improving the ones that can be modified.

Once cirrhosis is established, the rate of HCC development is 1–6% per year. HCV infection is responsible for a substantial proportion of the increase in HCC incidence and mortality recently observed in most Western countries [20]. Although experimental evidence raises the possibility that HCV might operate through direct pathways in promoting malignant transformation of hepatocytes, it is generally believed that HCC associated with chronic hepatitis C develops through a general pathway of increased liver cell turnover, induced by chronic liver injury and regeneration, facilitating the development and manifestation of multiple and stepwise genetic alterations [21].

HCV genome organisation and polyprotein processing

HCV has been classified as a member of the genus *Hepacivirus* that together with the genera *Pestivirus* and *Flavivirus*, and the as of yet unassigned species GB virus A, GB virus B, and GB virus C/hepatitis G virus belongs to the family *Flaviviridae* [22]. HCV genomes can be grouped into at least six genotypes that differ in their nucleotide sequence by 31–34% [23–25]. Furthermore, within an HCV genotype, several subtypes can be defined that differ in their nucleotide sequences by 20–23%. The variability of HCV genomes is due to the high error rate of the viral RNA-dependent RNA polymerase (RdRp), which has been calculated to be in the range of about 10^{-4} [26].

The genome of HCV is a single-stranded RNA of positive polarity that has a length of approximately 9.6 kb (Fig. 2A). The genome encodes a large polyprotein of about 3,000 amino acids that is expressed *via* an internal ribosome entry site (IRES) formed by domains II to IV in the 5' non-translated region (NTR) (Fig. 2B). The 3'-NTR has a tripartite structure composed of an about 40 nt-long variable region downstream of the HCV coding region, a poly (U/UC) tract of heterogeneous length, and a highly conserved 98 nt sequence named X-tail (Fig. 2B). Both domains I and II in the 5' NTR, as well as the X-tail in the 3' NTR are essential for RNA replication. An extra *cis*-acting RNA element (CRE) has been identified in the 3'-terminal coding region of NS5B [27] (Fig. 2B). This CRE (designated 5BSL3.2) is required for RNA replication through a RNA–RNA interaction with a sub-region designated as SL2 in the X-tail [28].

As for the other members of the flavivirus family, the large polyprotein encoded by the HCV genome is processed by cellular and viral proteases to generate at least 10 viral proteins [29–32] (Fig. 2C) that will be described in brief.

The core protein resides at the very N-terminus of the polyprotein and is released from the precursor by two cleavages. The first one separates core from E1 by signal peptidase and the second removes the E1 signal peptide by signal peptide peptidase, releasing mature core with an apparent molecular weight of about 21 kDa [33, 34]. The core protein is the major constituent of the nucleocapsid that may form by self-assembly assumed to be triggered by interaction with structured RNA [35, 36]. Interestingly, in transfected and infected cells core localises to lipid droplets and recent data suggest that these droplets play a very important role in virus particle assembly [37, 38].

E1 and E2 are heavily glycosylated type I transmembrane proteins [39] with an N-terminal ectodomain and short C-terminal hydrophobic transmembrane domains. The HCV envelope glycoproteins assemble as non-covalent heterodimers [40] with the glycans playing an important role for virus infectivity [41]. Two hypervariable regions (HVRs) have been identified in E2 with HVR1 residing in the N-terminal 27 amino acid residues

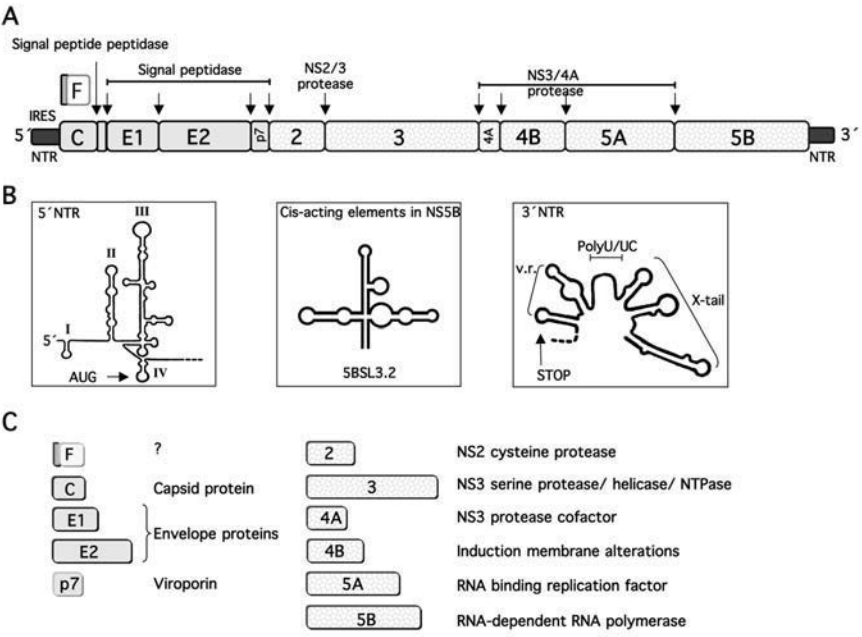

Figure 2. Organisation of the HCV genome, essential *cis*-acting RNA elements and functions of viral proteins. (A) Structure of the HCV genome. Cleavage sites and involved proteases are indicated by arrows. (B) RNA elements in the viral genome. The 5' NTR with its 4 domains is shown in the left box, the CRE in the NS5B coding region in the middle box and the 3' NTR that is composed of the variable region (v. r.), the poly (U/UC) tract and the 3' terminal X-tail is shown in the right box. (C) Polyprotein cleavage products and their functions in the viral replication cycle. No functions could be ascribed to the F-protein.

[42]. Although the sequence variability of HVR1 is high, the chemico-physical properties of the amino acid residues at each position and the conformation of HVR1 itself are highly conserved among the different genotypes indicating an important role of HVR1 during virus adsorption and entry [43, 44]. Based on recent comparison of 391 inter- and intra-host E2 sequences derived from 17 subjects infected with HCV, a new HVR was identified, termed HVR3 [45].

The small polypeptide p7 is composed of two transmembrane regions connected by a short cytoplasmic loop [46, 47]. When expressed in a heterologous system, p7 localised to the ER and mitochondrial membranes [47–49]. P7 appears to form an ion channel [50], presumably essential for infectivity *in vivo* [51] but dispensable for RNA replication [52, 53]. Very recently, it was shown that p7 is essential for efficient assembly and release of infectious virions and p7 promotes virus particle production in a genotype-specific manner [54]. Virus infectivity, however, is largely independent of p7 [53].

The integral membrane protein NS2 is dispensable for RNA replication [52, 55], but it is essential for virus production in cell culture and infectivity *in vivo* [54, 56]. NS2 contains an N-terminal transmembrane domain and a C-terminal cysteine protease domain with His-143, Glu-63 and Cys-184 forming the active site [57–59]. Enzymatically active NS2 is a dimer with 2 composite active sites [60].

NS3 is a multifunctional protein, containing a serine protease located in the N-terminal one-third and an RNA helicase/NTPase located in the C-terminal two-thirds of the protein. These enzymatic activities have been well characterised, and high-resolution structures have been solved [61–63]. Full NS3 protease activity requires activation by NS4A that intercalates into the NS3 N-terminal protease domain. In addition, an N-terminal transmembrane segment in NS4A tethers the NS3-4A enzyme complex to intracellular membranes [64]. The NS3-4A serine protease is responsible for polyprotein cleavage in the region C-terminal of NS3, and this activity is essential for the generation of the viral replication complex. These properties make NS3 an ideal target for antiviral therapy.

The NS3 helicase is a member of the superfamily 2 DExH/D-box helicases [65], which are capable of unwinding double-stranded RNA, or of single-stranded RNA regions with extensive secondary structure in an ATP-dependent manner. The helicase activity may be involved in initiation of RNA replication, contribute to the processivity of the replicase complex and may be involved in the dissociation of the replicative form into positive- and negative-strand HCV RNA.

NS4B is a highly hydrophobic nonstructural protein inducing membranous vesicles that serve as a scaffold for the assembly of the HCV replication complex [66–69]. NS4B appears to be palmitoylated at two C-terminal cysteine residues. Moreover, NS4B can oligomerise, which may contribute to the induction of membrane curvature [70].

NS5A is an RNA binding protein required both for RNA replication and for virus particle assembly. It is composed of an N-terminal amphipathic alpha-helix serving as a membrane anchor [71, 72] and three distinct domains that are separated by two trypsin-sensitive low complexity sequences. Domain I appears to be involved in RNA binding; domain II contributes to RNA replication and particle assembly; domain III is largely dispensable for RNA replication and is primarily required for assembly [73, 74]. NS5A is expressed as a basally phosphorylated and a hyperphosphorylated form (p56 and p58, respectively). Phosphorylation is mediated, at least in part, by casein kinase I alpha [75, 76]. Interestingly, partial inhibition of NS5A hyperphosphorylation enhances RNA replication, arguing that replicase activity may be regulated by the phosphorylation status of NS5A [77].

The key enzyme responsible for RNA amplification is the NS5B RdRp [29]. This viral enzyme has been extensively studied, and several high-resolution X-ray crystal structures have been described [78, 79]. NS5B has the classical finger, palm and thumb subdomains with the special feature that

the finger and thumb subdomains have extensive interactions, resulting in a completely enclosed active site. RdRp activity seems to be modulated by interaction with other viral proteins (NS3 and NS5A) as well as by cellular factors, especially cyclophilins [80]. It is assumed that the antiviral activity of cyclosporine is due to a sequestration of cyclophilin [81].

In addition to the polyprotein, HCV appears to encode a small protein, designated ARFP (alternative reading frame protein), or F (frame shift), or core+1 that is produced by internal initiation of translation of an alternative reading frame or by ribosomal frame shifting within the core gene [82]. This protein is dispensable for RNA replication in cell culture and *in vivo* and its role in the viral replication cycle, if any, is largely unknown [52, 82, 83].

Anatomy of the HCV particle

The basic biophysical properties of the HCV particle were initially deduced from infectivity studies in chimpanzees. The results indicated that HCV forms enveloped particles with an average diameter of about 50–70 nm [84, 85]. These properties are similar to the ones observed with HCV particles produced in cell culture [86]. Very recently, a 3D reconstruction model of HCV-like particles, produced in insect cells was proposed. By analogy to the high-resolution structures of Dengue virus and tick-borne encephalitis virus envelope glycoproteins, HCV E1 and E2 heterodimers may form dimers that are oriented parallel to the surface of the virus particle [87].

HCV particles have the propensity to bind to different serum components, such as lipoproteins [88] and immunoglobulins [89], explaining the heterogeneity of the particles in terms of size, density and sedimentation coefficients. Association of HCV particles with immunoglobulins has been reported, and these complexes exhibit an intermediate density of approximately 1.17–1.21 g/ml. In contrast, most HCV particles present within serum from an immunoglobulin-deficient patient banded at a density of less than 1.08 g/ml [90]. This virus could be immunoprecipitated with antibodies against apolipoprotein-B (apoB) and apolipoprotein-E (apoE), which are components of low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL). Additionally, it has been found that blocking VLDL assembly reduces HCV production in cultured hepatocytes [91]. Finally, in patients HCV particles have been detected as part of very large lipoprotein complexes, designated lipo-viroparticles [92]. All these findings are in line with the assumption of a close association of circulating HCV particles with LDL/VLDL. The assumption is corroborated by the dependence of HCV infection on LDL-receptor and SR-BI, which are the receptors for LDL and HDL, respectively [93–95].

Thus far we do not know how and when virus particles associate with lipoprotein components. However, since HCV assembly takes place in close proximity of lipid droplets [37, 38], which serve as a lipid source for the

assembly of VLDL particles, we hypothesise that association of structural proteins with one or several components of the VLDL pathway takes place during assembly. Moreover, HCV may exploit the VLDL pathway to assure particle secretion.

Hepatitis C virus replication cycle

The replication cycle starts with virus binding *via* the envelope glycoprotein E1/E2 complex to its cognate receptors presumably in a consecutive manner (reviewed in [29, 96, 97]) (Fig. 3). Virus particles may be trapped by interaction with heparan sulphate and LDL receptor, followed by binding to SR-BI. Current evidence suggests that these interactions precede engagement with CD81, followed by interaction with claudin-1 [98, 99]. HCV enters by receptor-mediated endocytosis, which requires passage through a low-pH compartment [100]. Nevertheless, virus particles are resistant to low-pH treatment, arguing that during interaction with one or several entry molecules, the viral glycoproteins acquire a pH-sensitive conformation [101]. Upon liberation of the RNA genome into the cytoplasm, translation takes place at the rough endoplasmic reticulum (rER) where polyprotein processing occurs (Fig. 3). After or during proteolytic cleavage, a membrane-associated replication complex forms designated the membranous web, which is the site of viral RNA amplification [69, 102]. Newly synthesised positive-strand RNAs are copied from negative-strand RNA intermediates, and positive-strand RNA is either re-used for RNA translation, or serves as template for negative-strand RNA synthesis, or is packaged into nucleocapsids. Assembly occurs in close proximity to lipid droplets where core protein accumulates [103, 104]. The viral replicase appears to be recruited, presumably *via* core, to lipid droplets where the viral RNA genome may be transferred from the replicase to the core protein. Since lipid droplets are closely surrounded by ER membranes that may contain the HCV glycoproteins, nucleocapsids forming in close proximity to lipid droplets may acquire their envelope *via* budding into the ER lumen. This process may be connected to VLDL assembly [91] and HCV particles may be released, eventually associated with components of VLDLs, *via* the constitutive secretory pathway.

Experimental systems

The propagation of HCV in cultured cells as well as the development of efficient animal models has been a challenging task that has been overcome in the past few years by the establishment of productive cell-based replication systems as well as small animal systems. In the following we will provide only a very short overview about the available models; the interested reader is referred to a recent review [105].

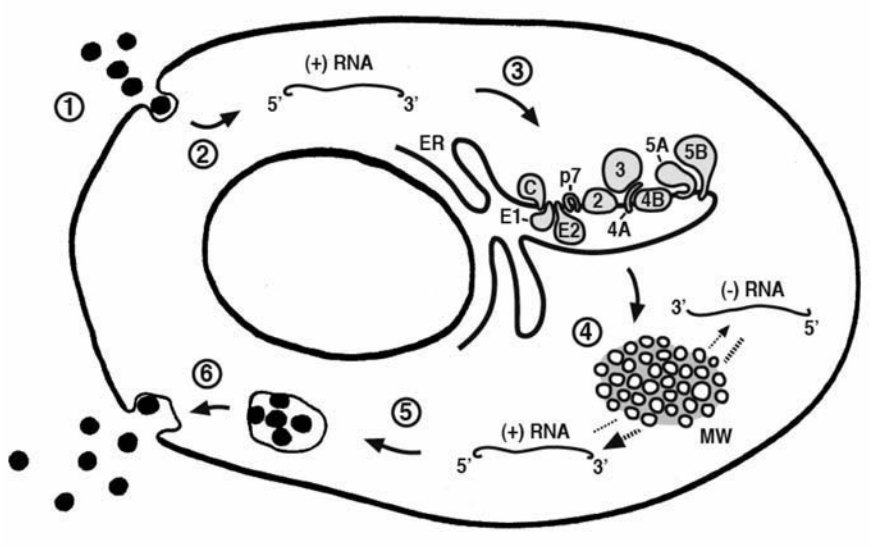

Figure 3. Schematic representation of the HCV replication cycle. After binding to a permissive host cell (1), HCV enters most likely by receptor-mediated endocytosis (2). The viral RNA is liberated into the cytoplasm and translation occurs at the ER membrane (3). The polyprotein is processed and a membranous replication complex, designated the membranous web (MW), is induced (4) where viral RNA is amplified. Positive strand RNA progeny is encapsidated (5) and virus particles may be released from the cell by the constitutive secretory pathway (6).

Animal models

Thus far, the only animal that can be infected reliably with HCV is the chimpanzee (*Pan troglodytes*) [106]. In spite of ethical concerns and high costs, chimpanzee experiments have provided invaluable information such as the first successful transmission of the non-A, non-B hepatitis agent from human serum to chimpanzees [107–109], the establishment of a high-titred serum pool from which the first HCV genome was cloned [110], or the demonstration of infectivity of molecular HCV clones [56, 111].

Because the clinical course of disease in chimpanzees and humans is similar, studies of experimentally infected animals have also shed light onto the role of the immune response in controlling virus infection [112]. Comparable to the human situation, a significant proportion of infected animals is unable to clear the virus and contracts a persistent infection with fluctuating virus titres and eventual sporadic occurrence of liver inflammation. Although it is not yet clear how the virus escapes the immune response, an increasing body of evidence shows that a vigorous immune reaction correlates positively with virus elimination as well as liver cell damage. The latter observation is in keeping with the notion that chronic hepatitis C is mainly sustained by the immune reaction.

Attempts to establish a robust small animal model thus far are of limited success [113, 114]. However, some progress has been made, of which the most promising is based on the xenotransplantation of primary human hepatocytes into immunodeficient (SCID) transgenic mice, overexpressing the murine urokinase-type plasminogen activator (uPA) in the liver [115]. Upon engraftment of human hepatocytes early after birth, the animals develop chimeric livers composed of murine and human hepatocytes. These mice can be productively infected with HCV, with virus replication being confined to the human hepatocytes.

Cell culture systems

In spite of the availability of infectious molecular clones of HCV genomes, they could not be propagated in cultured cells. A major breakthrough in the development of a robust and reliable cell culture system has therefore been the replicon system [52] (Fig. 4A). In this system, the region encoding core to NS2 was replaced by a selectable marker (neomycin resistance) expressed *via* the HCV IRES, whereas the HCV nonstructural genes were expressed *via* a heterologous IRES. Upon transfection of the human hepatoma cell line Huh-7, a low number of neomycin-resistant colonies was obtained that carried high amounts of autonomously replicating HCV RNA. High level replication was due to the selection for both more permissive Huh-7 cells and replication enhancing mutations (cell culture adaptive mutations) [116]. Owing to its unprecedented level of HCV RNA replication and antigen expression, the replicon system became a very important tool to study the intracellular steps of the viral replication cycle and provided the first functional cell-based platform for screening of antiviral agents that target the RNA replication process. However, this system did not support the production of infectious virus particles [117–119], which is due to the adaptive mutations that interfere with virus particle assembly and infectivity *in vivo* [120].

The first system allowing studies of the HCV infection process was based on HCV pseudoparticles (HCVpp) [121, 122]. These particles are composed of retroviral nucleocapsids surrounded by an envelope lipid bilayer into which authentic and entry-competent E1-E2 glycoprotein complexes are embedded (Fig. 4B). Since the early steps of infection are orchestrated by the envelope proteins, HCVpps are a very valuable surrogate system to study HCV infection and neutralisation.

Production of infectious HCV particles in cell culture only became possible with the availability of a molecular clone capable of high-level replication in cultured cells without requirement for cell culture adaptive mutations. This clone was isolated from a **J**apanese patient with **f**ulminant **h**epatitis (hence the acronym JFH-1) [123]. Subgenomic JFH-1 replicons are several orders of magnitude more efficient than replicons derived from other HCV molecular clones [124, 125]. Most importantly, transfection of

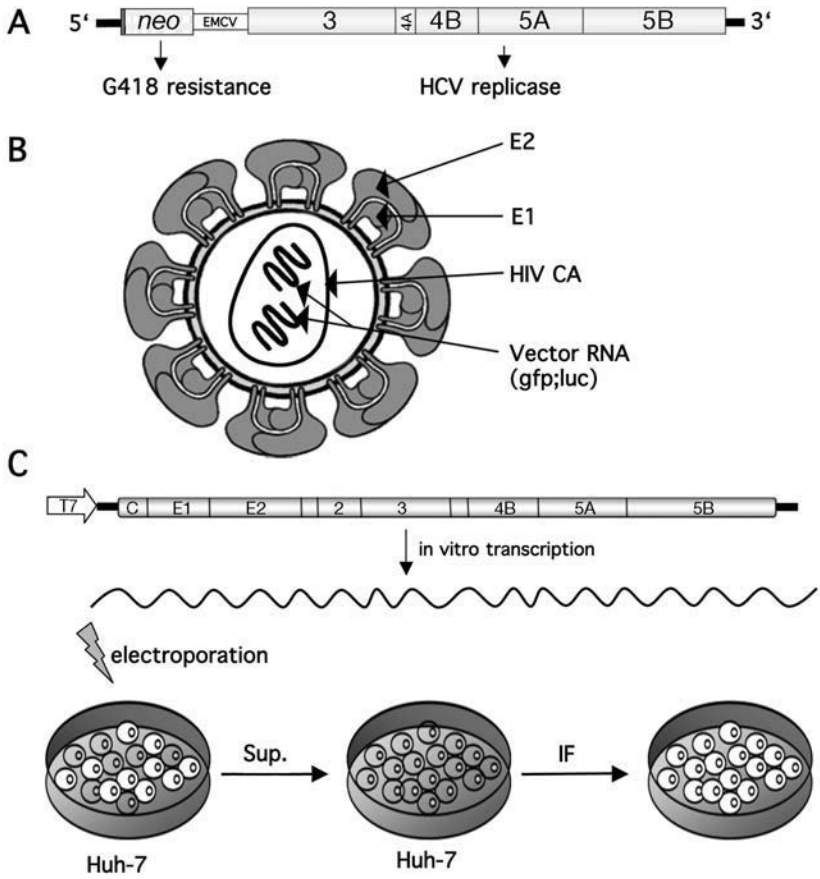

Figure 4. Experimental models to study HCV replication in cell culture. (A) Structure of a subgenomic HCV replicon composed of the 5' NTR (thick line), the gene encoding the neomycin phosphotransferase (*neo*), the IRES from the encephalomyocarditis virus (EMCV), the HCV replicase genes and the 3' NTR. (B) Architecture of a HCVpp. It is composed of a lipid envelope containing authentic HCV glycoprotein complexes and the capsid (CA) of HIV containing a retroviral vector RNA with an integrated reporter gene (most often either the gfp or the luciferase gene). (C) Schematic representation of the HCVcc (cell culture) system. *In vitro* transcripts of the JFH-1 genome generated by using T7 RNA polymerase are transfected into Huh-7 cells by electroporation. Supernatants (Sup.) harvested from the tranfected cells are used for infection of naïve Huh-7 cells. Infected cells are detected by HCV antigen-specific immunofluorescence (IF).

the full-length JFH-1 genome into Huh-7 cells leads to the production of HCV particles that are infectious for naive Huh-7 cells [86, 126], for chimpanzees [86] and for SCID/uPA mice with human liver xenografts [127] (Fig. 4C). Virus titres attained with JFH-1 are rather low, in the range of 10^3 to 10^4 tissue culture infectivity dose 50 ($TCID_{50}$) per ml [128], but the system was improved dramatically in several ways: First, by the identification of highly

permissive Huh-7 cell clones [126]; second, by the construction of chimeric JFH-1 genomes [128, 129]; third, by cell culture adaptation of JFH-1 and JFH-1 chimeras or the development of a genotype 1a isolate that supports virus production although virus titres attained with this isolate are still very low [130–132]. Additional improvements are the construction of replication-competent reporter virus genomes carrying, e.g., the luciferase gene or a gene encoding a fluorescent reporter. The latter is applicable to study virus infection in live cells as well as interactions between virus genomes.

HCV and the immune system

A key player that blocks the induction of the early innate antiviral defence appears to be the NS3-4A protease that can interfere with the dsRNA-induced activation of IFN regulatory factor 3 (IRF-3) [133]. Within a cell dsRNA is recognised by several molecules including Toll-like receptor 3 (TLR3), the RNA helicases retinoic acid inducible gene I (RIG-I) and mda5 (also called Helicard) [134]. Upon activation by dsRNA binding, these molecules recruit adaptor proteins, which are TRIF (also called TICAM-1) in case of TLR3, and Cardif (also called IPS-1, MAVS or VISA) in case of RIG-I. These adaptors trigger a signalling cascade resulting in the transcriptional activation of IFN-β. It was shown that both TRIF and Cardif can be cleaved by the NS3-4A protease [135–137]. The block of this pathway may contribute to the establishment of a persistent HCV infection.

In addition, several studies suggest that HCV proteins interfere with one or several steps of the effector phase of the IFN system, e.g., by blocking Jak/Stat signalling or inhibition of individual effector proteins such as PKR [138]. However, it remains to be determined whether these observations, most often made in artificial *in vitro* or cell-based systems also operate *in vivo*.

The adaptive immunity also appears to be undermined by HCV without globally affecting immune response to other infectious agents. It is generally accepted that the vigour and the breadth of the immune response mounted upon HCV infection determine the outcome of infection [139]. Therefore, a successful immune response targets multiple MHC Class I-restricted epitopes in the HCV polyprotein and induces rather large numbers of HCV-specific $CD8^+$ T-cells. In addition, a strong $CD8^+$ T-cell response is usually accompanied by a strong and multi-specific $CD4^+$ T-cell proliferative response and permanent loss of this response is a strong predictor for persistence. Resolved infections usually leave a durable memory response lowering the chance of persistence upon re-infection. The reason why the strong initial T-cell response is blunted at later stages of HCV infection is not understood. One possibility, however, is that the interference of HCV with the innate immune response results in $CD4^+$ T-cell impairment, e.g., due to the lack of important cytokines required for T-cell activation. Since

T-cell help is important for $CD8^+$ T-cells, the degree of helper cell impairment would be a critical determinant of virus elimination or persistence. The extent to which B-cell responses contribute to viral clearance thus far is not known. It is generally assumed that T-cell immunity is the primary determinant in controlling HCV infection.

Diagnosis of HCV infection

Diagnosis of hepatitis C is based on serological assays, which detect HCV-specific antibodies (anti-HCV), and on molecular assays, which detect HCV RNA [140]. Current enzyme immunoassays (EIAs) are highly sensitive as well as specific and represent the primary diagnostic tool. HCV RNA detection by real-time RT-PCR is now standardised, reliable and reproducible, and offers a broad dynamic quantitation range. HCV becomes positive by RT-PCR as early as 1–2 weeks after infection and 4–6 weeks before anti-HCV seroconversion. HCV RNA testing is used to confirm active infection in anti-HCV-positive individuals and to diagnose acute hepatitis C or chronic hepatitis C in the rare immunocompromised patients that do not develop anti-HCV antibodies. However, the principal role of HCV RNA testing is in the tailoring and monitoring of antiviral therapy. Determination of HCV genotype is important for the selection of the optimal antiviral regimen.

Liver biopsy allows determining the necro-inflammatory activity (grading) and the degree of fibrosis (staging) as well as to recognise or exclude coexisting liver pathology (such as alcoholic liver disease, iron overload or NAFLD). Liver biopsy is strongly recommended before the initiation of antiviral therapy.

A number of non-invasive tests are currently being explored to predict liver fibrosis [141, 142]. These are based on different combinations of blood tests, transient elastography (FibroScan®) [143] or magnetic resonance imaging [144]. While promising, current prediction methods remain limited with respect to the differentiation of intermediate fibrosis stages and thus may replace liver biopsy only in selected patients.

Current and future therapy

Current standard therapy consists of pegylated interferon-α (PEG-IFN-α), administered once weekly by subcutaneous injection, combined with ribavirin, which is taken orally on a daily basis [145]. Both drugs operate through incompletely understood, likely direct antiviral and immunomodulatory mechanisms [146]. Standard treatment duration is 48 weeks for genotype 1 and 24 weeks for genotypes 2 and 3. Current efforts are aimed at tailoring doses and treatment duration to the individual patient based on baseline parameters (e.g., genotype, viremia, fibrosis stage, degree of

steatosis) and on-treatment viral kinetics (viremia at 4, 12 and 24 weeks) [147–151]. Hence, therapy may be abbreviated in selected patients with favourable baseline parameters and a rapid virologic response (i.e., negative HCV RNA after 4 weeks of treatment), while others may benefit from prolonged treatment.

In general, treatment is currently recommended for patients with chronic hepatitis C (i.e., persistently [>6 months], elevated aminotransferase levels and anti-HCV as well as RNA positivity in serum) and findings of fibrosis and at least moderate degrees of inflammation and necrosis on liver biopsy. For other patients, decisions will have to be made on an individual basis.

Factors associated with a favourable treatment response are infection with genotypes 2 or 3, low baseline viremia, and low fibrosis stage. For unknown reasons, African Americans respond poorly to current treatment [152, 153]. In addition, overweight has been associated with poor treatment outcomes [154]. Novel and very promising genomic and proteomic approaches are aimed at predicting treatment outcomes on an individual basis [155, 156].

Contraindications to therapy with PEG-IFN-α and ribavirin include decompensated liver cirrhosis, autoimmune diseases, uncontrolled depression or psychosis, pregnancy, the lack of a reliable method of contraception (teratogenicity of ribavirin), cardiopulmonary diseases, severe leuko- or thrombocytopenia, and conditions that impair compliance with therapy. Ribavirin is in principle contraindicated in patients with creatinine clearance <50 ml/min and should be used only with very close monitoring under these circumstances.

Adverse effects of PEG-IFN-α include, among others, flu-like symptoms, fatigue, leuko- and thrombocytopenia, weight loss, irritability or depression, thyroid dysfunction, hair loss, rash, seizures, and sleep disturbances. The most frequent adverse effect of ribavirin is haemolytic anaemia.

Patients who become HCV RNA negative during therapy, and remain so for more than 6 months after the end of treatment, have a sustained virologic response (SVR) and usually remain negative. Patients who are HCV RNA negative at the end of treatment, but become positive again at 6 months of follow-up are classified as relapsers, and patients who do not respond to treatment at all as nonresponders. Genotype 1-infected patients who do not experience a ≥ 2 $\log_{10}$ reduction of viremia at 12 weeks (early virologic response) will fail to achieve an SVR [157, 158]. Treatment should be discontinued in these patients.

With the above treatment, 40–50% of genotype 1- and about 80% of genotype 2- and 3-infected patients achieve an SVR [158–160]. Overall, about 50% of patients with chronic hepatitis C can be cured with the current treatment. For these patients, ongoing efforts are aimed at tailoring treatment to the individual needs in order to improve tolerability. For the other patients and for the important proportion of patients who cannot tolerate current treatment, new therapeutic strategies need to be developed.

In principle, each step of the HCV life cycle represents a target for antiviral intervention [161]. Specific inhibitors of the biochemically and structurally well-characterised NS3-4A serine protease and NS5B RdRp are currently being developed as antiviral agents, and the first candidates have already been evaluated in clinical trials [162, 163]. Serine protease inhibitors seem particularly promising, as they not only block viral polyprotein processing, but may also reverse the inhibition of innate immune sensing by HCV. In addition, drugs affecting host factors involved in HCV replication are being explored as antiviral agents. The genetic variability of HCV, allowing the rapid development of antiviral resistance, represents a major challenge to the clinical development of specific inhibitors. Therefore, combination therapy will be necessary for therapeutic success [164]. With the increasing number of new compounds targeting different steps of the HCV replication cycle, we can expect that the number of patients that can be cured will increase further.

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Comparative Hepatitis
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Hepatitis A infection

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Abstract

Hepatitis A is a ubiquitous disease of man with acute, self-limiting liver inflammation and a low mortality rate, also known as 'traveller's hepatitis', 'hepatitis epidemica', and 'infectious hepatitis'. The causative infectious agent is the hepatitis A virus belonging to the family of the *Picornaviridae*, genus *Hepatovirus*. The route of transmission is mainly faecal-oral. Hepatitis A is an acute hepatitis that does not become chronic like hepatitis B and C, and it is no risk factor for the development of hepatocellular malignancies. The severity of clinical manifestation is often age related with predominantly inapparent infection in young individuals to more severe courses of infection in the adults and elderly. Hepatitis A is most predominant in countries with poor hygienic conditions and is therefore sometimes attributed as 'traveller's hepatitis'. Potent vaccines for protection and disease prevention are available and the ambitious medical aim – eradicating HAV by aggressive vaccination strategies – appears realistic.

Introduction

Hepatitis A is a ubiquitous disease of man with acute, self-limiting liver inflammation and a relatively low mortality rate. The disease is predominantly transmitted by the faecal-oral route and in extremely rare cases by blood donation from a proband with a hepatitis A infection with viremia.

The causative infectious agent is the Hepatitis A Virus (HAV). HAV is a member of the picornavirus family (*Picornaviridae*) which contains the genera *Aphthovirus*, *Cardiovirus*, *Enterovirus*, *Erbovirus*, *Hepatovirus*, *Kobuvirus*, *Parechovirus*, *Rhinovirus*, and *Teschovirus* [1]. Infections with different viruses belonging to this family are spread in humans and a lot of other mammals.

HAV is the only member of the species *Hepatovirus* and has the international Virus Code 00.052.0.03.001 [2, 3]. The genetic variability is comparatively low, and recognised wild-type isolates are the strains HM175 (wild-type, accession number M14707), HM175/43C (accession number M59809),

AGM27 (simian origin, accession number D00924), and CR326. Of further genomic/scientific importance are the isolates LU38, LY6, LA, GBM, MBB, AH 1-3, and FH 1-3 [4].

HAV is virulent for humans and higher primates ('Old' and 'New World'). The major tissue tropism is within the liver; there is also some minor tissue tropism for intestinal epithelia. A considerably persistent productive infection over a period of up to months – especially of liver cells – is common. The speciality of the HAV/*Hepatovirus* within the *Picornaviridae* is that this virus shows a comparably low level of RNA replication compared to most of the other picornaviruses.

Previously, HAV was classified as *Enterovirus* type 72. Later it has been shown by several studies that it can be distinguished from other picornaviruses with regard to nucleotide and amino acid sequences and growth in cell culture. Thus it is closely related to poliovirus (genus *Enterovirus*), coxsackievirus (genus *Enterovirus*) and echovirus (genus *Parechovirus*) which are of relevance for human infections.

HAV was first discovered by immune electron microscopy in 1973 [5] and isolated in cell culture in 1979 [6]. The characterisation of the HAV capsid proteins was performed in 1978 [7, 8] and the genome was characterised as single-stranded RNA in 1981 [9]. HAV has only one major serotype [10, 11] as only one neutralisation site is immunodominant [12]. There is no serological cross-reaction between HAV and other viruses causing hepatitis.

The virion is known to be very stable, resistant to elevated temperature and acidic pH conditions. It also has a high resistance to chemicals and environmental influences. It has been found to survive for days to months in experimentally contaminated freshwater, seawater, wastewater, and soils as well as in live oyster and cream-filled cookies [13].

HAV is an important pathogen; the infection is a common form of acute, usually self-limiting viral hepatitis in many parts of the world and causes worldwide about 1.5 million cases of acute clinical hepatitis each year.

'Acute hepatitis' is most commonly defined as a hepatitis lasting no longer than 6 months. A chronic disease manifestation as in other forms of infectious or non-infectious hepatitis is not observed in hepatitis A. It is not only a disease in 'developing countries' but accounts for about 25% of all cases of hepatitis in the 'developed world', whereas many cases have to be attributed to travelling activities into regions with high prevalence for HAV infection. Outbreaks occur periodically in several countries. The incidence of hepatitis A is closely related to socioeconomic development. The main vehicles of transmission are faecally contaminated food and water [14].

Viral structure and replication

Hepatitis A virus shares the key properties oft the picornavirus family (Tab. 1). The virion is an icosahedral nuclear capsid with a diameter from

Table 1. Hepatitis A virus *versus* poliomyelitis virus: Comparison of biological properties

Family	*Picornaviridae*	*Picornaviridae*
Genus	Hepatovirus	Enterovirus
	Hepatitis A Virus (HAV)	Poliovirus (types 1–3)
Serotypes	1	3
Morphology	- Non-enveloped virus - Icosahedric symmetry - Capsids consist of 4 different viral proteins (VP 1–4) - VP 1–3 are surface proteins - 12 Capsomeres per nucleocapsid - Diameter 27 nm - stable to acidic conditions	- Non-enveloped virus - Icosahedric symmetry - Capsids consist of 4 different viral proteins (VP 1–4) - VP 1–3 are surface proteins - 12 Capsomeres per nucleocapsid - Diameter 28–30 nm - stable to acidic conditions
Genome	- Virion contains 29.0% nucleic acids - One molecule of linear ss (+) RNA - Length of genome 7500 nt - 5'-end with hairpin structure (with genome associated protein (VPg)) - 3'-end with poly (A) - One open-reading frame, coding for one precursor protein	- Virion contains 29.2% nucleic acids - One molecule of linear ss (+) RNA - Length of genome 7400 nt - 5'-end with hairpin structure (with genome associated protein (VPg)) - 3'-end with poly (A) - One open-reading frame, coding for one precursor protein
Lipids	- Virion contains no lipids	- Virion contains sphingosin; approx. 0.1% lipids
	- Genome *per se* is infectious	- Genome *per se* is infectious

27–32 nm with cubic symmetry and composed of 30% RNA and 70% protein [5] (Fig. 1). The capsid possesses four major structural components: VP0, VP1, VP2, VP3. The molecular weights range from 21–33 kD. The surface proteins VP1 and VP3 are major antibody-binding sites. The capsid contains both precursor VP0 and product VP2. Early studies have shown the existence of a very small protein (7–14 kD). It was not possible to identify it as VP4 by immunologic reactivity or sequence analysis. In other picornaviruses such as poliovirus VP4 is approximately 7 kD and is myristoylated at the N-terminal glycine. In poliovirus VP4 is found on the interior capsid surface and may contribute to particle assembly and stability or to binding and entry of virus into cells [15]. VP4 has not been conclusively demonstrated in HAV particles. If it exists it is much smaller than other picornaviral VP4 molecules and not myristoylated [16].

The nucleocapsid contains 12 capsomeres. Like other picornaviruses, HAV has no envelope (Fig. 1).

First cDNA clones have been produced in 1983 [17] and first infectious cDNA clones of HAV were successfully generated in 1987 [18], making this virus accessible to molecular biology. The virion contains one copy of linear, positive-sense, single-stranded RNA with a relatively low G+C content of 37.9%. The HAV genome is 7.5 kb in size and can be divided into three

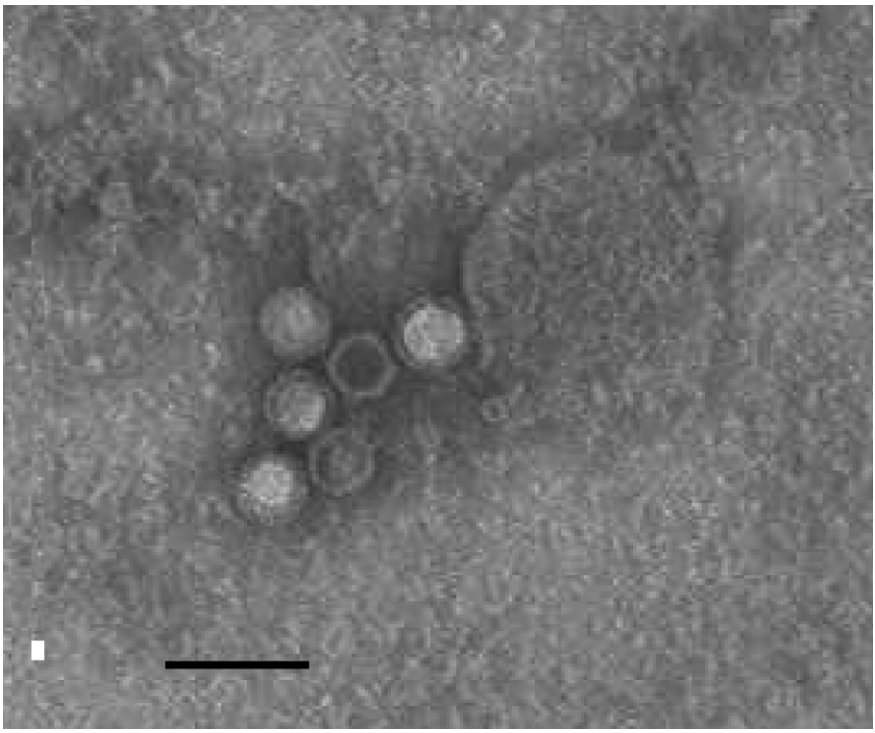

Figure 1. Electron microscopic image of HAV (Hepatitis A virus/strain HM 175); the bar represents 60 nm.

parts: (i) a 5' non-coding region (NCR) that comprises approximately 10% of the genome, (ii) an open reading frame that encodes all of the viral proteins and (iii) a short NCR [16]. RNA sediments at 33 S and has a buoyant density of 1.33 g/mL [9]. With RNA extracted from cells or transcribed from cloned amplified cDNA cultured cells can be transfected [9, 18]. RNA also has been translated *in vitro* demonstrating that the viral genome follows the same translation strategy as the monocistronic genomes of the other picornaviruses [19]. The 5' untranslated region functions as an internal ribosome entry site to which the genome-linked protein VPg is covalently linked. The virus enters the cell by acid-independent receptor-mediated endocytosis. Genomic replication occurs within the cytoplasm, and the corresponding replicase is a virus-encoded 3D RNA-dependent RNA polymerase which generates partially double-stranded replicative intermediate RNA. The egress mechanism after virion assembly within cytoplasmatic membrane vesicles is still unknown and a matter of current research.

Although 3–7 genotypes of HAV have been characterised [20, 21], only one major serotype can be defined so that there appears to be no variation detectable by serology in these neutralisation sites.

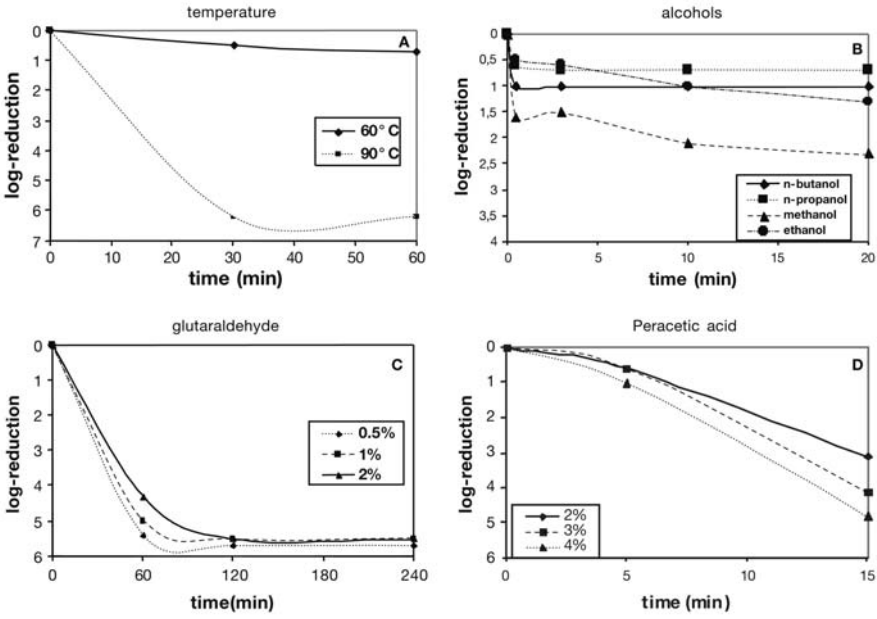

Figure 2. Inactivation of HAV/(Hepatitis A virus) strain HM175. A) by heat/temperature, B) with different alcohols, C) with glutaraldehyde, and D) with peracetic acid (MH Wolff and S Probst, unpublished data)

Virus stability

One of the main properties of HAV is its high stability not only with regard to environmental influences but even chemicals. Like many other viruses, HAV can easily survive low temperature and freezing. Even in fresh or salt water it persists at least up to 12 months. It can survive several hours on human hands and several days on environmental surfaces indoors, which can lead to transmission especially at places with poor hygiene [22–24].

Like most picornaviruses which are stable between pH 3 to 9 over a period of many hours, HAV survives acid treatment of lower pH. When subjected to pH 1 at room temperature the infectivity was retained for up to 5 h [25]. This makes it understandable that the virus, after transmission and passing the stomach, can survive in the faeces in which its infectivity can be retained for weeks even under dried conditions. HAV is also associated with food-borne outbreaks. In this connection the temperature stability is of high interest. HAV is relatively resistant to heat [26]. The infectivity is not or almost not affected within 1 h, if virus is incubated at 60 °C. Increased temperatures over 60 °C – e.g., at 90 °C – lead to a fast and significant inactivation over several log-steps, respectively (M.H. Wolff, S. Probst, unpublished results; Fig. 2a). By autoclaving, ultraviolet irradiation (1.1 W at a

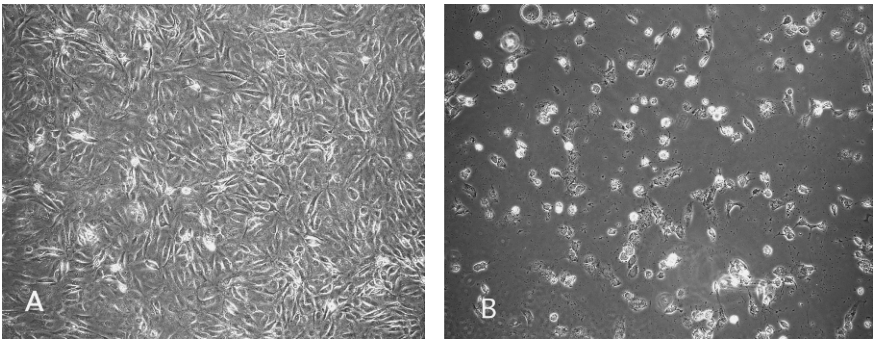

Figure 3. HAV: Cytopathic effect (CPE) in FRhK- 4 cells: typical CPE occurs 7 days *post infectionem*: A) Control (mock-infected), B) HAV-infected: typical CPEs

depth of 0.9 cm for 1 min) or formalin treatment (3% for 5 min) the virus will be destroyed rapidly [27, 28]. Against chemicals, especially such which are contained in disinfectants, HAV is relatively resistant. Inactivation by glutaraldehyde or peracetic acid (Figs 2c and 2d) is possible in a proper time and concentration, whereas alcohols are not able to decrease the infectivity in a suitable time (Fig. 2b).This is problematic because hand disinfectants usually contain alcohols. In our hands none of the tested commercially available disinfectants showed a log 10 reduction factor which satisfies the requirements of the corresponding German authorities (BGA/DVV; M.H. Wolff, S. Probst, unpublished results), internationally representative as key opinion leaders within this field.

Propagation in cell culture

It is still difficult to grow HAV in cell culture. Especially the isolation of wild-type virus from the faeces of a patient often fails. HAV shows a strong tissue tropism. Permissive cell lines are the AGMK, FRhK-4, and BSC-1 cell-line. There are several virus strains which are used for research; the cytopathic HAV variant HM 175 characterised by T. Cromeans et al. (1989) [29] is frequently used for studies. This strain can be propagated in foetal rhesus monkey kidney cells (FRhK-4). On these cells a typical cytopathic effect (CPE) is visible 10–14 days after infection (Fig. 3).

Transmission

Transmission of HAV usually occurs by the faecal-oral route either by direct close contact with infected persons or by ingestion of virus-contaminated water or food. In these cases the amount of virus incorporated plays an

important part. Infected people excrete 10^{12}–10^{14} virus particles per gram of stool but already an infection dose of only 10 to 100 virus particles leads to infection. In so far HAV is a potent pathogen which has been responsible for many food-borne outbreaks. The food implicated in these outbreaks mainly is shellfish. Infections are also described after consumption of contaminated raw oysters, mussels and clams [30–32]. Even if the food is heated, infections are possible because – as mentioned above – the used temperature and heating time usually is not sufficient to inactivate the virus. Infections have also been caused by salads, fruits, vegetables as well as dairy products, sandwiches, baked products [33, 34] and cream-filled cookies [13]. But there are also studies describing the transmission in homosexual men and it is even asserted that homosexual men are the most important risk group for the transmission of HAV infection [35]. Even though HAV is an enterovirus and usually transmitted by faecal-oral route there are single observations which show a transmission by blood or blood products [36, 37] so that also cases of infection in individuals with intravenous drug addiction have been reported. There is also a risk for infection for people working with waste water management.

Pathogenesis and clinical features

In contrast to hepatitis B and C which contribute to approximately 80% of all cases of human hepatocellular carcinoma, hepatitis A infection is not a predisposition for hepatic malignancies [38].

After ingestion HAV passes the stomach because of its pH stability, and can enter the small intestine and reach the liver *via* the *vena portae*. The further stages of the infection route starting in the gastrointestinal tract and resulting in hepatitis are still not completely understood. Yet some observations are well documented: replication site are the hepatocytes (most predominant 1–2 weeks after infection) and a viremia (typically 10^4 virus particles per mL blood) is observed during the incubation time of approximately 2–6 weeks after infection. At the same time infectious virus reaches the intestinal tract in the bile and occurs in the faeces [16]. The damage of the hepatocytes resulting in hepatitis might be caused by the cytotoxity of HAV, like with other picornaviruses which exit the host cell by disintegration. Most obviously it is mainly of immunological origin (necrotic, apoptotic): *in vitro* studies have shown evidence of cell-mediated lysis of HAV-infected cells by natural killer cells and by HLA-restricted, HAV specific $CD8^+$, cytotoxic T-cells that were cloned from liver biopsies obtained during acute hepatitis A in patients with hepatocellular necrosis [39, 40]. This immunological process is generally initiated in the infected host mostly 3–4 weeks after infection.

The isolation of cytotoxic T lymphocytes from liver tissue of patients with acute hepatitis A infection gives some evidence that the hepatocellular

damage might primarily be due to a cellular immune response. In the acute phase necrotic, inflammatory changes are observed predominantly in the periportal region also containing infiltrates of plasma cells. HAV antigen can further be found within the hepatocytes and Kupffer cells [41]. Acute viral hepatitis A, B, and C cannot be discriminated by macromorphological and histopathological features (light microscopy). The histopathology of acute viral hepatitis is comprehensively described in textbooks of pathology and can be revisited there.

The incubation period ranges from 2–7 weeks and the course of hepatitis A may be either inapparent or subclinical, which means anicteric. Especially in children the disease is milder and in 80–95% of cases inapparent. In young children HAV infection is usually asymptomatic whereas symptomatic disease occurs more commonly among adults with icterus in more than 70% of the infected individuals. Pregnancy does not seem to have any impact on incidence and severeness of the disease. Despite of this, for the patient's and newborn's safety, a vaccination with an inactivated vaccine can be taken into consideration prior to intended pregnancy in case of persons with risk factors for hepatitis A. In less than 10–20% of the cases clinical signs such as icterus (serum bilirubin >3 mg/dL), flu-like symptoms, fever, fatigue, myalgia, polyarthritis, nausea, abdominal discomfort, jaundice, and glomerulonephritis (autoimmunological) are obvious. Symptoms generally subside when the disease enters the icteric phase (intrahepatic cholestasis). Often a pale colour of the faeces is observed and a darker colour of the urine (bilirubinuria). The disease is clinically divided into three phases: (i) the unspecific 'prodromal phase', the (ii) 'icteric phase', and (iii) the 'reconvalescence phase'. Within the last phase there is typically an aversion against consumption of alcohol and fatty food which is known as 'posthepatic syndrome'. Mostly the disease is self-limiting within a period of some weeks, although – in rare cases – prolonged courses of infection of 1–2 years have been reported.

Rare complications of hepatitis A include relapsing hepatitis, cholestatic hepatitis, and fulminant hepatitis. Chronic infection with HAV does not occur and infection induces life-long immunity. The mortality rate is very low, in children about 0.1% and in adults 0.3–2.1%. The mortality rate can be higher in immunosuppressed patients.

Laboratory diagnostics

Historically, immune electron microscopy of stool and serum specimens played a role in diagnostics of HAV infection. Due to the rapid decline of virus titres during infection, these approaches were only successful during the very early phase of infection/incubation period.

The diagnosis of hepatitis in general is made by biochemical tests of transaminases, especially ALT (ALAT; alanine aminotransferase) and also

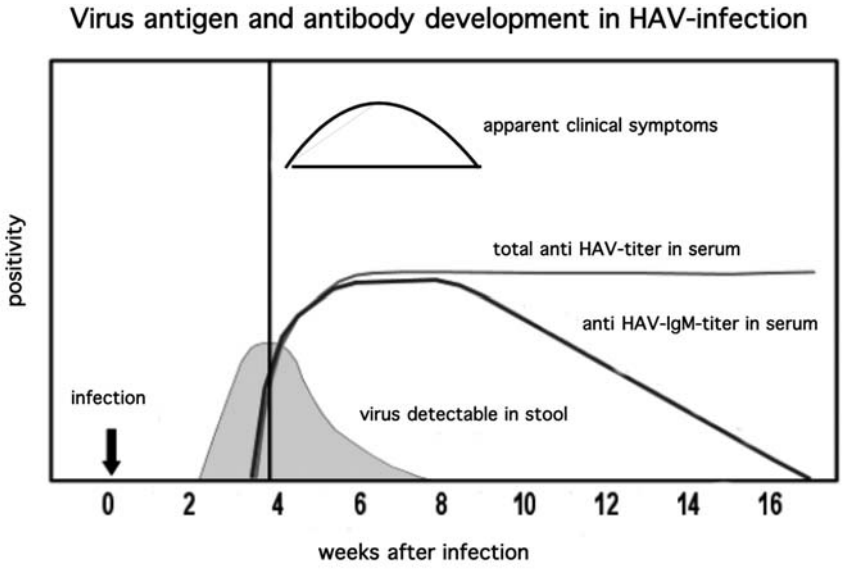

Figure 4. Development of specific virus antigen and antibody titres during typical course of hepatitis A infection

AST (ASAT; aspartate aminotransferase) and should be performed in patients at risk for acute hepatitis A infection. In acute hepatitis A infection they reach values of log 2 of the upper reference level and the quotient AST (ASAT)/ALT (ALAT) is typically lower than 1 in case of HAV infection. These enzymes are a very sensitive marker of hepatocellular damage. It should be mentioned that in fulminant cases of liver failure transaminase titres may even decline again due to the lack of intact liver tissue (hepatocellular 'burned out phenomenon'). But with these biochemical assessments, as well as with clinical observation, a differentiation between the different hepatitis viruses as responsible infectious agent is not possible. Therefore specific assays for anti-HAV IgM and anti-HAV IgG have to be used. Although infectious virus occurs in high amounts in faeces and could be easily detected by immune electron microscopy the direct proof in stool specimens is not practical, because the virus excretion reaches its maximum already in the late phase of the incubation period (Fig. 4).

The ELISA/EIA IgM-anti-HAV should be/become positive during the apparent phase of infection and might stay positive for up to 2 years in case of severe infection. Interestingly, in rare cases the total amount of serum IgM (serum electrophoresis) is upregulated in HAV infection, a phenomenon which is not sufficiently understood and might also cause unspecific cross-reactions with other (infectious) antigens, which has to be taken into diagnostic consideration. On the other hand, a lacking ELISA IgM-anti-HAV titre does not exclude an apparent acute hepatitis A in rare cases.

During the incubation period serum IgM-anti-HAV is generally negative. Positive IgM-anti-HAV results have to be confirmed by an ELISA/EIA determination of total anti-HAV antibodies.

In the early phase of infection (incubation period) HAV antigen can be determined in stool and serum specimen *via* antigen ELISA/EIA or corresponding molecular diagnostic approaches such as RT/PCR. After an infection – whether clinically apparent or inapparent – the anti-HAV antibody status generally remains positive life-long and appears to offer a sufficient protection against re-infection with HAV.

New molecular approaches for diagnostics of hepatitis A infection are currently under evaluation, including the attempt to obtain a better understanding of hepatitis A pathobiology itself [42].

Protection and vaccination

Several inactivated or live attenuated vaccines against HAV have been developed, and currently four inactivated vaccines are internationally available. Cell tropism and attenuation are reviewed by J. Graff et al. [43]. The vaccines are manufactured from a cell culture-adapted virus strain (e.g., HAV strain H2) propagated in human fibroblast (attenuation procedure: serial passages from monkey kidney cells to human fibroblasts) followed by purification from cell lysates, formalin inactivation and adsorption to aluminium hydroxide adjuvant [44]. The vaccines are given parenterally as a two-dose series, 6–18 months apart. They are highly efficacious and provide long-lasting protection [45, 46].

A life vaccine is especially widely used and accepted in the Peoples' Republic of China with, e.g., a cumulative vaccination coverage of over 89% in Jiaojiang City among children which were 1–15 years old [44]. Especially from the broad vaccination attempts in the Peoples' Republic of China, the protective efficacy of the H2-life-vaccine seemed excellent. The attenuated H2-vaccine was introduced in China in 1992, followed by the introduction of the inactivated HM-vaccine (1993) and the inactivated CR326-vaccine (1994) in many countries worldwide.

Non-responders, like in case of hepatitis B vaccination, are generally not observed and it appears that a sufficient protection against infection can be achieved for a period of (over?) 10 years after a sufficient vaccination with a two-dose application vaccination regimen.

There are also combination vaccines available containing inactivated HAV and recombinant HBV. This vaccine is given as a three-dose series using a 0, 1, 6 month vaccination schedule. Further, a combination vaccine against hepatitis A and *Salmonella typhi* is available.

There is convincing evidence that the vaccine confers herd immunisation if the main spreaders of HAV are targeted for immunisation. This should encourage countries to start mass vaccination programmes against HAV,

especially as pharmacoeconomic considerations are beginning to demonstrate that such a strategy could be a highly cost-effective way of controlling this disease.

It is even conceivable to eradicate HAV. This should even be easier to achieve than the attempt of polio eradication as HAV vaccines seem to confer more durable immunity than polio vaccines do. Physicians should consider routine vaccination of children 12–23 months of age based on recommendations from the Centers for Disease Control and Prevention (CDC, Atlanta, USA) [47].

Further research on optimising the vaccination and developing new vaccines – especially under the perspective of reducing the sometimes occurring (mostly mild) adverse reactions – are ongoing.

Post-exposure immunotherapy

A post-exposure immunotherapy with HAV hyperimmunoglobulin preparations (sera) of human origin can be successful in preventing infection or generating a milder course of infection. These preparations should be administered to the patient as soon as possible after potential exposure/infection, 5–10 days after exposure at the latest. In the last two decades the availability of such preparations unfortunately decreased significantly, mostly due to the activities and options related to active vaccination. Empirically, the dose aimed by physicians is 0.02–0.12 mL/kg body weight of the hyperimmunoglobulin preparation as mentioned above administered i.m.

Therapeutic options

Hepatitis A should be regarded as a preventable medical threat, and sufficient precautions of prevention should be taken into consideration as there are no specific therapeutic options against acute hepatitis A. This should especially be considered before travelling into endemic regions.

In severe cases the patient should be hospitalised and the consumption of any drugs should be reduced to the medically necessary minimum. Further, there should be no alcohol consumption, the patient should strictly stay in bed and coagulation has to be monitored and – in case of coagulopathias – to be treated.

On the other hand – fortunately – many cases of hepatitis A infection are inapparent or have a mild course of infection and/or are self-limiting so that no therapeutic intervention is necessary.

In case of live-threatening fulminant acute hepatitis and/or hepatic encephalopathia due to acute liver failure, which is an uncommon but potential event in hepatitis A infection, liver transplantation is the *ultima ratio* for therapeutic intervention. Complementary, selective gut decontami-

nation by antibiotics might be taken into consideration in order to reduce toxin production within the GI-tract. The frequency of patients requiring liver transplantation for HAV has decreased from 0.7% to 0.1% in the UNOS database in the time period from 1988 to 2005. The transplant/death ratio is reported within the range of approximately 0.6 and 0.9 due to different scores and studies [48].

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Comparative Hepatitis
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Hepatitis E infection

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Abstract

Hepatitis E – formerly called 'enterically transmitted non-A non-B hepatitis' – is transmitted by the faecal-oral route. The Hepatitis E Virus (HEV) is a positive-sense single-stranded RNA virus and has great similarities to the caliciviruses. Virus replication appears to be limited to the hepatocyte. The disease is especially endemic and/or epidemic on the Indian sub-continent. Epidemics are mostly waterborne infections. Also in other 'developing regions' outbreaks of HEV infection are observed. In industrialised countries this disease only plays a minor role in hepatitis infections. HEV causes epidemics, endemics and sporadic cases of acute hepatitis. The incubation period for hepatitis E varies from 2–9 weeks. The course of disease is usually mild and self-limiting and resolves within a 2 weeks period. Fulminant cases of infection are rare. HEV infection does not induce chronic courses of hepatitis or liver disease. In clinically apparent cases of infection jaundice, pruritus, clay-coloured faeces and generalised lymphadenopathia may be observed. Fatal infections of fulminant hepatitis E are rare. Pregnant women appear to be exceptionally susceptible to severe disease forms, with an excessive mortality of infected mothers of about 20% in this group. HEV infections appearingly induce mostly life-long immunity to re-infection. To date there is no therapy against HEV infection available. The attempts in generating a vaccine against HEV infections are promising. Improving the socioeconomic situation – including hygienic conditions – is the most effective measure of disease prevention.

Introduction

Hepatitis E, formerly called 'enterically transmitted non-A non-B hepatitis' is transmitted by the faecal-oral route. The Hepatitis E Virus (HEV) is a still unassigned genus with great similarities to the caliciviruses. The morphology of the non-enveloped virion is spherical and the genome consists of a polyadenylated positive-sense single-stranded, linear, 1 segment RNA of an about 7.5 kb. Replication of the virus appears to be limited to the hepatocyte. Also non-human primates are susceptible for HEV infection. The disease is especially endemic and/or epidemic on the

Indian subcontinent. Epidemics are mostly waterborne infections. Also in other 'developing regions' (e.g., Asian-Pacific Region, former Soviet Union, Middle East, northern/western parts of Africa) outbreaks of HEV infection are observed. In industrialised countries this disease only plays a minor role in hepatitis infections. The pathophysiology of HEV infection is still poorly understood. HEV causes epidemics, endemics and sporadic cases of acute hepatitis. The incubation period for hepatitis E varies from 2–9 weeks (average 40 d). During acute infection a mostly short viremic period is observed. A persistent intestinal carrier stage or persistent viremia has not been reported. The course of disease is usually mild and self-limiting and resolves within a 2 weeks period. Fulminant cases of infection are rare. HEV infection does not induce chronic courses of hepatitis or liver disease. Clinically apparent cases of HEV infection are most often seen in young to middle aged adults (15–40 years old). Clinically HEV infection is indistinguishable from the other forms of viral hepatitis. In clinically apparent cases of infection jaundice, pruritus, clay-coloured faeces and generalised lymphadenopathia may be observed. Fatal infections of fulminant hepatitis E are rare. HEV exhibits its highest virulence within the third trimester of pregnancy. Pregnant women appear to be exceptionally susceptible to severe disease forms, with an excessive mortality of infected mothers of about 20% reported in this group. There is also a high frequency of cases of intrauterine deaths and abortions in case of maternal infection. HEV infections appearingly induce mostly life-long immunity to re-infection. In case of diagnostics serological and molecular approaches are combined for early and appropriate results. Until now there is no therapy against HEV infection available. The attempts in generating a vaccine against HEV infections are promising. Improving the socioeconomic situation including hygienic conditions is the most effective measure of disease prevention.

Virology

The Hepatitis E Virus (HEV: HEV-like virus) is a non-enveloped virus particle with a diameter of 30–34 nm. Its Virus Code is 00.084.0.01.001. According to the International Committee on Taxonomy of Viruses (ICTV) HEV is still officially unassigned. However, the genus is named 'Hepevirus' and the family '*Hepeviridae*' in certain databases (i.e., the NCBI taxonomy database). The morphology is spherical and the virion structure very similar to that of the caliciviruses [1]. The serologically related smaller particles of 27–30 nm in size are often found in faeces of patients with acute hepatitis E infection and are recently presumed to represent degraded viral particles.

Molecular analysis of the HEV genome has shown that it is a polyadenylated positive-sense single-stranded, linear, 1 segment RNA of an about

7.5 kb with short 5'- and 3' non-coding regions which contain three separate open reading frames (ORFs) [2]. The G+C content is 54.4–57.9% and the buoyant density 1.29 g/mL, respectively. The replication strategy of HEV is poorly understood until now, apart that it has been shown that the transcriptase and replicase are virus-encoded RNA-dependent polymerases [1]. Replication of the virus may be limited to the hepatocyte. It has been postulated by Reyes et al. [3] that after the virus enters the hepatocyte, the positive-sense HEV RNA is translated to produce the non-structural proteins needed for the generation of negative-stranded RNA ('antigenomic RNA'). This negative-stranded RNA may then serve as a template for synthesis of positive-sense RNA and subgenomic RNAs, which encode the production of the structural proteins in order to encapsidate the positive-sense RNA for the assembly of new viral particles. It is yet unclear if there might be additional replication within the gut.

Atypical isolates which differ from the type strain have been reported (minor differences in resistance to inactivation, slight differences in immunoreactivity), but it is generally accepted that only one serotype of HEV has been identified up to now [4]. Nevertheless, isolates obtained from geographically more distant regions tend to show higher differences in their amino acid sequences. These observations showed that the RNA polymerase region apparently is the most conserved region within the HEV genome. Tendencies for spontaneous mutations are rare and a homologous immunity can be postulated as second infections with HEV have not been reported so far.

Also non-human primates such as *Cynomolgus* macaques and owl monkeys, marmosets, *Aotus* monkeys and chimpanzees can be experimentally infected with HEV and an acute form of hepatitis can be induced within these species [5].

The propagation of the virus in cell culture is still problematic which complicates virological research on this infectious agent. Recently a Chinese isolate was reported to be successfully cultivated in human lung carcinoma cells (Huang et al., 1999 [6]; unconfirmed observation).

HEV sequences are accessible for the strains 'Mexico' (M74506), 'SAR-55' (M80581), 'K52-87' (L25595), 'US1' (AF060668), 'US2' (AF060669), 'genotype 4' (AJ272108), 'Hyderabad India' (AF076239), 'TK15/92' (AF051830), 'fulminant hepatitis' (X98292), 'Uigh 179' (D11093), 'HeBei' (M94177), and the Type Strain (M73218). The accession numbers are given in brackets.

History

In the years 1955/1956 the first outbreak of an epidemic, enterically transmitted 'non-A, non-B hepatitis' was reported from Delhi, India [7]. This form of hepatitis became (or already was?) epidemic and endemic in India

and in 1980 Wong et al. reported the first evidence for the existence of a 'non-A, non-B' viral agent as causative infectious agent [8]. In 1983 Balayan et al. published deeper insights into this infective agent including the successful experimental transmission of the infectious agent to non-human primates [9]. After extensive research on the structure of the particle and the genome thereafter, Goldsmith et al. developed the first ELISA test system for hepatitis E diagnostics [10], the fundamental base for diagnostic assessment of the disease.

Epidemiology

It is not unlikely that HEV infection has a considerably long history within the group of the 'non-A, non-B' viral hepatitis transmitted by the enterical pathway [11]. There are still discussions on HEV-like cases of viral hepatitis from endemic regions especially on the Indian sub-continent which are reported to be serologically distinct from HEV so that there are postulations for subtypes or even 'the sixth human hepatitis virus' with a comparable pathophysiology like HEV infection [12]. HEV infection is only a sporadic form of infectious hepatitis in 'developing countries'.

Disease outbreaks in endemic/epidemic regions are predominantly waterborne infections due to poor hygienic environments. HEV infection occurs endemically and/or epidemically predominantly on the Indian subcontinent. Also outbreaks in 'developing countries' of the Asian-Pacific Region, the former Soviet Union, the Middle East and the northern and western parts of Africa have been reported. It appears that HEV infection is originally an infection of the 'Old World' although also infections in Mexico [13] and seropositivity in Venezuela [14] have been reported. Sporadic infections within the 'developed world'/industrialised countries are rare and mostly imported to these regions by international travelling or immigrants from endemic regions. As reliable and demographically representative epidemiological data on positive anti-HEV seroprevalence are sparse – especially for the Indian subcontinent where HEV appears to be most prevalent – it should be highlighted that positive anti-HEV seroprevalence was reported to be even as high as 25% in blood donors in Egypt [11], where HEV infection is of lower endemic importance than, e.g., on the Indian subcontinent. This should reflect the importance of HEV infections in highly endemic regions.

Clinics

The pathology and pathophysiology of HEV infection is still poorly understood. Hepatitis E, or formerly called 'enterically transmitted non-A non-B hepatitis' is transmitted by the faecal-oral route. HEV has not been isolated

from food and no standardised method is currently available for routine analysis of food concerning HEV.

The incubation period for hepatitis E varies from two to nine weeks and is on the average approximately 40 days. As assessed in non-human primates it is most obvious that the virus is shed in the faeces after its passage from the liver tissue into the bile and into the duodenum, consecutively. During acute infection a mostly short viremic period is observed, mostly at the time-point when jaundice becomes obvious. In a study of Nanda et al. [15] prolonged phases of viremia were reported in up to 15% of the cases of acute HEV infection. One patient is reported where viremia began as early as one week before the onset of jaundice and lasted for a further 4 months. Nevertheless, a persistent intestinal carrier stage or persistent viremia has never been described.

The course of disease is usually mild and self-limiting and resolves within a 2 week period. Fulminant cases of infection are rare. HEV infection does not induce chronic courses of hepatitis or liver disease such as cirrhosis or hepatic neoplasia. Clinically apparent cases of HEV infection are most often seen in young to middle aged adults of 15–40 years old. In younger individuals and children infections tend to be anicteric. To date, HEV infection is the most common form of clinically apparent sporadic viral hepatitis in the young adult population in 'developing countries' when we focus on the 'Old World'. From the clinical perspective HEV infection is indistinguishable from the other forms of viral hepatitis. In clinically apparent cases of infection jaundice mostly lasts from one up to 3 weeks. Also pruritus, clay-coloured faeces and generalised lymphadenopathia may be observed during the course of disease. Fatal infections of fulminant hepatitis E are rare and in the range of 1–3% on the average. They are more often observed in pregnant women who appear to be exceptionally susceptible to severe disease forms where they can occur in up to 20% of HEV infection with a lethal course of infection. Anyhow, HEV exhibits its highest virulence within the third trimester of pregnancy; the reasons for this are not sufficiently understood until now. There is also a high frequency of cases of intra-uterine deaths and abortions in case of maternal HEV infection. Surviving neonates of infected mothers often show a clinically apparent acute HEV infection.

An altered status of hormones and immunity are observed during pregnancy but the actual cause of this high mortality during pregnancy is still unknown. It is suggested that diminished cellular immunity – indicated by a decrease in CD4, an increase in CD8 cell counts and a consecutively lowered CD4/CD8 cell ratio – and a high level of steroid hormones that influence viral replication/expression during pregnancy appear to be the most plausible reason for severity of this disease during pregnancy [16, 17].

There is much evidence that HEV infections induce a good and mostly life-long immunity to re-infection by HEV.

Diagnostics

The virus can be visualised by electron microscopy, e.g., in faecal samples.

Today, most of the diagnostics is based on immunological methods on the antibody level. Specific IgM antibodies to HEV occur early – mostly at the time point of onset of symptoms/jaundice – and regularly in HEV infection and can persist over a considerably long period of time of up to months after the peak of the infection [18]. Also specific IgG anti-HEV antibodies occur early and regularly peak approximately 2–3 weeks after clinical manifestations are observed and remain for at least 2 years detectable. Patients with a delayed IgG response were reported [19]. Faecal HEV RNA regularly becomes positive before the onset of clinical symptoms and before serum HEV RNA becomes detectable (start of positivity goes almost in parallel with IgM/IgG seroconversion). Due to these facts molecular PCR techniques play an important complimentary role in up to date HEV infectiological diagnostics.

Of course, specific liver parameter such as, e.g., serum alanine aminotransferase (ALAT), bilirubin etc., are altered such as in other forms of viral hepatitis.

Disease management and prevention

Improving the socioeconomic situation – including hygienic conditions – with an optimal waste water management programme are the most effective measures of disease management of preventing HEV infections. Most infections appear being due to the consumption of contaminated 'freshwater'. There is much evidence that even intra-familial person-to-person transmission only plays a very minor role under acceptable hygienic conditions which reflects that the disease on its own is not extremely contagious.

The virus particle appears being relatively labile and can be inactivated by heating up to 90–100°C over a period of 10 min or even by cycles of freeze-thawing.

To date there is no specific therapy against HEV infection available. Further, approaches of passive immunotherapy with hyperimmunglobuline sera – even with high specific anti-HEV IgG titres, deriving from endemic areas – did not lead to clinical improvement of the clinical situation.

A vaccine against HEV infection in humans is not yet available on the market; however, development of a candidate vaccine is in progress with a high visibility of success [20]. Research on this issue started with the early successful approaches in cynomolgus monkeys performed by Tsarev et al. in 1994 [21]. They discovered that immunisation of *Cynomolgus* macaques with a 55 kDa recombinant HEV fusion protein deriving from the second ORF of HEV protected the animals against experimental infection with HEV. These results are promising for the development of a vaccine.

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Comparative Hepatitis
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Bacterial infections of the liver

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Abstract

Bacterial infections of the liver can be categorised into three entities: acute bacterial hepatitis, bacterial liver abscesses and granulomatous liver disease caused by bacteria. A broad spectrum of bacteria has been implicated in different forms of hepatic infections, and a wide variety of systemic bacterial infections affect the liver during the course of infection. Clinical symptoms, causative pathogens and therapeutic approaches overlap widely. Most bacterial infections that affect the liver cause secondary hepatitis with only discrete clinical and laboratory findings. Standard diagnostic procedures including physical examination, imaging and microbiological cultures will usually be sufficient to detect and ascertain the bacterial causes of hepatitis. Therapy of bacterial infections of the liver usually includes antimicrobial chemotherapy according to standard guidelines for the underlying disease and the identified pathogen as well as additional invasive therapy which may be required for certain manifestations.

Introduction

The liver can be affected by a wide variety of systemic infections. In bacterial infections most common forms of liver involvement are pyogenic abscesses, acute hepatitis and granulomatous liver disease.

Bacteria can reach the liver in several ways: Hematogenous spread of bacteria into the liver during bloodstream infection *via* the portal vein or the hepatic artery, direct spread of bacteria from a contiguous focal infection, *via* the biliary tree, or following internal or external trauma. These infections usually manifest as so-called pyogenic abscesses that are clinically indistinguishable from other abscesses such as the amoebic liver abscess (caused by *Entamoeba histolytica*).

While viruses are the most common causes of acute hepatitis, infections due to bacteria such as *Neisseria meningitidis*, *Salmonella typhi*, *Brucella* spp. or *Campylobacter* spp., have also been associated with this clinical

entity. Infections due to bacteria such as *Mycobacterium* spp., T*ropheryma whipplei*, *Treponema pallidum*, *Coxiella burnetii* or *Rickettsia* spp. are among the major causes of granulomatous liver disease.

This chapter reviews the different forms of liver diseases that can be caused by bacteria and summarises the clinical features and therapeutic options for the most common forms and pathogens.

Routes of infection

There are in general four mechanisms by which bacterial infections of the liver develop: haematogenous, biliary, contiguous spread, and *via* direct inoculation.

Haematogenous seeding of bacteria can occur *via* the portal vein or the hepatic artery from the systemic circulation and are frequently associated with peritonitis, diverticulitis or generalised bloodstream infection or sepsis. In the pre-antibiotic era, the haematogenous route was the most common method of infection, and infection usually originated from appendiceal pathology [1]. Today bacterial infections of the liver are most commonly associated with biliary disease [2, 3].

Underlying conditions leading to bacterial infections of the liver *via* the biliary tree are most commonly cholangitis, cholecystitis, or malignancies such as cholangiocarcinoma [3].

Translocation of bacteria from infectious processes in close proximity of the liver such as appendicitis, diverticulitis and other inflammatory processes of the intestine are less frequent, although contiguous spread from the lung, kidney, colon or the stomach has been reported [2, 4–6].

There are two potential mechanisms of direct infection: iatrogenic following invasive procedures such as ERCP, percutaneous liver biopsy [7] or abdominal surgery [8] accounting for approximately 7% of all pyogenic abscesses of the liver, or *via* penetrating trauma through the skin or secondary to accidental ingestion of sharp objects such as fish bones [9] or toothpicks [10] accounting for approximately 5% [3].

Cryptogenic infections where the route of infection and/or the underlying condition cannot be identified account for 40–99% of abscesses based on data from several studies [2, 4, 11, 12].

Clinical entities

Bacterial infections of the liver can be categorised into three entities: acute bacterial hepatitis, bacterial (pyogenic) liver abscesses and granulomatous liver disease caused by bacteria. While this differentiation remains largely theoretical in most infections because clinical symptoms, causative pathogens and therapeutic approaches overlap widely, differ-

ences in outcome have been reported in patients with liver infection due to *Listeria monocytogenes* [13].

Bacterial hepatitis

Among the various causes of acute hepatitis, bacterial infections are relatively rare. Bloodstream infections and severe sepsis or septic shock can cause hepatic ischaemia that can present as an acute hepatitis. A variety of organisms has been described as causative agents of hepatitis, including *Bartonella henselae*, *Borrelia burgdorferi*, *Brucella abortus*, *Chlamydia* spp., *Coxiella burnetii*, *Francisella tularensis*, *Legionella pneumophila*, *Leptospira interrogans* s.l., *Listeria monocytogenes*, *Mycobacterium* spp., *Salmonella* spp., *Treponema pallidum*, and *Yersinia pestis* [14].

Bacterial abscesses

Pyogenic abscesses are the most common form of bacterial infections of the liver and develop in 5–22 patients per 100,000 hospital admissions [2, 3, 15–17]. Bacterial abscesses account for about 75% of all liver abscesses in industrialised countries, while in countries of the Third World parasitic abscesses are more frequent [18]. Liver abscesses frequently develop *via* direct spread following obstruction of the biliary tree or infections of the gastrointestinal tract such as diverticulitis or peptic ulcer disease but can also be associated with abdominal surgery or malignancies in the close proximity of the liver. Consequently, incidence is highest in patients between 50 and 60 years [2, 19, 20]. Most studies did not identify any significant differences in gender and did not detect any clear geographic patterns [19, 20]. The incidence of bacterial liver abscesses has been increasing over the past decade. Mortality has decreased considerably during that time, mostly due to advances in imaging and antimicrobial chemotherapy [21].

The most important differential diagnosis is liver abscess due to other causes, most commonly amoebic liver abscesses caused by infection with *E. histolytica*, even though approximately 2.5% of amoebic abscesses also yield bacteria [12, 22].

Granulomatous hepatitis due to bacterial infections

Involvement of the liver, mostly in the form of granulomatous liver disease can be an important manifestation of certain bacterial infections. The differential diagnosis includes diseases caused by a number of viral (e.g., HAV HCV, CMV, EBV), or parasitic (e.g., *Leishmania*, *Schistosoma*, *Toxocara*,

Clonorchis) agents which are discussed elsewhere in this book, as well as a variety of non infectious causes such as drug induced liver disease, primary biliary cirrhosis, neoplastic and malignant disorders, as well as systemic diseases such as sarcoidosis.

Clinical features

Most bacterial infections affecting the liver cause secondary hepatitis with mild to moderate elevation of liver enzymes (mainly ALT, AST, alkaline phosphatase) and only discrete clinical symptoms. Many systemic bacterial infections only involve the liver to a relatively minor extent and are therefore not primarily classified as hepatitis; however, some infections such as leptospirosis (Weil's disease) or syphilis may present with the classical signs and symptoms of an acute hepatitis.

Clinical symptoms such as abdominal pain, (typically in the right upper quadrant) and fever may present with an acute onset and severe complications such as pneumoperitoneum following the rupture of a gas containing abscess have been described [23]. More often, however, symptoms are non-specific and can include general weakness, headache, myalgia, and nausea [2, 24]. Patients typically present with slowly progressive vague constitutional symptoms such as fatigue, anorexia and fever [23–26]. Time between the onset of symptoms and diagnosis averages 2 weeks but intervals of greater than 4 weeks have been reported [27]. Depending on the size and the location of the abscess, more specific symptoms such as nausea, diffuse abdominal discomfort, pleuritic chest pain, referred right shoulder discomfort caused by lesions near the diaphragm, or biliary obstruction and mild jaundice caused by abscesses compressing the biliary tree may be present [24, 28–30]. Only between 10% and 37% of patients present with the classical triad of jaundice, fever and tenderness in the right upper quadrant [2, 11, 31]. In a recent series fever was the most common symptom, presenting in approximately 90% of cases, followed by abdominal pain (70%), nausea (43%), weight loss (38%), pleural effusions (33%) and hepatomegaly and jaundice (20–28%) [26, 31].

Physical examination generally reflects the clinical symptoms, highlighting the non-specific nature of the presentation in many patients [2, 26, 31].

Leukocytosis (present in 70–88% of patients [32, 33]), two- to three-fold increased alkaline phosphatase and elevation in the serum C-reactive protein (CRP), often in combination with anaemia, decreased serum albumin and elevated bilirubin are the most commonly seen laboratory abnormalities [2, 3, 24].

In contrast, liver function tests can be normal or show only slight to moderate deviations from the normal values. Alkaline phosphatase (AP) is most commonly elevated, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are elevated mostly in patients with primary or

secondary biliary involvement, while albumin and prothrombin time are often within normal limits.

Due to the non-specific nature of clinical (and sometimes also laboratory) findings, diagnosis of bacterial hepatitis usually requires a high degree of suspicion. Most of the important differential diagnoses such as amoebic liver abscess, cholecystitis and pyelonephritis cannot be ruled out on clinical signs alone. Since there are a variety of underlying conditions that can lead to abscesses in the liver, diagnosis may be missed initially, especially in abscesses occurring in the wake of abdominal surgery or other invasive procedures where symptoms such as pain and fever may well be related to the underlying condition.

Mortality rates have decreased substantially over the past several decades, with recent studies reporting rates of 11–31% [26].

Diagnosis

Standard diagnostic procedures including physical examination, imaging (ultrasound, CT or MRI scan) and microbiological cultures will usually be sufficient to detect and ascertain the bacterial causes of hepatitis.

Chest X-ray is not very sensitive, but may reveal an elevated right hemidiaphragm and pleural effusion [4]. Diagnosis can usually be made by abdominal ultrasound, where single or multiple abscesses can be detected [21]. In some cases, ultrasound may also help to establish the origin of infection, for example obstruction of the biliary tree. Abscesses too small to be detected by ultrasound can be detected using computed tomography (CT) with contrast magnetic resonance imaging (MRI) [34]. These studies can also be helpful in establishing the primary site of infection. Series comparing imaging techniques found ultrasound slightly less sensitive (94% *versus* 100%) that CT with contrast [35]. Further invasive tests such as ERCP may be indicated depending on the underlying conditions [36]. Also nuclear imaging to locate metastaic foci and ensure adequate drainage may be warranted in patients failing to achieve defervescence [11, 24].

Aspiration of the abscess(es) should be attempted if possible, in order to obtain material for microbiological and cytological examination, and also as part of the treatment in larger abscesses.

Abscess material for microbiological examination should be collected in a sterile tube and transported to the laboratory as soon as possible. In addition, some material can be inoculated into blood culture bottles. Blood cultures (2–3 sets) should be obtained to complete microbiological diagnosis. During the bacteremic phase most infections can be diagnosed by blood cultures, which are positive in about half of the cases but may yield different pathogens than abscess materials recovered from the same patient [26, 27].

Cytologic examination of smears from the abscess material is warranted to exclude underlying malignancies.

Therapy

Therapy of bacterial infections of the liver usually consists of antimicrobial chemotherapy according to standard guidelines for the underlying disease and the identified pathogen. Due to increasing resistance especially in *enterobacteriaceae*, culture of the causative pathogen(s) should always be attempted and susceptibilities of identified bacteria should always be ascertained. In some cases, such as fulminate leptospirosis, full supportive therapy in the intensive care unit may be necessary.

Although there are some reports of cure by antibiotic treatment without surgery [28, 37], drainage of abscesses is usually essential [24].

Percutaneous transhepatic drainage is a relatively low risk procedure that has replaced open surgery as the method of choice [2, 5, 28, 38, 39]. Percutaneous catheter drainage (PCD) and percutaneous needle aspiration (PNA) should be attempted if there is one large and accessible abscess. These methods had a high success rate (70–90%) while mortality was relatively low [2, 39]. In comparison of both techniques, Yu and co-workers concluded that intermittent needle aspiration is probably as effective as continuous catheter drainage for the treatment of pyogenic liver abscess and recommended it should be considered as a first line approach due to the additional advantages of procedure simplicity, patient comfort, and reduced price [39].

Due to the equivalent efficacy of PCD/PNA to surgical therapy and the higher mortality of the latter (up to 30%), most authors recommend an open surgical approach only in selected cases [39, 40]. Recent studies also describe endoscopic drainage of pyogenic liver abscesses with suspected biliary origin and conclude that ERCP enables not only demonstration of communications between the biliary tract and liver abscesses, but also that an internal drainage of the cavity is feasible and safe [41]. In contrast, others found that for large liver abscesses of more than 5 cm, open surgical drainage provides better clinical outcomes than PCD in terms of treatment success, number of secondary procedures, and hospital stay with comparable morbidity and mortality rates [42].

Since abscesses are often polymicrobial, initial therapy utilising broad-spectrum antibiotics with anaerobic coverage, is recommended. Antimicrobial agents recommended as first-line drugs include beta-lactam/beta-lactamase inhibitor combinations (for example, ampicillin/sulbactam or piperacillin/tazobactam) in combination with a third generation cephalosporin, or a fluroquinolone or in cases of nosocomial infections and/or pretreated patients, carbapenems (for example, imipenem, meropenem) [43, 44]. Other factors such as underlying conditions or probable source of infection can also influence the decision on initial antimicrobial therapy.

Therapy should be adjusted as soon as results from blood cultures and from cultures of the abscess are available.

Pathogens

A broad spectrum of bacteria has been implicated in different forms of hepatic infections, and a wide variety of systemic bacterial infections affect the liver during the course of infection.

Acute (peri)hepatitis can be caused by bacterial pathogens including *Bartonella henselae*, *Borrelia burgdorferi*, *Brucella abortus*, *Chlamydia* spp., *Coxiella burnetii*, *Francisella tularensis*, *Legionella pneumophila*, *Leptospira interrogans* s.l., *Listeria monocytogenes* [13], *Mycobacterium* spp., *Neisseria gonorrhoeae* [45] and *N. meningitides* [46], *Salmonella* spp. [47, 48], *Treponema pallidum*, and *Yersinia pestis* [14].

Bacterial pathogens can be recovered from about 60% of abscesses [5] and may vary considerably depending on the source of the abscess. Abscesses are polymicrobial in 20–50% of cases and some authors have suggested that solitary abscesses are more frequently polymicrobial than multiple abscesses [2, 17, 49, 50]. The most frequently encountered aerobic bacteria are *Enterobacteriaceae* such as *Escherichia coli* [51] and *Klebsiella pneumoniae* [24, 52] including extended spectrum beta-lactamase (ESBL) producing strains [53]. Gram-positive organisms such as staphylococci [54] (including community-acquired methicillin-resistant *Staphylococcus aureus* [55]) and streptococci [56–58] including *S. pneumoniae* [59] have been isolated from the liver as well. The percentage of anaerobic microorganisms varies between 10% and 54% in different series [2, 4, 24, 60, 61]. In addition, a variety of other organisms have been reported as causative agents of liver abscesses including *Actinomyces* spp. [62, 63], *B. henselae* [64], *Capnocytophaga* spp. [65], *Rhodococcus equi* [66], and *Yersinia enterocolitica* [67].

Bacterial infections that usually manifest as granulomatous liver disease include infections due to *Mycobacterium* spp., *Burkholderia pseudomallei* [68], *Brucella* spp. [69, 70] *Listeria* spp. [13, 71, 72], *Tropherryma whipplei* [73], *T. pallidum* [74, 75], and *C. burnetii* [76, 77].

Gram-negative bacteria

Enterobacteriaceae

Gram-negative rods, especially *Enterobacteriaceae* are the most common bacteria isolated from liver abscesses. Prior to the mid 1980s *Escherichia coli* was the predominant pathogen [2, 35, 49]. More recently the spectrum shifted to *Klebsiella pneumoniae*, which is now the most commonly isolated pathogen from pyogenic abscesses [24, 78] with the first cases due to ESBL producing strains reported recently [52]. This shift was first observed in Taiwan [28], followed by other Asian countries such as Japan [5, 25, 79], Singapore [38, 80], Korea [4], India [81], Hong Kong [39] as well as Europe (Spain [82], UK [83]), Australia [84] and the United States [24, 85–87].

Concomitant bacteraemia is reported in about half the cases of bacterial abscesses [2], but some authors reported blood stream infection (BSI) in up to 95% of patients with liver abscesses due to *K. pneumoniae* [88]. Extrahepatic abscesses have been described in only 7–12% of these patients [11, 28, 89]. Secondary abscesses are most often encountered in the eye [88, 90] but a variety of other sites has been described such as the lung/pleura [25, 79], kidney [89] prostate [39, 79], CNS/meninges [28, 79, 85, 89], bones [28] and skin and soft-tissue [28, 81, 91]. Diabetes mellitus and alcoholism have been identified as significant risk factors for developing metastatic infections from pyogenic liver abscesses [49]. In liver infections due to *K. pneumonia*, recent studies found the K1 genotype to be associated with metastatic complications [51].

In contrast to other *Enterobacteriaceae*, *Salmonella* spp., mainly *S. typhi* rarely cause hepatic abscesses, even though there have been some reports including one recent case of salmonellosis with septic shock and liver abscess in a diabetic and cirrhotic patient [47]. Even in immunocompromised patients such as patients with HIV/AIDS where bacteraemia due to non-typhi *Salmonella* is more frequent than in immunocompetent hosts, focal complications involving the liver have been rarely described [92].

Generally, in patients with typhoid fever, the usual histologic finding of the liver is nonspecific reactive hepatitis and other manifestations such as hepatic granuloma are rare complications of typhoid fever [48]. Systemic infections due to *Salmonella* spp. often involve the liver, mainly during the bacteremic phase of infection. In almost half of the infections due to *S. typhi* hepatosplenomegaly and severe impairment of liver functions can be observed. There have been more than 150 cases of salmonella hepatitis reported and incidences range from less than 1% to 26% of patients with enteric fever. While extreme hepatic dysfunction is a rare complication in *Salmonella* hepatitis, typhoid nodules with marked hyperplasia of reticuloendothelial cells is usually observed. Specific antimicrobial therapy is usually effective but severe clinical courses with mortality rates up to 20% have been reported [47, 93, 94].

Neisseria meningitidis/Neisseria gonorrhoeae

The liver can be affected during the bacteraemia that may occur during meningococcal or gonococcal infections.

Acute perihepatitis due to *N. gonorrhoeae* (one etiology of the Fitz-Hugh-Curtis syndrome) is mainly encountered in female patients with pelvic inflammatory disease and can develop even months after the primary infection [45]. The incidence of this complication of pelvic inflammatory disease was relatively low (4%) in recent series [95].

Recently cholestatic hepatitis has been reported in a child in Turkey following chronic meningococcaemia [46]. Hepatitis developed four days after the initial presentation that included fever, arthralgia and a maculopapular rash, while the patient was treated with penicillin consistent with the suscep-

tibilities of the *N. meningitidis* isolates recovered from the blood cultures. Diagnosis was made by clinical and laboratory findings, and other causes of hepatitis were ruled out [46].

Burkholderia pseudomallei
Infections due to *Burkholderia pseudomallei* are usually associated with multiple abscesses or and/or granulomatous liver disease. Several cases have been reported and close attention to the patients' travel history, underlying co-morbidities (such as diabetes, renal disease and alcohol abuse), and the presence of concomitant splenic abscess are important to enable early detection of melioidosis. The treatment of choice is intravenous ceftazidime for at least 14 days or more. An adequate duration of maintenance oral therapy, with amoxicillin-clavulanate or trimethoprim-sulfamethoxazole for 12–20 weeks, is necessary to prevent relapse [66].

Bartonella henselae
B. henselae (originally designated *Rochalimaea henselae*) usually affects the regional lymph nodes leading to abscesses (cat scratch disease, CSD). In highly immunocompromised patients a granulomatous infection of the liver can develop as a second stage of the disease. Atypical presentation of *B. henselae* infection is reported in approximately 25% of patients including paediatric patients [62]. Endocarditis, granulomatous hepatitis, hepatosplenic infection or osteomyelitis are the most common manifestations of bartonellosis apart from CSD [107].

Brucella spp.
Brucella spp. are usually ingested with raw milk or cheese manufactured from the milk of infected animals. Hepatitis has been reported as a clinical manifestation of infection with *B. melitensis* [108]. Localised manifestation of Brucellosis in the liver (hepatic brucelloma) are rare and less than 50 cases have been reported including manifestations in children [67, 109, 110].

Francisella tularensis
Tularemia caused by *Francisella tularensis* usually manifests as an ulceroglandular or a typhoial illness. In the latter there are few localised signs and it is often associated with a mild hepatitis [116].

Gram-positive bacteria

Staphylococcus spp., *Streptococcus* spp., *Enterococcus* spp.
Gram-positive organisms such as *S. aureus*, coagulase-negative staphylococci such as *S. haemolyticus* [96], beta-hemolytic streptococci, *S. milleri* [97] and *Enterococcus* spp., are recovered less frequently from bacterial

infections of the liver than Gram-negative pathogens [2, 24, 28]. In addition, these organisms are more likely to be recovered from monobacterial secondary abscesses in patients where the primary focus of infection is usually located outside the abdomen.

In recent studies, *S. pneumoniae* has been reported as the cause of liver infections mainly in immunocompromised hosts. Alcoholism, HIV infection, splenectomy, connective tissue disease, steroid use, diabetes mellitus, and intravenous drug use remain common risk factors for invasive pneumococcal infections [58].

Listeria monocytogenes

Listeria monocytogenes causes sepsis and meningitis in immunocompromised patients and a devastating maternal/fetal infection in pregnant women [13, 99, 100]. Various outbreaks demonstrated that *L. monocytogenes* can cause gastroenteritis in otherwise healthy individuals and more severe invasive disease in immunocompromised patients [70, 101, 102].

Maternal listeriosis usually results in a nonspecific febrile illness that is rarely diagnosed prepartum (about one third of patients have no symptoms at all) [9, 100] but may lead to spontaneous abortion, stillbirth, death of the newborn within hours after birth, or neonatal sepsis or result in granulomatosis infantiseptica, characterised by microabscesses and granulomas in the liver and spleen [13, 100].

In adults there are a number of atypical clinical forms of listeriosis (5–10% of cases), including endocarditis (the third most frequent form), myocarditis, hepatitis, colecystitis, localised abscesses [100]. To date there are 35 cases of hepatic listeriosis described in the literature, with clinical presentations including solitary liver abscesses, multiple abscesses and diffuse or granulomatous hepatitis, which differ in clinical course and outcome [13]. Details of the clinical features, diagnostic modalities and treatment options have recently been reviewed in detail by Scholing and colleagues [13].

Anaerobic bacteria

The incidence of anaerobic bacteria recovered from liver abscesses varies greatly between series. Even in studies that specifically tried to culture anaerobic microorganisms incidences between 10% and 54% are reported, while more recent series report incidences between 10% and 22% [2, 4, 24, 59, 60]. Recovery of anaerobic bacteria, especially from mixed infections, is sometimes challenging, so the incidence of these organisms may well be under-reported. Anaerobic bacteria are more likely to be recovered from abscess aspiration and are generally not detected in concomitant blood cultures. *Bacteroides fragilis* is the most commonly seen anaerobe in most series [4, 24, 98].

Other bacteria

Leptospira icterohaemorrhagiae
While several *Leptospira* spp. can cause disease in man, *L. icterohaemorrhagiae* is the causative agent of Weil's disease. Characteristic symptoms and jaundice make this illness an important differential diagnosis of viral hepatitis and similar hepatic illnesses have been described in patients infected with hantaviruses [103, 104].

Treponema pallidum
Syphilis, a sexually transmitted disease, can affect the liver in several ways. If untreated, *T. pallidum* can cause acute hepatitis in the second stage of disease, while gummata can also be present in the liver during the tertiary phase of syphilis [73]. The liver is also regularly affected in congenital syphilis [105]. Recently syphilitic hepatitis has also been described in liver-transplant patients [106].

Mycobacterium tuberculosis
The liver is affected most commonly in miliary tuberculosis spreading from the lung *via* the hepatic artery [111] but other routes of infection such as lymphatic spread have been reported [112, 113]. Focal isolated tuberculosis of the liver has been reported mainly in immunocompromised patients such as patients with HIV/AIDS [114].

Hepatic tuberculosis may present as an abscess or a more diffuse form of granulomatous liver disease. Clinical features, diagnosis and management of this rare manifestation have recently been reviewed in detail [115].

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Comparative Hepatitis
ed. by Olaf Weber and Ulrike Protzer

Comparative hepatitis: Diseases caused by adult parasites or their distinct life cycle stages

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Abstract

There are a variety of parasites – protozoans, cestodes, trematodes, nematodes or pentastomides – which reside in the liver or invade this organ and are responsible for inflammation resulting in hepatitis. Among the Protozoa *Entamoeba histolytica* is of high importance. In the cyst fluid of the liver abscess purulent components are visible, but also some amoeba, so-called magna forms. Furthermore the amastigote stages of *Trypanosoma cruzi* and *Leishmania donovani* – both are Protozoa which possess a kinetoplast – and the schizonts of *Plasmodium* spp., which are the causative pathogens of malaria, have to be mentioned as a putative cause of parasite-induced hepatitis. Among cestodes *Echinococcus granulosus* and *E. multilocularis* preferentially reside in the liver. Within the trematodes *Schistosoma* spp., *Clonorchis sinensis*, *Opisthorchis viverrini*, *Dicrocoelium dendriticum* and the juvenile stages of *Fasciola hepatica* have to be considered. Among the large group of human nematodes, only *Capillaria hepatica*, migrating nematode larvae (such as larva 2) and the adults of *Ascaris lumbricoides*, larvae of *Strongyloides stercoralis*, hookworms, *Toxocara canis* and microfilariae of different filariae play a role in inducing liver inflammation.

Hepatitis caused by protozoa

Entamoebiasis: Entamoeba histolytica *[1–6]*

Taxonomy

Subregnum:	Protozoa
Phylum:	Amoebozoa
Class:	Lobosea
Order:	Amoebida
Family:	Entamoebidae
Genus:	*Entamoeba*

Morphology

The life cycle of *Entamoeba histolytica* comprises two morphologically distinct developmental stages: the vegetative stages (minuta and magna forms), also known as trophozoites, and the cyst stage (Figs 1 and 2; Figs 1–3 and Figs 5–17 see pp 174–177).

Trophozoites are uninucleate cells with a diameter of 20–40 μm and a typical nucleus (spherical, central karyosome); they show pronounced gliding movements by formation of a single apical pseudopodium; cysts are spherical, quadrinucleate and have diameters from 10–20 μm.

Transmission, multiplication and incubation period

Entamoeba histolytica is usually transmitted by the faecal-oral route by ingestion of infectious cysts (contaminated food), but transmission through relevant homosexual practices is also possible.

Multiplication of *Entamoeba histolytica* takes place only in the intestine. After ingestion of the typically quadrinucleate cysts, which unlike the trophozoites can remain infectious for months outside the body and are not destroyed by the acid contents of the stomach, the uninucleate trophozoites capable of multiplication develop in the small intestine. In the lower colon there is renewed encystment with two subsequent nuclear divisions. An infected person can pass up to 500 million cysts per day. The incubation period of amoebiasis is very variable. The time between infection and the occurrence of clinical symptoms can be a few days but in some cases even several years. The average incubation period for an amoebic liver abscess is up to 5 months.

Epidemiology

Natural infection with *Entamoeba histolytica* is limited to man and a few Old World monkey species. People in tropical and subtropical countries in which *Entamoeba histolytica* is endemic are usually associated with low socioeconomic status and poor hygienic conditions; travellers to such countries and male homosexuals are endangered.

The prevalence of *Entamoeba histolytica* depends primarily on the number of cyst passers and the hygienic conditions. Under the hygienic conditions which are standard in Western Europe the infection usually does not or is poorly spread.

Diseases and symptoms

The pathogenicity of *Entamoeba histolytica* is based primarily on the abil-

ity to destroy host tissue and cells. Surface receptors, cysteine proteinases and pore-forming peptides of the amoebae play a decisive role in this. The virulence of individual amoeba isolates can vary depending on the level of expression of these molecules. Antigen variability has not yet been shown for *Entamoeba histolytica*.

Any infection with *Entamoeba histolytica* is called entamoebiasis. Most infections of preexposed persons are asymptomatic. Oral uptake of cysts leads mainly to intestinal manifestations but also, as a result of spread of the amoebae to other organs, to extraintestinal manifestations. In the latter case there occur usually bloody diarrhoea and severe illness, accompanied by fever and weight loss (Fig. 3).

Intestinal amoebiasis
Intestinal amoebiasis is linked with enteritis or colitis of varying severity. Typically, ulcerative mucosal lesions with diarrhoeal stools containing blood and mucus (Fig. 3). Amoeboma may occur as tumour-like granulomatous inflammatory reaction of the colon caused by *Entamoeba histolytica*.

Extraintestinal amoebiasis
Magna forms of the amoebae can spread to other organs by haematogenous dissemination and lead to abscess formation. In more than 90% there are extraintestinal manifestations in the liver (Fig. 4, p. 164). In rare cases there can be complications such as peritonitis after perforation of an amoebic liver abscess or migration from the liver into the lung or pericardium.

Immune response

Infections with *Entamoeba histolytica* regularly induce a specific B- and T-cell response. Even in the case of asymptomatic infections serum antibodies to *Entamoeba histolytica* can be detected in more than 80% of cases.

Differential diagnosis and diagnosis

In the case of enteritis or colitis other intestinal infectious agents should always be ruled out. In the case of marked mucosal lesions ulcerative colitis should be considered. An amoebic liver abscess should be differentiated from a bacterial abscess. Further differential diagnoses are echinococcosis or a necrotic tumour.

The diagnostic workup of invasive amoebiasis depends on the site of manifestation of the illness: a) In the case of intestinal amoebiasis rectoscopic or colonoscopic detection of appropriate mucosal lesions and direct detection of the pathogen are the primary measures. The latter is performed by examination of the stools (Figs 1 and 2) or by histological detection of

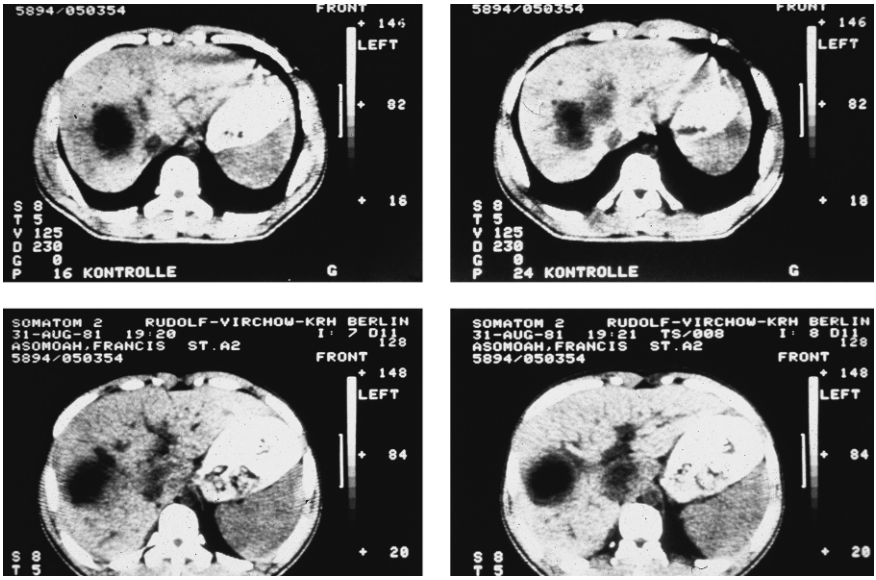


Figure 4. Computer tomographies of a liver with an abscess due to *E. histolytica*

amoebae in biopsy material; b) In extraintestinal amoebiasis imaging procedures such as ultrasound and computed tomography are used to detect appropriate organ manifestations and structural defects. At the same time serological detection of specific antibodies to *Entamoeba histolytica* is an important, often pivotal diagnostic tool.

To distinguish it from other apathogenic intestinal amoebae (e.g., *Entamoeba dispar*), particularly in the case of asymptomatic infections, *Entamoeba histolytica* should also be characterised immunologically or genetically.

Immunological characterisation using monoclonal antibodies to specific epitopes of *Entamoeba histolytica* is also available as well as the genetic characterisation on the basis of specific DNA sequences, e.g., within the rRNA gene.

The genome of *Entamoeba histolytica* is currently being sequenced. It probably comprises 20 megabases which are distributed over 14 chromosomes.

Treatment

Infection with *Entamoeba histolytica* must always be treated.

Asymptomatic intestinal *Entamoeba histolytica* infections should be treated with diloxanide fuorate or paromomycin for 10 days. All *Entamoeba histolytica* infections with intestinal or extraintestinal clinical manifestations

should be treated first with metronidazole (30 mg/kg body wt/day divided into three daily doses for 10 days) to kill amoebae which have already penetrated the tissues, followed by treatment with diloxanide fuorate or paromomycin to eliminate the remaining intraluminal forms.

Resistance

Drug resistance to metronidazole has been suspected in individual cases, but has not yet been scientifically confirmed.

Prevention

Work on a vaccine against *Entamoeba histolytica* is in progress. Prophylaxis currently consists of filtering, disinfecting or cooking all food which might be contaminated with infectious *Entamoeba histolytica* cysts.

Notifiable disease status

Entamoebiasis is not notifiable; however, occurrence in schools, refugie-camps, etc., have to be announced to Public Health Authorities.

Leishmaniasis: Leishmania *species [5–9]*

Taxonomy

Subregnum:	Protozoa
Phylum:	Euglenozoa
Class:	Trypanosomatidea
Order:	Trypanosomatida
Genus:	*Leishmania*
Species (Old World):	*L. donovani*, *L. infantum*, *L. major*, *L. tropica*, *L. aethiopica*, etc.
Species (New World):	*L. chagasi*, *L. mexicana*, *L. amazonensis*, *L. brasiliensis*, *L. panamensis*, *L. peruviana*, etc.

Morphology

The so-called amastigote form of the parasite is found in cells of the reticuloendothelial system of the vertebrate host (Fig. 5). It is spherical or ovoidal, measures about 2–3 μm in diameter and has only a short retracted

flagellum. The nucleus and kinetoplast stain strongly with the Romanovsky stain, which gives the organism its characteristic appearance. The so-called promastigote form is found in the sandfly and in culture medium. It has an anterior flagellum and devides by binary fission (Fig. 6).

Transmission, multiplication and incubation period

Transmission is done by sandflies thus being a zoonosis originating from dogs or rodents or occurs from man to man. Further transmission from man to man may be done by blood products. The amastigote form is taken up from the blood or tissue of the mammalian host by the female sandfly and is transformed there into the infectious promastigote form. Visceral leishmaniasis (VL) needs weeks to months (until clinical signs occur, while cutaneous leishmaniasis (CL) is manifested within days by bad-healing skin lesions).

Epidemiology

Leishmaniasis is usually a zoonosis. Reservoir hosts are wild or peridomestic carnivores (e.g., dogs), rodents and also man. Endangered are people living in endemic areas and travellers returning from endemic areas, foreign residents, migratory workers, asylum seekers, refugees from endemic regions as well as people infected with HIV (opportunistic infection). The annual incidence of CL is around 10 million cases; that of VL about 400,000 cases (about 50% of these in India and 90% of these in the state of Bihar). The numbers are increasing on account of deforestation (Brazil), irrigation projects and urbanisation (Middle East), as well as the collapse of infrastructure (Afghanistan). VL occurs in the Indian subcontinent, Pakistan, Nepal, Central and Southwest Asia, China, North and Subsaharan Africa, Near and Middle East, Mediterranean countries and Central and South America.

Diseases and symptoms

Visceral leishmaniasis VL (Kala azar)
Outside endemic areas patients with VL often remain undiagnosed for a long time. The primary symptom – fever – is often misdiagnosed, blood changes such as anaemia, leucopenia or thrombopenia (or pancytopenia) and hepatosplenomegaly are often misinterpreted. In the case of infection with *Leishmania infantum* (in countries of the Mediterranean Coast) or *Leishmania chagasi* (South America) subclinical courses and spontaneous recoveries are more common than clinically manifested disease. Infections with *Leishmania donovani* on the other hand are usually clinically manifest and induce a high mortality rate. The disease can begin insidiously or

abruptly. The patient presentation depends to a large extent on the duration of the disease. It usually consists of fever, abdominal symptoms associated with an enlarged spleen, weight loss, cough or diarrhoea.

Post kala azar dermal leishmaniasis (PKDL)
This nodular or hypopigmented macular skin eruption may occur after successful treatment of VL caused by *L. donovani*.

Immune response

Inborn resistance is based on the ability of the macrophages to kill the intracellular *Leishmania* stages by nitric oxide production. The parasite in turn has developed the ability to ward off this danger by specific inhibition and to multiply inside the cytoplasm instead vacuoles.

Acquired immunity is initiated *via* macrophages and dendritic cells which present the *Leishmania* antigens to T-lymphocytes. This results in either an effective cellular or ineffective humoral immune response. The effective TH1 response leads to activation of the macrophages while the TH2 response inhibits the macrophages with regard to their ability to kill the *Leishmania* stages. The cellular immune response of the host determines the manifestation of the disease (clinical – subclinical, visceral – cutaneous – mucocutaneous, etc.).

Differential diagnosis and diagnosis

Excluded must be numerous diseases in which occur fever, anaemia, leucopenia, thrombopenia and hepatosplenomegaly, particularly bacterial sepsis and malignant diseases.

Visceral leishmaniasis
Many types of non-healing wounds.

Serology
Direct agglutination test (DAT), immunofluorescence antibody tests (IFAT), enzyme linked immunosorbent assays (ELISA). The sensitivity of the serology is >90%. In HIV-infected persons, depending on the HIV stage antibodies may no longer be detectable despite the presence of clinically manifest VL. Only about 50% of the HIV infected patients with VL test positive in routine serological testing. The problem with serological diagnosis of VL lies in the specificity of the available test systems and in the fact that individuals with asymptomatic infection and people who have been treated show positive serological results for a long time (serological 'scar').

Direct detection of the parasite
Direct detection of the parasite is performed in:

Venous blood – fluorescence microscopic detection of *Leishmania* stages in the buffy coat layer (sensitivity up to 70%; the immunodeficiency of AIDS patients means that peripheral monocytes are more likely to be infected than in immunocompetent infected patients; therefore particularly high sensitivities are to be expected);

Examination of material obtained by puncture of the spleen, the liver or the bone marrow – the smears are stained with Giemsa, Wright or Leishman stain (sensitivity: spleen >95%, liver or bone marrow 70–85%) (Fig. 5). Inoculation of biopsy material into suitable culture medium and culture. In positive cultures microscopically visible motile promastigote cells can be expected within 2 weeks. The culture step can further increase the sensitivity (by up to 10%).

Diagnostic procedures for determining the severity of disease
Blood count, plasmatic coagulation, liver values, protein electrophoresis, ultrasound of the abdomen. In advanced, complicated courses: procedures to detect secondary bacterial infections (pneumonia, tuberculosis, dysentery).

Treatment

Visceral leishmaniasis
Liposomal amphotericin B (AmBisone®): belongs to the group of the lipid-associated amphotericins. These are taken up by macrophages which thus carry the amphotericin B directly to the site of infection. Very high levels are achieved in the liver and spleen. They are less toxic than amphotericin B. On the basis of the criteria efficacy and side-effect profile, liposomal amphotericin B is currently the best drug for treatment of VL. However, the price is a problem.

Pentavalent antimony compounds (sodium stibogluconate [Pentostam®] contains 100 mg SbV/ml, meglumine antimoniate [Glucantime®] contains 85 mg SbV/ml): the toxicity is associated with the cumulative dose of SbV. Myalgia, arthralgia, nausea, abdominal symptoms (possibly indicating pancreatitis), hepatitis, headaches, reversible peripheral neuropathy, disturbances of the stimulus conduction system of the heart. Further drugs (second line drugs): There are a large number of further drugs (e.g., paromomycin, ketoconazole/itraconazole, allopurinol) which are used under certain conditions.

HIV patients
Liposomal amphotericin B initially has a high cure rate (100%) compared with antimony compounds (50%), but recurrences occur here, too. Drugs for relapse prophylaxis are given.

Resistance

Pentavalent antimony compounds: primary resistance is found in about 1% of the patients in African endemic areas and in about 10–30% in India.

Prevention

There are no vaccines available. There is no chemoprophylaxis. Repellents and clothing are useful as protection against the diurnally (early evening) active vectors. Mosquito nets impregnated with insecticide (permethrin) protect against nocturnally active vectors during sleeping.

Strategies for prevention and control of the disease

These strategies are concentrated on control of the regionally significant reservoir hosts (e.g., dogs) and of the vectors, as well as active case finding and treatment.

Notifiable disease status

*Leishmania*sis is not a notifiable disease according to the German Law for the Protection Against Infectious Diseases (*Infektionsschutzgesetz*).

Chagas disease: Trypanosoma cruzi *[5, 6]*

Taxonomy

Subregnum:	Protozoa
Phylum:	Euglenozoa
Class:	Trypanosomatidea
Order:	Trypanosomatida
Genus:	*Trypanosoma*
Species:	*Trypanosoma cruzi*

Morphology

Infection of man and numerous reservoir hosts in a sylvatic cycle (e.g., *via* armadillo, opossum, dog, cat, monkeys, rodents) takes place through the bite of blood-sucking reduviid bugs, including those of the genera *Triatoma* and

Rhodnius. In contrast to the African trypanosomes, however, transmission is not done with the saliva but through the trypomastigote stages contained in the faeces. The bugs pass these before or during their blood meal and they are either carried into the bite wounds by the bugs or rubbed into the wounds by the hosts through scratching. In the blood these trypomastigote stages appear C or S shaped and reach a length of 15–20 μm (Fig. 7). They very soon invade the host cells (particularly macrophages, muscle and nerve cells) and multiply directly in the cytoplasm by constant binary fission, appearing first as amastigote, later as epimastigote and finally again as trypomastigote stages. These infected host cells are also called pseudocysts (Fig. 8), but rupture during the multiplication phase of the parasites so that whole organs (e.g., heart) suffer from substantial loss of tissue. The multiplication takes place very gradually over several years and goes largely unnoticed by the body – on account of the mainly intracellular location of the parasite. If a bug takes up trypomastigote stages with the blood, these attach to its midgut wall, transform into epimastigote forms and multiply intensively through longitudinal binary fission. When they reach the bug's hind gut they are transformed to the infectious, trypomastigote (= metacyclic) form.

Transmission, multiplication and incubation period

Route of infection
Percutaneously through rubbing in trypomastigote stages contained in liquid bug faeces which are passed during or before the usually nocturnal feeding (= stercoral infection). Furthermore infection may occur by blood transfusion or breast milk.

Incubation period
5–20 days to occurrence of oedema (e.g., of the eyelid, Chagoma).

Prepatent period
1–2 months.

Patent period
Can need years in chronic courses.

Epidemiology

Countries of occurrence are: rural regions with bugs in California, Southern USA; South and Central America: Mexico to Argentina and Northern Chile.

Diseases and symptoms

Symptoms of the disease (Chagas' disease, American trypanosomiasis): An acute and a chronic phase of the disease are distinguished. After inoculation the parasites first multiply at the site of infection, producing a severe inflammatory reaction (chagoma). The area around the eye is often affected: the cheek, the eyelids and conjunctiva of the affected eye become red and swollen (Romaña's sign). The pre-auricular lymph nodes may become swollen.

After an incubation period of 1–3 weeks the haematogenous spread of the parasites begins, which can be characterised by fever, lymph node swelling, hepatosplenomegaly, oedema of the face and the legs. Myocarditis or meningoencephalitis occasionally occurs, particularly in children, and can lead to death even in the acute phase. Otherwise all these symptoms subside in the course of weeks or months. Note: The parasites are more easily observed in the blood during this phase. They are C- or S-shaped and reach about 15–20 μm in length; their kinetoplast is very large and located at the pointed posterior end of the cell. These forms are distinct from the apathogenic stages of the species *T. rangeli*, which are also transmitted by bugs.

During the chronic phase of the disease, which can be completely asymptomatic for years (so-called intermediate phase), parasites only rarely appear in the blood as they multiply mainly intracellularly, until the consequences of the organ destruction become manifest as Chagas' disease. The disease affects particularly the myocardium and the stimulus conduction system, with corresponding severe ECG changes, but also the visceral hollow organs. As regards the pathogenesis it is not yet clear whether the damage is caused by the direct action of the parasites or by an autoimmune mechanism. The cardiomyopathy, which affects mainly the right ventricle, leads *via* bouts of acute and chronic infection and fibrosis to cardiomegaly and often to a so-called apical aneurysm. The congestive cardiomyopathy is usually fatal within a few months. The lesions of the conducting fibres cause conduction disturbances and arrhythmias which can lead to sudden cardiac death. In the oesophagus and colon, but also in other hollow organs, destruction of the nerve cells of Meissner's and Auerbach's plexus leads to loss of tone and motility with enormous dilation (megaoesophagus, megacolon) with corresponding complications of dysphagia and constipation. These patients can die of aspiration pneumonia or ileus.

Differential diagnosis and diagnosis

Direct detection of trypomastigote forms in the blood is easiest during the acute stage. In fresh preparations they stand out through their motility. The details can be evaluated best in the stained smear (Fig. 7). A certain amount of concentration can be achieved with a thick drop but the mor-

phology is usually considerably impaired. Often more effective methods of concentration are required for detection, such as differential centrifugation or the microhaematocrit method. The Lanham method is less suitable for *T. cruzi*. The simple method of Strout is very effective: 10–20 ml of blood is allowed to clot, the serum is centrifuged first for 5 min at 150 g (removal of remaining erythrocytes), then for 10 min at 600 g; the motile trypanosomes are concentrated in the sediment. In the acute stage (particularly in acute meningoencephalitis) trypomastigote stages can occasionally also be found in the cerebrospinal fluid.

In the chronic phase of the disease direct detection is not usually possible. However, in up to 50% of the cases **xenodiagnosis** is successful. This is done by allowing 40 parasite-free, laboratory-bred reduviid bugs (3rd and 4th nymph stage) to feed on the patient (alternatively membrane feeding). After 30 or 60 days the faeces or gut of the bugs are then examined for the presence of epimastigote and trypomastigote parasites. In some cases isolation during the chronic phase is also possible through repeated haemocultures (in different culture media). Finally, isolation on rodents is also possible. In regions where *T. rangeli* is widespread, differentiation from this apathogenic trypanosome species is necessary. Finally, amastigote stages can sometimes be found in biopsies of various organs such as skeletal muscle, myocardium (catheter biopsies), intestine, liver etc.; however, histological examination is not suitable for specific diagnosis.

In the **chronic stage** serological tests are often the only indication of an infection. Numerous tests (IFT, ELISA, CBR, IHA, direct agglutination) detect IgG antibodies in more than 95% of the patients; in the acute stage and in congenital infections IgM antibodies are usually present. Tests may be negative in the first weeks after infection; false positive reactions can occur in patients with leishmaniasis and *T. rangeli* infections. PCR, which has already been used successfully for diagnosis, will probably allow very sensitive detection of *T. cruzi DNA* (in blood and in biopsies) in the chronic stage, too, in the future.

Treatment

The most effective treatment in both the acute and the chronic stages is nifurtimox 8–10 mg/kg body wt (children 15–20 mg/kg body wt) daily for 3–4 months. Benznidazole 5 mg/kg body wt (children 10 mg/kg body wt) daily for 60 days is also effective. In the chronic stage, damage that has already occurred is no longer influenced by the treatment.

Prophylaxis

Indoor control of bugs with insecticides.

Malaria : Malaria parasites, Plasmodium *species [5, 6, 10]*

Taxonomy

Subregnum:	Protozoa
Phylum:	Alveolata
Class:	Haematozoa
Order:	Haemosporida
Genus:	*Plasmodium*
Species:	*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*

Transmission, multiplication and incubation period

The malaria parasites are Protozoa belonging to the group Sporozoa (Apicomplexa) which are transmitted from man to man only (no animal reservoir) by the female mosquitoes of the genus *Anopheles* during the blood meal. Infection is, however, also possible through infected blood transfusions (the merozoites remain infectious in cooled citrated blood for at least 14 days), as well as through use of shared injection needles by drug addicts. Connatal malaria can occur as a result of transplacental or perinatal transmission of merozoites. A special form of natural transmission is so-called airport malaria which can occur in airport workers or people living near airports in case they are bitten by 'imported' mosquitoes.

Transmission of infectious stages (sporozoites) with the mosquito saliva is followed by asexual multiplication (exoerythrocytic schizogony) in cells of the liver parenchyma and in the RES (reticuloendothelial system; Fig. 9). It is 1– rarely 6 weeks (depending on the species) before the asexual stages being produced in the liver (merozoites) invade the red blood cells. The time up to their first appearance in the blood is termed the **prepatent period**, while the time to appearance of the first clinical symptoms (not identical) is defined as the **incubation period**.

After the merozoites have penetrated into the red blood cells the parasite appears first as a small unicellular trophozoite which has the appearance of a signet ring under the light microscope as its large central food vacuole does not stain and pushes the cell nucleus to the edge. The trophozoites grow by cell division and an increase in cytoplasm to form schizonts which finally occupy most of the erythrocyte and, depending on the species, consist of 8–32 new merozoites. These are released when the erythrocyte bursts and invade new erythrocytes. In the red cells these asexual multiplication processes are repeated (erythrocytic schizogony) (Figs 10 and 11), synchronised by host influences and lead finally to a rhythmic (species-specific) release of numerous merozoites and digested haemoglobin (pigment). It appears to

be particularly the free merozoites rather than the pigment which produce the typical fever following their release. The pigment, which appears brown-black under the light microscope, is stored in the endothelia of numerous organs (brain, kidney, liver, etc.) and is considered a marker for an existing or past infection.

As in this chapter, the main aim to deal with those stages multiplying in the exo-erythrocytic liver cells further descriptions of the erythrocytic stages are neglected.

Incubation period
P. falciparum: 8–24 days; *P. vivax*: 12–18 days; *P. ovale*: 10–17 days; *P. malariae*: 18–42 days.

Prepatent period
The minimum duration of exoerythrocytic schizogony (= appearance of the first stages in the blood): *P. falciparum*: 5 days (mean 8–12); *P. vivax*: 8 days (mean 13–17); *P. ovale*: 8 days (mean 13–17); *P. malariae*: 13–17 days (mean 18–37).

Patent period
P. falciparum: with treatment about 4–6 weeks; without treatment maximum 18 months. *P. vivax*: 5–7 years; *P. ovale*: up to 2 years; *P. malariae*: up to 30 years or more.

Epidemiology

Warm, humid countries between the latitudes of 40° North and 30° South. In Germany there are around 1,000 malaria cases per year with *falciparum*

Figure 1. Light micrograph (LM) of a minuta form of *E. histolytica*

Figure 2. LM of cysts of *E. histolytica*

Figure 3. Slimy, bloody diarrhoea as result of an infection with *E. histolytica*

Figure 4, see page 164

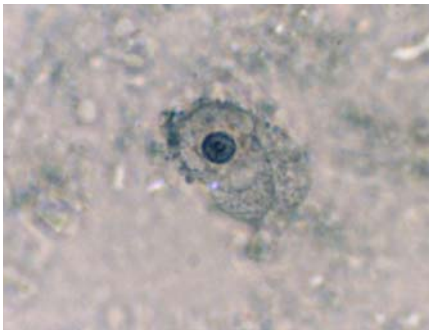
Figure 5. Light micrograph of amastigote stages of *Leishmania donovani* in and around a macrophage

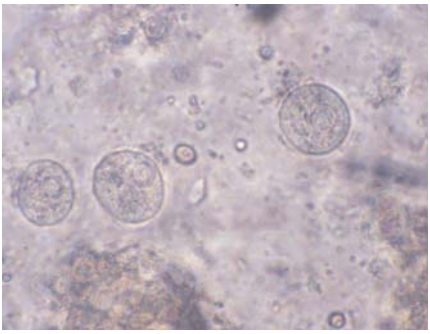
Figure 6. Scanning electron micrograph of a dividing promastigote of *Leishmania* sp.

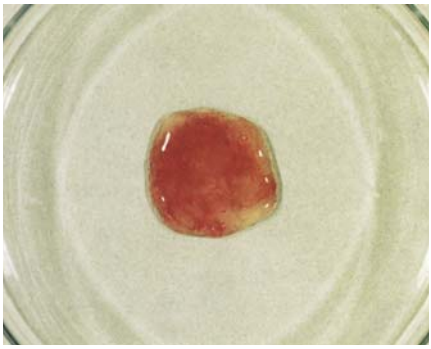
Figure 7. Light micrograph of a blood smear preparation of *T. cruzi* trypomastigotes

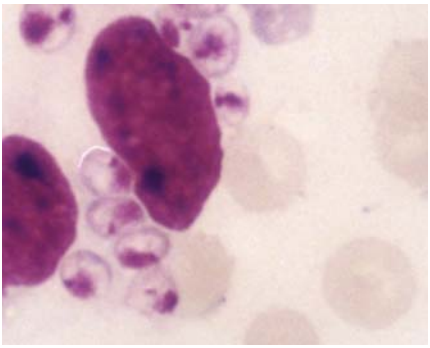
Figure 8. Section through a muscle cell with a pseudocyst containing *T. cruzi* stages

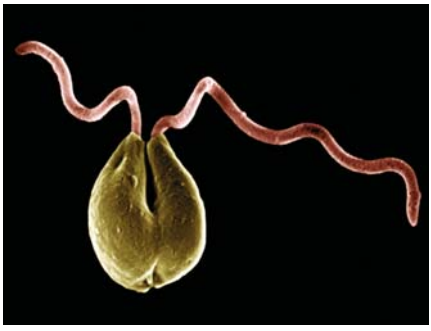
Figure 9. Light micrograph of a section through a liver schizont of *Plasmodium falciparum*

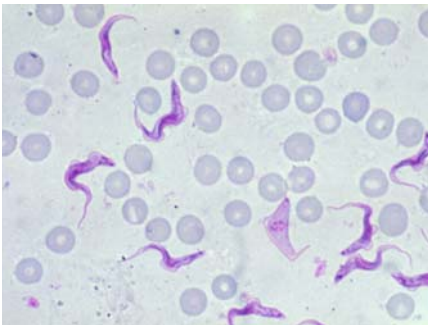
1 

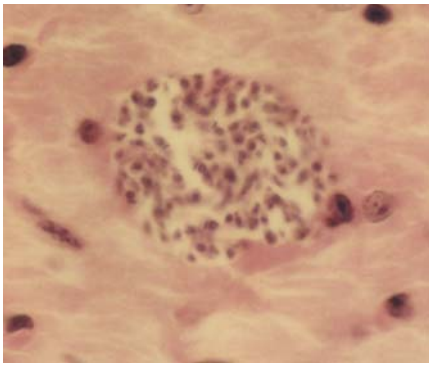
 2

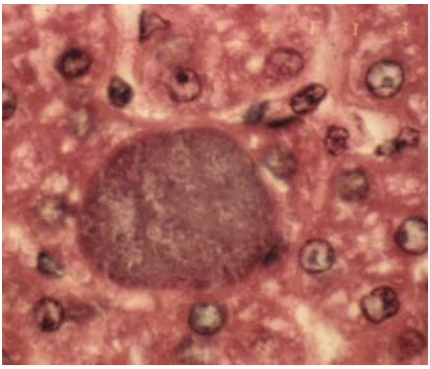
3 

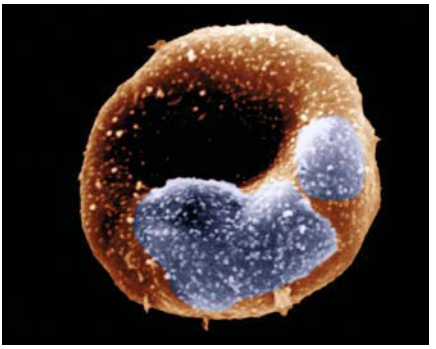
 5

6 

 7

8 

 9



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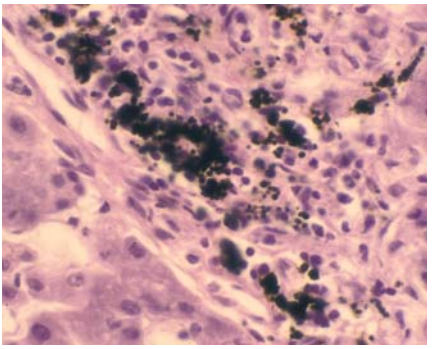

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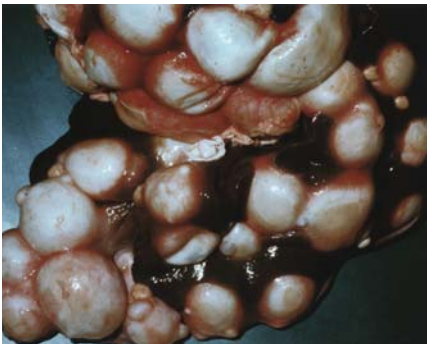

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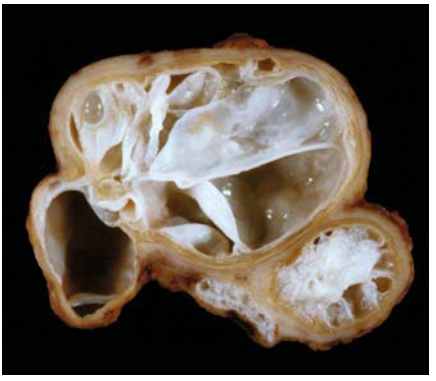

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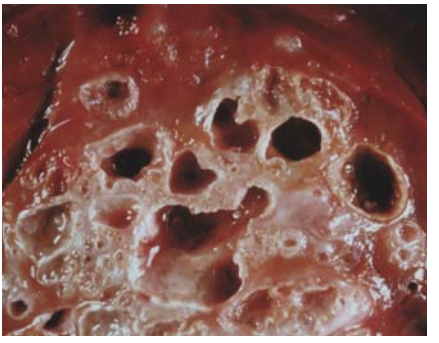

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malaria accounting for 70%. Around 90% of these infections are imported from Africa. The lethality of this disease during the last 10 years has been about 4%. Prognostically unfavourable factors are lack of or inadequate chemoprophylaxis, failure of doctors and patients to recognise the symptoms (**misdiagnoses**: influenza, hepatitis, encephalitis, pyelonephritis, febrile diarrhoeal diseases, etc.) and the patient's age. In patients over 60 years of age the lethality is 16%. Worldwide more than 200 million people are infected and about 2–5 million people die every year (in tropical countries mostly children).

The malaria parasites affecting man have no significant reservoirs. With the exception of a few monkey species exclusively infected people serve as the source of infections of the mosquitoes. In endemic areas the inhabitants have developed a semi-immunity after repeated infections which often permits only mild clinical symptoms. Certain diseases such as sickle cell anaemia (HbS), thalassaemia or glucose-6-phosphate dehydrogenase deficiency protect against the effects of *falciparum* malaria as the parasites are either unable to invade the erythrocytes or do not develop properly once they have done so.

Diseases and symptoms

Four different species of malaria parasite may occur in endemic areas; they differ markedly with respect to their blood stages and with regard to the clinical symptoms they cause:

Figure 10. Scanning electron micrograph of a red blood cell infected by two schizonts of *Plasmodium falciparum*; note the surface knobs of the red blood cells and the protruding schizonts

Figure 11. Light micrograph through a capillary blocked by *P. falciparum* infected red blood cells plus pigment

Figure 12. Scanning electron micrograph of an adult *Echinococcus granulosus* worm

Figure 13. Liver showing several cysts of *E. granulosus*

Figure 14. Section of three differently advanced cysts of *E. granulosus*, two of which show septa-like chambers containing small brood capsules. The septa-like structures may induce the bicycle-spoke appearance in computer tomography

Figure 15. Light micrograph of adult worms of *Echinocoocus multilocularis* (right) and *Echinococcus granulosus*. Note the size difference

Figure 16. Scanning electron micrograph of the scolex of an adult *E. multilocularis*

Figure 17. Human liver with numerous hollows due to alveolar caverns of *E. multilocularis* metacestode cysts

a) *Plasmodium vivax*: *P. vivax* malaria, benign tertian malaria;
b) *P. ovale*: ovale tertian malaria;
c) *P. malariae*: quartan malaria;
d) *P. falciparum*: *falciparum* malaria, malignant tertian malaria.

Most symptoms and pathology of malaria are related to haemolysis of erythrocytes and their effects on different organs. These are reviewed in detail in the below mentioned references.

After inoculation of sporozoites into a new human host they with the next bite, they enter cells of the RES within 30 seconds to 2 min. In the salivary gland of the mosquitoes these sporozoites have developed a surface coat which protects them from the human immune system. In the case of *P. vivax* and *P. ovale* sporozoites can remain dormant in liver cells for several months or even years in the form of hypnozoites or dormozoites before they begin to multiply and lead to relapses. The new attacks attributable to hypnozoites are referred to as relapses and signify the re-infection of previously parasite-free blood. These relapses occur mainly with *P. vivax* and can vary in length depending on the strain (delay of up to 24 months) after completion of the primary parasitaemia in 7–28 days. The recurrence of clinical symptoms in cases where there were always a small number of parasites in the erythrocytes but the number did not lead to disease is described as recrudescence. In *P. malariae* the recurring fever attacks which can go on for years (see below) are attributed to this phenomenon of recrudescence.

The laboratory findings include normal or decreased white cell counts, decreasing platelet counts, a rapidly increasing erythrocyte sedimentation reaction and increasing anaemia. The potentially fatal course of *falciparum* malaria compared with the other forms is due to the fact that the parasite-containing erythrocytes attach to the capillary walls (see above), which is due among other things to the 'knobs' on the erythrocyte surface visible under the electron microscope. This results in impairment of the microcirculation with stasis, acidosis, perivascular oedema and petechial bleeding. Depending on the vascular region primarily affected, **signs of severe and increasing organ lesions** begin to appear on the 4th to 5th day of illness: in the brain as cerebral malaria with severe headaches, clouding of consciousness and, as a result of the increasing cerebral oedema, seizures, extensive neurological deficits, unconsciousness, coma with fatal outcome. Cerebral malaria is the most common cause of death in *falciparum* malaria. Severe organ lesions can also occur in the kidney leading to dialysis-dependent renal failure (renal malaria), in the lung with interstitial and alveolar oedema, in the cardiovascular system with rhythm disorders and myocardial dilatation, in the liver with hepatic jaundice in addition to the haemolysis as a sign of increasing impairment of organ function, and finally in the gastrointestinal tract with diarrhoea as the cardinal symptom. Severe haemolysis which is not explained by the disintegration of the parasitised erythrocytes

alone can result in black water fever with renal failure and threatening tubular necrosis.

Differential diagnosis and diagnosis

The large number of symptoms resulting from the organ lesions explains the frequent **misdiagnoses** which are often fatal for the patient if the doctor does not consider malaria and initiate a competent diagnostic work-up soon enough.

Detection of the parasites in the thick or thin blood film is decisive. The thick film leads to an approximately 20- to 40-fold concentration, depending on the thickness, and is therefore more suitable for the detection of low levels of parasitaemia (<0.1%). However, its evaluation and particularly differentiation of the different types of plasmodia is more difficult; it is often only possible to distinguish between *falciparum* and non-*falciparum* plasmodia.

Detection of plasmodia or their components is also possible with various newer methods such as fluorescence microscopy of haematocrit capillaries (QBC = quantitative buffy coat analysis) or blood films, detection of circulating antigens and numerous varieties of DNA *in situ* hybridisation and PCR. These methods are important tools for research and epidemiological studies. However, they are by no means a substitute for parasitological blood tests (stained smear and thick film). These are fast, cheap and universally available. They also have a high sensitivity even compared with the molecular biological methods and the unachieved specificity of the gold standard. Deaths due to malaria are almost always due to the fact that these tests were not performed or performed too late.

Prophylaxis

General measures

Keeping away mosquitoes by spraying skin and clothing with repellents (Autan®, Viticks-Cool® plus, etc.), sleeping under mosquito nets, spraying insecticides (pyrethrum derivates) in bedrooms and on mosquito nets.

Chemoprophylaxis

Chemoprophylaxis is always advisable when travelling to malaria regions and can considerably reduce the risk even in areas with resistant parasites. The choice of prophylaxis will depend on the destination, season, length of stay and type of travel and should be made individually also taking into account age, pregnancy, previous illnesses, intolerances and any other drugs being taken. In the case of insufficient prophylaxis in areas with resistant parasites a therapeutic dose of a reserve drug (e.g., mefloquine) should

also be carried which can be taken in the event of symptoms suggestive of malaria and if medical help is not available (emergency or standby treatment). However, this should only be an emergency measure until medical assistance can be obtained. Carrying a standby drug alone without taking prophylactic medication can be considered in the case of very short exposure, very low risk of malaria and intolerance of malaria prophylaxis. **Attention**: If the prophylaxis is taken irregularly or if there is vomiting or diarrhoea the effectiveness of the prophylaxis may be compromised.

Treatment

Quinine
Quinine is the main alkaloid of the Cinchona bark in Peru known as effective febrifuge against intermittent fever since the early 17th century. It is used especially for the therapy of chloroquine- and multidrug-resistant *Plasmodium falciparum* infections. The combination with tetracycline is used in cases of severe resistance.

Chloroquine (Resochin® and other preparations)
Chloroquine is the drug of choice for tertian and quartan malaria (resistance of *P. vivax* has occasionally been observed). According to the WHO standard regimen an initial dose of 600 mg chloroquine base (children 10 mg/kg body wt) corresponding to 4 tablets of Resochin® 250 mg (1,000 mg chloroquine diphosphate) is given, followed by 300 mg base (children 5 mg/kg body wt) after 6 h and on the 2nd and 3rd day. In tertian malaria this is followed by treatment with primaquine 15 mg (children 0.25 mg/kg body wt) daily for 14 days to destroy the tissue schizonts responsible for relapses (**caution**: danger of haemolysis in patients with glucose-6-phosphate dehydrogenase deficiency).

Amodiaquine
Amodiaquine was invented between 1941 and 1945 as an antimalarial drug and was introduced in 1975. Its antimalarial activity is comparable to that of chloroquine.

Halofantrine
Halofantrine was developed within the Walter Reed Army Institute for Research antimalarial drug development program in 1984. It was introduced in 1988. However, because of severe cardiovascular side effects, halofantrine is no longer used.

Proguanil (Paludrine®)
In areas with partial resistance against chloroquine, proguanil (Paludrine®) can be used in addition to chloroquine prophylaxis. Thereby an additional protection can be achieved. However, as mono product proguanil cannot be used, the dosage for prophylaxis is 200 mg per day.

Mefloquine (Lariam®)
Mefloquine has been marketed since 1977. The activity is directed against multidrug-resistant *Plasmodium falciparum*. For prophylaxis 250 mg (1 tablet) is given per week. This starts 1–3 weeks before and ends up to 4 weeks after the visit of a malaria area. Therapy starts with the initial dose of 750 mg (= 3 tablets), followed by a further 500 mg (2 tablets) after 6–8 h; after a further 6–8 h 250 mg (= 1 tablet) is given.

Doxycylin
Doxycyclin alone is not suitable for therapy. It can be used for prophylaxis in areas with mefloquine resistances as an alternative to atovaquone/proguanil. The dosage is 100 mg per day 1–2 days before, and up to 4 weeks after, the visit to a malaria area.

Sulfadoxin-Pyrimethamine (Fansidar®)
Sulfadoxin-Pyrimethamine (Fansidar®) is not suitable for prophylaxis. For therapy it is used especially in Africa.

Atovaquone/Proguanil (Malarone®)
Atovaquone/Proguanil (Malarone®) is a fix combination for prophylaxis and chemotherapy inclusive the standby self-medication of uncomplicated *P. falciparum* infections. For prophylaxis 250 mg atovaquone and 100 mg proguanil (= 1 tablet) are given daily for 1–2 days up to 7 days. For therapy 1,000 mg atovaquone and 400 mg proguanil (= 4 tablets) are given in a single dose on three consecutive days.

Artemisinin
Artemisinin has been used so far for the treatment of malaria in at least 3 million people. The advantage of this drug is its rapid action against cerebral malaria. The combination of artemether/lumefantrine (Riamed®, Coartem®) is not suitable for prophylaxis but is recommended for standby self-medication. The dosage is 80 mg/480 mg (= 4 tablets) initially, followed by further 4 tablets after 8 h, and 4 tablets two-times daily on days 2 and 3 (in total 24 tablets).

Hepatitis caused by cestodes

Echinococcosis, Echinococciasis, Hydatidosis: Echinococcus granulosus *[5, 6, 11–17]*

Other name of the agent of disease

Small dog tapeworm

Taxonomy

Subregnum:	Animalia
Phylum:	Platyhelminthes
Class:	Cestoda
Order:	Cyclophyllidea
Family:	Taeniidae
Genus:	*Echinococcus*
Species:	*Echinococcus granulosus*

Morphology

The rather short tapeworm with its 2–5 mm long strobila lives in the intestine of the dog and some other carnivores, is provided with 37–42 μm long large and 29–34 μm long small hooks at the rostellum, 3–4 (–6) proglottids and a uterus with lobed lateral sacculations (Fig. 12). The cystic larva (metacestode) takes the form of a fluid-filled, generally unilocular cyst (hydatid) that grows by expansion (Figs 13 and 14), it may contain daughter cysts in its interior and in extreme cases it attains a diameter of up to 30 cm. Protoscolices bud from blood capsules of the inner germinal layer and may break free. In the fluid, these together with calcareous corpuscles form what is known as hydatid sand.

Transmission, multiplication and incubation period

Transmission to humans (and other intermediate hosts) takes place by means of oral ingestion of the eggs of *E. granulosus* originating from the faeces of dogs or other definitive hosts.

The adult forms of *E. granulosus* parasitising the intestine of the definitive host contain 500–1,000 taeniid eggs (32–39 × 24–26 μm) in their gravid proglottids. After the terminal proglottids with eggs are excreted with the faeces, they are orally ingested by an intermediate host. Here occurs hatching in the intestinal tract of the larva (oncosphere) present in the egg membrane, penetration of the wall of the intestine. Oncospheres spread

via the bloodstream to the liver, where the majority settle. Other oncospheres are transported further to the lungs and continue to develop there. Approximately 10–20% pass through the lung and then travel *via* the systemic circulation to a wide range of organs. The oncosphere develops into a vesicular structure that grows by means of expansion to form a cyst and inside contains protoscolices. The definitive host is infected by ingestion of such cysts. In the intestinal tract of the definitive host, each protoscolex matures into an adult tapeworm. The **incubation period** in humans to produce cysts is assumed to be over 5 years.

Epidemiology

Echinococcosis is primarily a zoonosis with a broad spectrum of definitive and intermediate hosts. The most important definitive host is the domestic dog, although the wolf, coyote, dingo, hyena, jackal and some other carnivorous mammals may also harbour the adult form of *E. granulosus*. Mammals with a herbivorous or omnivorous diet serve as intermediate hosts. Those that play the most important role are domestic animals such as cattle, sheep, goats, pigs and horses. A number of wild animals (e.g., buffaloes, bisons, antelopes, gazelles, elks and reindeer) may also host the metacestodes of this species. Humans constitute a dead-end intermediate host, as they generally do not allow transmission to the definitive final host.

Dog owners who feed their dogs scraps of raw meat are among those most at risk. In Germany, however, the risk is very low, as autochthonous infections are rare. Statutory meat inspection prevents meat containing cysts being sold as dog food.

As a zoonosis, the form of echinococcosis caused by *E. granulosus* is still distributed worldwide. It is particularly common, for example, in Mediterranean countries, broad regions of Central and South America and India. Dogs brought home from holiday destinations may therefore be infected. Cuddling of these animals may lead to human infections. *Echinococcus* eggs from the animals' coat or muzzle can also easily end up on carpets, chairs, etc., which may then again lead to infection of humans, too.

Diseases and symptoms

Cystic echinococcosis

Infestation of humans with metacestodes of *E. granulosus* is known as cystic echinococcosis or *E. granulosus* infection. The slow growth of the parasite explains why the disease generally does not become manifest for several years post infection. As a result of the expansive growth of the cyst, this form of echinococcosis presents as a space-occupying lesion predominantly in the liver or the lung (approximately 70–90% of cases; Fig. 14). With the

gradual expansion, major vessels and structures may become compressed and occluded. The clinical presentation is extremely varied, depending on the number, size and site of the cysts. Cystic echinococcosis may give rise to febrile secondary infections, abscesses or fistulisation. A major concern is cyst rupture, leading to secondary echinococcosis, as each of the released protoscolices may develop into a new cyst. The cysts usually grow slowly and are often detected by chance during routine investigations. There is a broad spectrum of symptoms, depending on the organ affected. Infestation of the liver, for example, causes a sensation of pressure in the upper abdomen and jaundice, whereas haemoptysis and expectoration of cyst contents with protoscolices may occur if the site of infestation is the lung.

Clinical differentiation is often made as follows, depending on the organ involved:

Hepatic cystic echinococcosis: Well-demarcated cyst(s) (Figs 13 and 14), usually appear with a characteristic honeycomb parenchymal pattern on an ultrasound scan. On this basis, the cyst(s) is/are classified and staged in stages 1 to 6 using a classification proposed by a WHO working group (WHO, 2001). If the cysts rupture into the abdominal cavity, this may lead to life-threatening peritonitis. Furthermore, seeding occurs in a process known as secondary echinococcosis. Compression of the efferent bile ducts leads to post-hepatic jaundice. If a cyst ruptures in the biliary tree, the cyst contents are discharged and, if small daughter cysts pass through the papilla, yellowish grape-like cysts will be detectable in the stools. Cholangitis often develops as a complication as a result of ascension of the pathogen.

Pulmonary cystic echinococcosis: Cyst(s) ranging in size from that of a tennis ball to that of a child's head is/are well-demarcated and may be mistaken for circular foci of varying origin on plain X-rays. Clinical symptoms occur if a cyst latches onto the bronchial system. High fever, eosinophilia and pulmonary infiltrates are characteristic clinical manifestations. The clear cyst fluid with membrane fragments and hydatid sand may be expectorated *via* the bronchial tree. Rupture of the cysts and collapse of the endocysts results in the water-lily sign as a result of air permeating between ectocysts and endocysts. Bacterial secondary infections are a common complication.

Manifestation in other organs: Any organ is suitable as a nesting site for the development of the metacestode. The morphological correlate is the same for all the large parenchymatous organs, namely a well-demarcated mass with a host capsule. If the bones and muscles are involved, however, the presentation is polycystic. Differentiation from *E. multilocularis* in bone is therefore difficult. Expert advice should be sought.

Immune response

It is currently not known why the humoral immune response is so slow to respond to longstanding infection and symptom manifestation. High titre

antibodies occur only in the event of cyst rupture and an allergic reaction. The cellular immune response in persistent infection is as yet poorly understood.

Differential diagnosis and diagnosis

Different differential diagnoses need to be considered for the individual stages of the liver cysts visualisable with ultrasound. Benign liver cysts may be confused with stage 1 of hepatic cystic echinococcosis. Calcified haematomas, calcified abscesses and also liver metastases have to be considered as possible differential diagnoses for stages 5 and 6. With pulmonary cysts, there is characteristic bulging of the circular foci, in which homogeneously clear contents, possibly with daughter cysts, are detectable with diagnostic imaging techniques. A typical presenting feature in other parenchymatous organs is the well-demarcated host capsule. With infection of bone, the presentation is polycystic, making differential diagnosis difficult.

A blood count generally shows only moderate eosinophilia. Once a cyst has burst, excessive eosinophilia is detectable. Immunoglobulin E levels are generally raised. The following techniques are used for detection of cystic echinococcosis.

Ultrasound and computed tomography provide characteristic morphological images of the lesion. In the liver, the cysts usually show a bicycle-spoke structure. The WHO classification 2001 characterises the stage of the disease. The cysts of *E. granulosus* are well-demarcated and are embedded in a host capsule known as the pericyst. The surrounding wall of the pericyst varies in thickness, depending on the organ involved. Calcifications are occasionally to be found. In the case of degenerated cysts, the tissue is compressed and the lesion may calcify completely.

A number of test methods have been developed for the detection of antibodies. The most commonly used are indirect immunofluorescence, indirect haemagglutination tests and ELISA. Serological tests generally allow differentiation of this disease from infestation with metacestodes of *E. multilocularis*. Negative results are nevertheless obtained in up to 50% of cases. This applies in particular to pulmonary cystic echinococcosis.

Macroscopic features are the characteristic cysts and daughter cysts. Diagnostic fine needle biopsy is contraindicated. If the cyst is accidentally punctured, protoscolices and hooks are detectable in the clear cyst fluid. This confirms the diagnosis. PAS-positive laminated membranes are found in degenerated cysts. A few DNA probes for *E. granulosus* have been described. They have not yet been validated on clinical specimens, however.

Treatment

In the past few years, there has been a considerable change in the strategies

for the treatment of hepatic cystic echinococcosis. Whereas surgery was previously considered the treatment of choice, this is nowadays reserved only for certain morphological cyst stages. When performing surgical procedures, it is recommended that the endocyst is opened up and the cyst wall is disinfected with 10–20% NaCl solution. With more radical procedures, the cyst(s) is/are enucleated. The cyst(s) can then be resected together with the host capsule.

For easily accessible cysts of the liver and particular cyst stages, a technique known as Puncture-Aspiration-Instillation-Reaspiration (PAIR) can be used. This involves ultrasound-guided puncture of the cyst, removal and analysis of the cyst contents, injection of a scolicidal solution (70–90% alcohol or 15–20% NaCl solution) and reaspiration after a brief incubation period. This method has a promising success rate. The treatment should take place at specialist centres.

Drug therapy of cystic echinococcosis with the benzimidazole derivatives mebendazole or albendazole is a key cornerstone of the treatment plan (WHO, 1996). Following curative surgery, treatment is recommended for 3 months. Drug therapy is essential before, during and after treatment with the PAIR method. Drug therapy alone has proved successful at various treatment centres, leading after a period of months or years to degeneration of the cyst and curing of the disease. Both anthelmintics are able to stop growth of the cysts' germinal layer and the protoscolices. A 3-month period of treatment with albendazole at a dosage of 10–15 mg/kg BW per day is recommended. Longer treatment periods may be necessary, depending on which organ is affected. The anthelmintic therapy is often commenced preoperatively. The logic behind this is that it will reduce the high pressure inside the cyst and so prevent the risk of seeding during surgery. This thinking is not backed up by studies, however, and the WHO recommendation that pretreatment with anthelmintics should be confined to just a few days before surgery therefore still applies (WHO, 1996). Treatment can be monitored on the basis of measured concentrations of the anthelmintics. The extent to which the dose of the drug correlates with effective control of growth of the parasite *in vivo* has not been proven, however.

Resistance

Resistance to anthelmintics has not been described to date.

Prevention

In order to prevent the infection being transmitted to humans, meat containing cysts must not be fed to dogs. If a dog is already infected, however, its faeces must be destroyed immediately and the infested dog must be given worming treatment under quarantine-like conditions. Praziquantel is the drug of choice for this purpose.

Alveolar echinococcosis: Echinocoocus multilocularis *[5, 6, 11–14, 17–20]*

Synonym

Small fox tapeworm

Taxonomy

Subregnum:	Animalia
Phylum:	Platyhelminthes
Class:	Cestoda
Order:	Cyclophyllidea
Family:	Taeniidae
Genus:	*Echinococcus*

Morphology

The 1.2–3.7 mm long tapeworm is mainly found in the intestine of the red fox (*Vulpes vulpes*) and dogs among some other carnivores, with approximately 31 μm long large and approximately 27 μm long small hooks on the rostellum, 4–5 (2–6) proglottids and a sacciform uterus (Figs 15 and 16). The larva (metacestode) of the alveolar type (Fig. 17) takes the form of a structure made up of multiple small vesicles that grows by infiltration by means of exogenous budding. Individual vesicles are mere millimetres in size, with a gelatinous matrix inside and protoscolices formed from the germinal layer.

Transmission, multiplication and incubation period

Transmission to humans (among other intermediate hosts) takes place by oral ingestion of the eggs of *E. multilocularis* originating from the faeces of red foxes or other definitive hosts. Contact to egg-contaminated hair of final hosts and consumption of wild berries (blueberries, strawberries, etc.) contaminated with fox faeces or inhalation of helminth eggs swirled up during work in fields are thought to be responsible, too.

The adult form of *E. multilocularis* is a parasite in the small intestine of the definitive host. The number of eggs formed in the gravid proglottids depends on the host species and the age of the infection. In young worms, it is 200–500 per segment but later declines dramatically. Excretion of taeniid eggs (32–39 × 24–26 μm) within the proglottids with the faeces of the definitive final host. After oral ingestion by an intermediate host, the larva (oncosphere) present in the egg membrane in the intestinal tract hatch, penetrate

the intestinal wall. Oncospheres spread *via* the bloodstream to the liver, lodge in the liver and develop into a multivesicular metacestode (permanently growing by means of exogenous budding). Protoscolices are formed internally. Infection of the definitive host occurs as a result of consumption of the intermediate host, only in the definite host parasites mature into adult worms. The **incubation period** in humans is assumed to be 10–15 years.

Epidemiology

The parasitosis caused by *E. multilocularis* is primarily a zoonosis, though with a distinctly narrower host spectrum than is the case with *E. granulosus* infection. The red fox (*Vulpes vulpes*) is the principal definitive host in Central Europe with infestation rates for *E. multilocularis* of way over 50% in the main endemic areas. The Arctic fox (*Alopex lagopus*) plays a key role in Arctic regions. In addition, other canines (domestic dog, wolf, raccoon dog, etc.) also serve as definitive hosts. Humans constitute a dead-end intermediate host. The spectrum of natural intermediate hosts includes in particular species of the rodent family Cricetidae, with the field mouse (*Microtus arvalis*) as the most important species in Central Europe. Other *Microtus* species, bank voles, muskrats, lemmings but also house mice, brown rats and other rodents may harbour the metacestodes of *E. multilocularis*.

Anyone who works in the farming and forestry industries in endemic areas should be considered to be at increased risk. Some professional associations reckon that an occupational disease claim for compensation can be made for alveolar echinococcosis.

The zoonosis caused by *E. multilocularis* is limited in its distribution to particular regions of the northern hemisphere. In Europe, these areas are Southern and Eastern France, Germany and Austria. The disease is also prevalent in the Czech Republic, Slovakia, Poland and Bulgaria as well as Turkey, from where the endemic area extends eastwards as far as Siberia and the Northern Islands of Japan. Its geographical distribution is likewise considered to include Alaska, Canada and the Central Northern states of the USA. As the prevalence in the definitive host populations increases, so the affected area expands, increasing the risk of infection for humans.

Diseases and symptoms

Alveolar echinococcosis: The primary site of infection with *Echinococcus multilocularis* in humans in 98% of cases is the liver (Fig. 17). The slow growth of the mass in the liver generally causes no symptoms. The disease is therefore often only diagnosed by chance. At advanced stages, systemic symptoms such as night sweats, weight loss and fatigue are experienced and are suggestive of a malignant disease. Additional symptoms may occur as a

result of compression of major vessels in the liver. The pathogen's growth by infiltration causes different signs and symptoms according to which organs are affected.

Abdominal organs are receptive to growth of the alveolar mass (drop metastases). Lymph nodes are also colonised. Haematogenous dissemination of detached germinal layer cells (e.g., undifferentiated cells act like tumour-cells) may lead to seeding in other organs. Such metastasis is favoured by immunosuppression. The spread of the parasitic mass at the time of diagnosis is currently described using an anatomical distribution pattern similar to the TNM system (PNM classification of the European Echinococcosis Working Group).

Pathogenicity, virulence and antigen variability

The chronic persistence of the parasitic lesion is as yet only little understood. There is TH2-weighted immunomodulation, as a result of which the parasitic infiltrate is presumably tolerated by the host organism for many years. Immunological control is assumed to play a role in the case of 'abortive' lesions. In such cases, the effective cellular immune response is able to kill the parasite. The infection is cured, leaving a calcified lesion. It is not known how often this happens. Studies on immunogenetic predisposition are available and provide initial indications of possible resistance to the pathogen in the presence of particular HLA-DR characteristics. Rapid progression and metastasis have been described in the immunosuppressed.

Immune response

A humoral immune response is generally detectable in cases of *E. multilocularis* infection. The cellular immune response is characterised by marked expression of IL-10 and partially explains the chronic persistence of the pathogen.

Differential diagnosis and diagnosis

The poor definition of the liver lesion with calcifications and central necrotic cavities is morphologically similar to the presentation of hepatocellular carcinoma. Early manifestations may give the impression of haemangiomas. Tiny intrahepatic calcifications are currently considered to be a manifestation of recovery from the parasitic disease, i.e., of the abortive form of alveolar echinococcosis.

Eosinophilia, which is rarely diagnostically informative, does not occur. Elevation of the immunoglobulin E level, on the other hand, is detectable

in active alveolar echinococcosis. Imaging and serological techniques have to be used for diagnosis.

Macroscopic and histopathological evidence of the *E. multilocularis* metacestode is conclusive. A diagnostic biopsy is not recommended on account of the risk of dissemination of larval tissue.

The alveolar liver echinococcus is seen on ultrasound and CT scans as a grape-like tumour with scattered marginal calcifications. It is poorly differentiated from the rest of the liver tissue. It is not uncommon to see central necrotic cavities that give the impression of a cyst. The irregular texture, the poor differentiation and scattered calcified deposits are indicative. Diagnostic considerations must include a malignant lesion. Depending on which organs are affected, different imaging techniques may be informative.

Specific antibodies are detectable in over 90% of cases, allowing differentiation from cystic echinococcosis. False-positive serological reactions do occur.

Various DNA probes are available. They have not yet been validated, however, for clinical diagnosis.

Therapy

Surgery

The treatment of choice is radical surgery for clearly circumscribed lesions. With curative surgery, therapy should be supplemented with subsequent drug treatment with albendazole or mebendazole for a minimum of 2 years. Recurrences, though only after some years, are common with palliative procedures.

In cases of extensive organ involvement and compression of major blood vessels, interventional measures are crucial. These include the insertion of stents, relief of pressure in necrotic cavities or sealing off newly formed cavities.

A liver transplant should be considered if the liver is additionally damaged by other diseases. If the liver is very extensively affected (even without spread to other organs), transplantation offers no additional advantage over chemotherapy alone. Long-term subsequent treatment with albendazole is necessary to prevent recurrences.

Chemotherapy

For inoperable lesions, chemotherapy on its own with albendazole or mebendazole leads to consolidation and regression by stopping the growth of the parasite. The action of both drugs is exclusively parasitostatic. On the basis of current knowledge, treatment therefore has to be continued for life.

Resistance

The action of benzimidazoles against *E. multilocularis* infection is exclusively parasitostatic. The cellular target molecules for benzimidazoles are β-tubulins as essential components of the cytoskeleton. Variable sensitivity of the *Echinococcus* β-tubulin to benzimidazoles could explain drug resistance.

Prevention

Consumption of raw wild berries has to be considered risky and should therefore be avoided. Individual prophylactic measures are nevertheless insufficient as a means of prevention. Control measures must be directed at reducing infestation within fox populations.

Strategies for disease prevention

The fox population in some regions of Southern Germany was treated for parasites by regularly leaving out bait containing praziquantel. This has been shown to result in a reduction in the high rate of infection of foxes with adult forms of *E. multilocularis*. It is, however, unclear what impact this will have on the potential infection of humans.

Hepatitis caused by trematodes

Fasciolosis, Fascioliasis: Fasciola hepatica *[5, 6]*

Other name of the agent of disease

Large liver fluke

Taxonomy

Subregnum:	Animalia
Phylum:	Platyhelminthes
Class:	Trematoda
Subclass:	Digenea
Order:	Echinostomata
Family:	Fasciolidae
Genus:	*Fasciola*

Morphology

Humans are infected predominantly with the large liver fluke (*Fasciola hepatica*), which grows to a size of 14 × 30 mm (Fig. 18; Figs 18–29, 31–34 see pp 202–205). The giant liver fluke (*F. gigantica*) occurs only exceptionally and with a limited geographical distribution, growing to up to 70 mm in length in its natural host. The surface of these worms is fortified by numerous scales (Fig. 19).

Transmission, multiplication and incubation period

Humans are infected as a result of oral ingestion of the metacercariae that are usually attached to plants. It is sufficient for such plants to be casually placed in the mouth.

The eggs of the adult flukes living in the bile ducts (Fig. 20) are excreted *via* the common bile duct with the faeces and need to reach water in order to develop further. This results in the development within 1–2 weeks inside the egg of a ciliated larva, the miracidium, which hatches from the egg membrane and actively seeks out the intermediate host (snails such as *Lymnaea*). Here, asexual reproduction involving a number of stages takes place, leading to the formation of cercariae. These leave the snail, swim around in the water, ultimately attach themselves to grasses and encyst to form the metacercaria, the infectious stage that can live for months, primarily under moist conditions.

Once orally ingested, the young flukes hatch in the small intestine, bore their way through the intestinal wall and migrate through the abdominal cavity to the liver. After 6–8 weeks' migration in the hepatic parenchyma, the flukes break through into the bile ducts, their final resting place (Fig. 20). Sexual maturity is reached approximately 10 weeks after infection (prepatency period).

Epidemiology

F. hepatica occurs worldwide and is particularly common in humid regions with a high rainfall that meet the biotope requirements of the amphibious intermediate host snails (*Lymnaea* spp., also known as mud snails). The main hosts are domestic and wild ruminants. Infections in other herbivores and omnivores are also not uncommon. Clinically and economically significant, often epidemic diseases may be triggered in domestic ruminants.

F. gigantica occurs in tropical zones of Asia and Africa, in South-East Asia and the Pacific region, often alongside *F. hepatica*. Isolated areas of distribution are found in the Middle East and in the Southern repub-

lics of the former USSR. The host spectrum is the same as that for *F. hepatica*.

Infection of humans with *F. hepatica* occurs worldwide and usually sporadically. Group infestations have been reported in England, France and North Africa. Approximately 400 cases per year had been documented in France between 1970 and 1982, with higher rates in some regions. Infestation of humans with *F. gigantica* is rare, though cases are known from the Pacific and Asian region.

Diseases and symptoms

Fascioliasis is an acute or chronic disease of the liver following infestation of humans with liver flukes. The degree of infestation in humans is usually only slight. Infections are therefore often clinically inapparent, particularly in the prepatent period. In the early stage, approximately 2 weeks after infection, larval migration gives rise to perihepatitis with systemic symptoms such as fever, exhaustion and loss of appetite. Leucocytosis, eosinophilia and elevated IgE levels are generally observed.

Once the adult parasites have colonised the bile ducts, inflammation and later fibrosis and calcification of the latter occur. The anaemia that occurs at a later stage of the disease is caused by the sustained consumption of blood by the flukes. Besides intermittent fever, the symptoms experienced by patients are anorexia, weight loss, pruritus and pain usually localised under the right costal margin. Occasional obstruction of the bile ducts by the migration of the worms leads to recurrent periods of jaundice. Ectopic sites of infection with the parasite are connective tissue and CNS.

Differential diagnosis and diagnosis

Conditions that must be ruled out are perihepatitis of other aetiology in the acute stage and cholestasis of other origin and gallstones at the chronic stage.

In the prepatent period lasting 10 weeks or more, serological methods (indirect immunofluorescence test, indirect haemagglutination and ELISA) can be used for aetiological clarification, although cross-reactions are likely, particularly with other trematodes (*Opisthorchis*, *Schistosoma* spp.). After the prepatent period, characteristic operculated eggs measuring 90×150 μm (*F. hepatica*) and 90×190 μm (*F. gigantica*) are detectable in the stools. The eggs are not regularly excreted. Repeated testing is therefore necessary and egg detection is not always possible. Egg detection on a single occasion is not sufficient, as liver fluke eggs consumed with contaminated beef liver (sausage) also pass through the intestine unchanged.

Treatment

Efficient treatment is achieved with triclabendazole (single dose of 10 mg/kg). Treatment with bithionol (2×20 mg/kg per day, every other day for 2 weeks) is also recommended. Furthermore praziquantel for 3 days when the daily dose is given (devided) at three intervals.

Resistance

Resistance in treatment of human fasciolosis is unknown at present. By contrast there are severe problems in veterinary medicine in treating *Fasciola hepatica* infections with all available common fasciolocidal drugs.

Prophylaxis

Plants growing on wet ground and in the vicinity of natural waters subject to occasional flooding may be contaminated with metacercariae and should not be placed in the mouth. Consumption of naturally growing watercress should be avoided.

Dicrocoelosis, Dicrocoeliasis: Dicrocoelium dendriticum *[5, 6]*

Other name of the agent of disease

Small liver fluke, lancet fluke, *Dicrocoelium lanceolatum*

Taxonomy

Subregnum:	Animalia
Phylum:	Platyhelminthes
Class:	Trematoda
Subclass:	Digenea
Order:	Plagiorchiata
Family:	Dicrocoeliidae
Genus:	*Dicrocoelium*

Morphology

The pathogens are the small liver fluke *Dicrocoelium dendriticum* and *D. hospes* which both have a smooth, not scaly surface (Figs 21 and 22). The parasites reach a size of 2×12 mm.

Transmission, multiplication and incubation period

Infection occurs as a result of accidental ingestion of infected second intermediate hosts, particular species of ant, such as *Formica* spp. in the case of *D. dendriticum* and *Camponotus* spp. in the case of *D. hospes*.

Larvae-containing eggs reach the outside world with the faeces of infected animals. They are ingested by terrestrial snails of the genera *Helicella* and *Zebrina* in the case of *D. dendriticum* and *Lymnicolaria* species in the case of *D. hospes*. Asexual reproduction in the snails gives rise to cercariae that are discharged by the snails, enclosed in balls of mucus. Ants, as the second intermediate host, eat these balls of mucus containing the cercariae. The infectious stages, metacercariae, form in the ants' coelom. Infestation of the hypopharyngeal ganglion alters the behaviour of infected ants, which then bite into plants in the evening as outdoor temperatures fall and are ingested orally in the morning, for example by a grazing animal as the definitive host. The young flukes are released from the metacercariae in the small intestine and migrate *via* the common bile duct to the small bile ducts. There they reach sexual maturity in 9–10 weeks and start to lay the typical operculated eggs (prepatent period).

Epidemiology

D. dendriticum occurs in the temperate zones of the Northern hemisphere. An increased prevalence is often observed in confined areas, as the liver fluke is dependent on the simultaneous presence of two intermediate hosts, terrestrial snails and particular species of ant, during its complicated development cycle. Its definitive hosts are a number of species of mammal, including ruminants and rabbits. High infestation rates are known in domestic ruminants in Southern Europe. *D. hospes* is confined to Eastern and Central Africa.

Dicrocoeliasis in humans occurs in correlation with infestation in animals. In Central Asia (Uzbekistan), for example, the prevalence in humans on the basis of autopsy reports is 0.28%, compared with between 20–30% in domestic ruminants.

Diseases and symptoms

Dicrocoeliasis is a chronic disease of the liver, particularly the biliary system, occurring rarely in humans following infection with the small liver fluke. The clinical symptoms are dependent on the worm burden. Colonisation of the bile ducts by the parasite leads to acute, and later chronic, cholangitis. A late sequela is the development of cirrhotic changes in the periportal region.

Symptoms observed in heavy infections are persistent right-sided upper abdominal pain, jaundice, enlargement of the liver and the spleen, alternating diarrhoea and constipation, flatulence, dizziness, vomiting, headache and, in advanced cases, anaemia.

Differential diagnosis and diagnosis

Cholangitis and cholestasis of other origin. Patent infections are diagnosed by detection in the stool of the operculated, dark brown eggs, measuring approximately 25×40 µm. The test is repeated to exclude the possibility of eggs that are merely passing through the intestine, having got there by chance as a result of eating the liver of infected animals.

Treatment

Praziquantel (3×25 mg/kg on a single day) is used as a chemotherapeutic agent.

Prophylaxis

Look out for the presence of ants when eating windfall fruit and naturally-growing plants. People should not chew on blades of grass.

Clonorchosis, Clonorchiasis: Clonorchis sinensis *[5, 6, 21, 22]*

Other name of the agent of disease

Lanceolate fluke, Chinese liver fluke, *Opisthorchis sinensis*

Taxonomy

Subregnum:	Animalia
Phylum:	Platyhelminthes
Class:	Trematoda
Subclass:	Digenea
Order:	Opisthorchiata
Family:	Opisthorchiidae
Genus:	*Clonorchis*

Morphology

The lanceolate parasite, *Clonorchis sinensis*, which is a transparent pink colour while alive, grows to a size of 3–5 × 8–15 mm (Figs 23 and 24). It has characteristic branched testes arranged in pairs on the posterior third of the body.

Transmission, multiplication and incubation period

Humans are infected by consumption of raw or inadequately prepared freshwater fish, particularly carp, the second intermediate host in the development cycle.

If the small, operculated worm eggs (maximum 35 μm in length) find their way into water with the faeces of infected vertebrates and are ingested there by snails (*Bulinus* and *Parafossarulus* species), asexual reproduction then takes place in this first intermediate host in the course of development *via* a number of stages. Cercariae that are released from the snails actively penetrate small freshwater fish (predominantly cyprinids), encyst in muscle and form metacercariae. After being consumed in raw fish, the young flukes hatch in the duodenum, migrate *via* the common bile duct to the distal bile ducts, attach themselves to the epithelium, mature into adult flukes and start to lay eggs after 2–4 weeks (prepatency). The parasite can survive in the definitive host for 20–25 years (patency).

Epidemiology

C. sinensis occurs throughout Asia from Indochina to Japan. Besides humans, cats, dogs, pigs, various small predators (mustelids) and rats are also infested, these animals constituting a reservoir that is difficult to control.

Endemic areas are Korea, Vietnam, Taiwan, China and Hong Kong, where prevalences of over 50% are common. In Japan, however, infestation of humans has today become a rarity.

Diseases and symptoms

Confined to Asia, clonorchiasis is a disease of the liver, primarily the biliary system, as a result of infection with the Chinese liver fluke. *C. sinensis* causes inflammatory and proliferative changes in the bile ducts, the severity of these changes being dependent on the number of flukes and the duration of the infestation. Infection is manifested initially in terms of exhaustion, loss of appetite and gastrointestinal symptoms such as diarrhoea and meteorism. Severe infections are followed by pain in the right epigastric region, hepatomegaly, fever, jaundice and occasionally urticaria. Massive infestation as

a result of repeated infections (more than 1,000 flukes per patient may be seen) leads to severe disorders of general wellbeing with dizziness, tremor, seizures and weight loss. Cirrhosis of the liver, oedema and ascites occur with persistent infections.

Pancreatitis and gallstones are common. Bacterial superinfections (usually *E. coli*) and cholecystitis are common complications.

Infection predisposes to cholangiocarcinomas, which occur in cases of long term infections.

Differential diagnosis and diagnosis

Cholangitis and cirrhosis of the liver of other origin need to be ruled out, depending on the stage and the severity of the infection. The clinical presentation resembles that seen in cases of infestation with other *Opisthorchis* species or *Dicrocoelium* spp.

The yellowish-coloured, operculated eggs, approx. 17 × 30 μm in size, are microscopically detectable in stools or duodenal fluid. Egg excretion may commence just 14 days after infection.

Because they are so small, the eggs are easily missed in stool specimens. Concentration techniques should therefore be used.

Serological techniques and PCR techniques have also proved useful for diagnostic purposes. Eosinophilia is observed in the acute stage.

Treatment

The disease is treated with praziquantel (3 × 25 mg/kg per day for 2 days) or albendazole (400 mg/day for 7 days).

As with most fluke infections, the introduction of praziquantel constituted a significant advance in the treatment also of clonorchiasis. The eggs disappear from stools and duodenal fluid a few days after treatment.

Prophylaxis

Thorough cooking or frying of fish is the most important prophylactic measure in endemic areas. Since changes to the eating practices of population groups that traditionally eat raw fish are achievable only in the long-term, attempts are being directed at making raw fish safe by treating it with gamma irradiation (0.15 kGy) to kill off the metacercariae. Human faeces should not be allowed to contaminate fish waters so as to prevent infection of the snails living there *via* the eggs.

Opisthorchosis, Opisthorchiasis: Opisthorchis felineus, O. viverrini *[1, 5, 6, 21–24]*

Other name(s) of the agents of disease

Liver fluke, cat liver fluke, oriental liver fluke

Taxonomy

Subregnum:	Animalia
Phylum:	Platyhelminthes
Class:	Digenea
Order:	Opisthorchiida
Family:	Opisthorchiidae
Genus:	*Opisthorchis*
Species:	*Opisthorchis felineus*, *O. viverrini*

Morphology

Dorsoventrally flattened hermaphrodite trematodes measure 7–25 mm in length and 2–5 mm in width. They have a blind-ending branched intestine and two suckers. Solid testes are located in the hind part of the body (Fig. 25).

Transmission, multiplication and incubation period

Man is infected almost only by eating raw or uncooked carp flesh. In some endemic areas fish from other families are also possible sources of infection.

The genus *Opisthorchis* belongs to the group of triheteroxenous helminths (having one definitive and two intermediate hosts). Developmental cycle starts with excretion of the eggs with the stools of the definitive host. Further development occurs only in freshwater by ingestion of miracidia containing eggs by the first intermediate host (snails: Bithyniidae and other families). Different larval stages develop in the snail (sporocyst, redia, cercaria). Cercariae are released and infect the second intermediate host (carp: Cyprinidae). Here development of infectious metacercariae occur. After ingestion of the metacercariae by the definitive host the bile ducts are colonised with adult worms. Egg excretion begins about 3–4 weeks after infection (= prepatent period).

The main multiplication phase of the liver fluke takes place at the larvae stage in the first intermediate host (snail). In the definitive host (man, mammals) the number of adult worms corresponds to the number of

infectious stages ingested (one metacercaria develops to one adult worm). Multiplication in the definitive host consists only in the production of eggs which must leave the body in order to undergo further development. An incubation period cannot usually be defined as the development of clinical symptoms depends on the number of – usually cumulatively – ingested metacercariae and the duration of the infection. In the case of simultaneous massive infection the first symptoms can be expected 1–3 weeks after infection.

Epidemiology

Man is the definitive (= final) host. In addition there is a broad spectrum of reservoir hosts in piscivorous mammals (Canidae, Felidae, etc.). People who eat raw or partially cooked (carp) fish or parts of these fish are at high risk. The distribution of opisthorchosis is limited to Eurasia. It occurs wherever eating habits allow infection. The range of *O. felineus* extends from Spain eastwards to Siberia, while *O. sinensis* occurs only in East Asia and *O. viverrini* only in South-East Asia (Thailand, Laos). Autochthonous *Opisthorchis* infection is now extremely rare in Western and Central Europe.

Diseases and symptoms

Opisthorchosis: The disease is caused by bile duct parasites whose infectious stages (metacercariae) are released in the duodenum from where they migrate directly to the bile ducts (occasionally also to the pancreatic duct). Adult worms cause dilation of the bile ducts with hypertrophy of the epithelium and fibrous wall thickening. The severity of the disease is determined by the duration (untreated up to 10 years) and the intensity of infection (as many as 12,000 worms have been found at autopsy). The acute phase (only with simultaneous intake of numerous metacercariae) is associated with fever, epigastric and upper abdominal symptoms and diarrhoea. The chronic phase is characterised by fever, colicy abdominal pains, sometimes bile duct obstruction, hepatomegaly. In addition there may be secondary bacterial infections, cholelithiasis, abscesses, cirrhosis, oedema, ascites and cholangiocarcinoma as complications.

Immune response

The immune response produced by liver flukes neither kills the parasites nor protects against reinfection.

Differential diagnosis and diagnosis

The differential diagnosis includes bile duct diseases of other origin with appropriate symptoms.

Microscopy
The only proof of the presence of opisthorchosis is detection of the operculated eggs measuring 28–35 μm in length and 11–19 μm in width in the stools or bile. Excretion of eggs begins about 3–4 weeks after infection (prepatent period). As the differentiation of eggs among *Opisthorchis* species and from those of numerous other species of small intestinal flukes is very difficult, the diagnosis should be made by specialised parasitological or tropical medicine laboratories.

Serology
No tests of high specificity and sensitivity are currently available. **PCR** was recently shown to help in diagnosis.

Treatment

The drug of choice is praziquantel (Biltricide®). Dosage: 40 mg/kg once or 25 mg/kg body weight 3 times on the same day.

Prevention

The only reliable protection against infection is avoiding eating dishes containing raw or partially cooked fish, particularly carp, which could contain infectious stages.

Strategies for prevention and control of the disease

In endemic areas campaigns comprising health education, mass anthelminic treatment and proper removal of excrement are needed. However, because of the existence of animal reservoir hosts extermination is practically unachievable.

Schistosomiasis, Schistosomosis, Bilharziasis: Schistosoma *species [5, 6, 17]*

Other name of agents of disease

S. intercalatum, *S. japonicum*, *S. mansoni*, *S. mekongi*
Bilharzia species, *Distomum bilharziase*, blood fluke, intestinal blood fluke, bladder fluke, Japanese blood fluke, coupled or paired worms

Taxonomy

Subregnum:	Animalia
Phylum:	Platyhelminthes
Class:	Trematoda
Subclass:	Digenea
Order:	Schistosomatida
Family:	Schistosomatidae
Genus:	*Schistosoma*
Species:	*S. mansoni*, *S. japonicum*, S. *mekongi*, *S. intercalatum*, *S. haematobium*

Morphology

Schistosomes are diecious, approximately 1–3 cm long flukes (Figs 26 and 27). The male carries the female in a ventral groove (*canalis gynaecophorus* or gynaecophoral canal). Two suckers are used for attachment to and movement within blood vessels (veins of the mesenteric or vesical plexus). The simple, blind-ending intestine is filled with ingested erythrocytes. The tegument consists of a syncytium with a double surface membrane. The eggs (mean size in length 65–170 μm according to species) each contain one larva (miracidium) and have a characteristic spine (see Diagnosis).

Figure 18. Light micrograph of an adult *Fasciola hepatica* as as seen from its ventral side

Figure 19. Scanning electron micrograph of the anterior region of an adult *F. hepatica* fluke showing the oral and the larger ventral sucker, the in-between situated two small genital openings and the typical scaly surface (by toothed tegumantal scales)

Figure 20. Cut off bile duct with adult *Fasciola* flukes

Figure 21. Light micrograph of an adult *D. dendriticum* fluke

Figure 22. Scanning electron micrograph of the anterior end of an adult *D. dendriticum* fluke. Note the smooth, not scaly surface

Figure 23. Light micrograph of an unstained adult *Clonorchis sinensis*

Figure 24. Scanning electron micrograph of the ventral side of an adult *C. sinensis* fluke. Note the smooth, unscaly surface

Figure 25. Light micrograph of an adult *Opisthorchis viverrini* worm. Note the solid posterior testes

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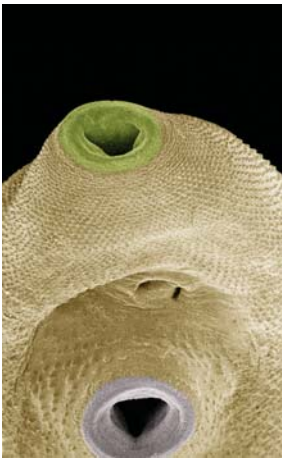
19 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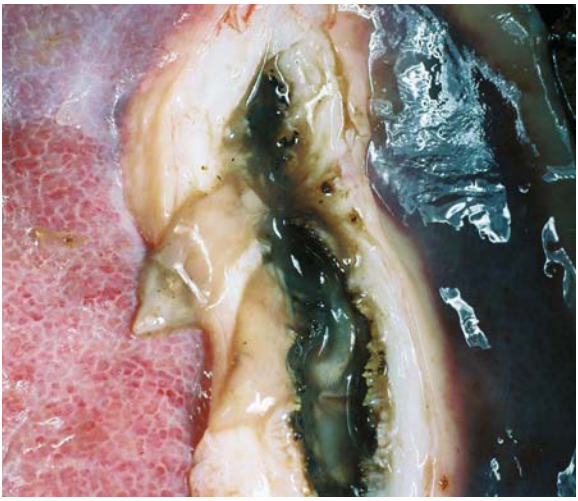
20 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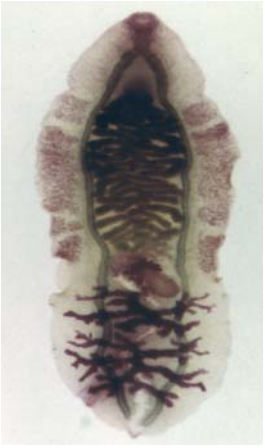
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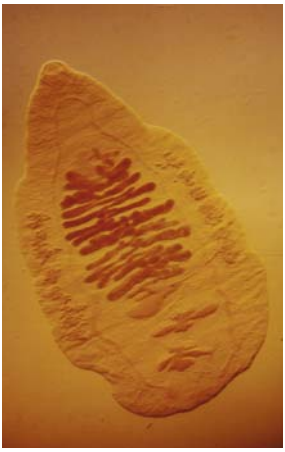
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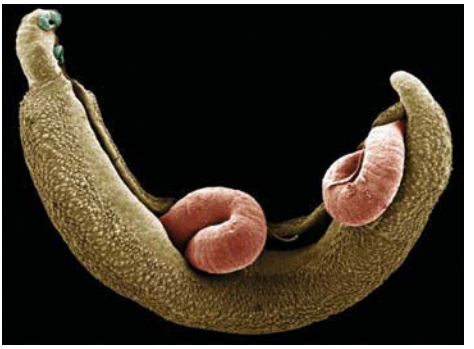

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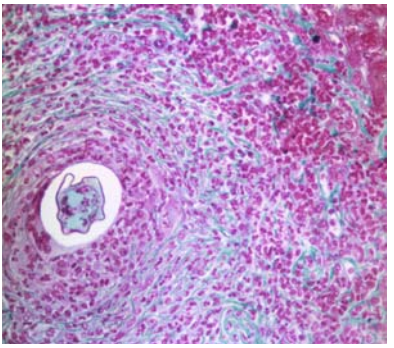

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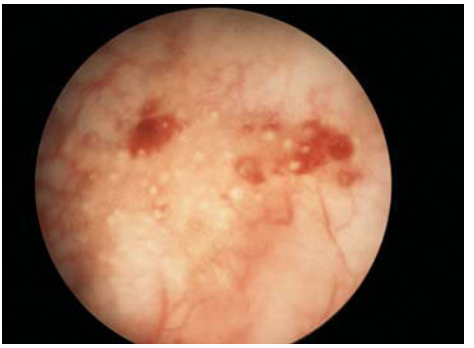

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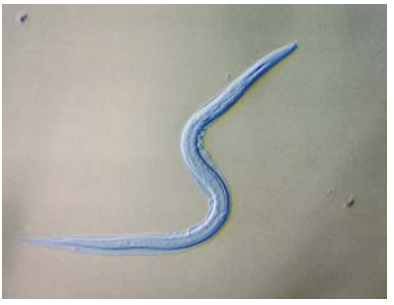

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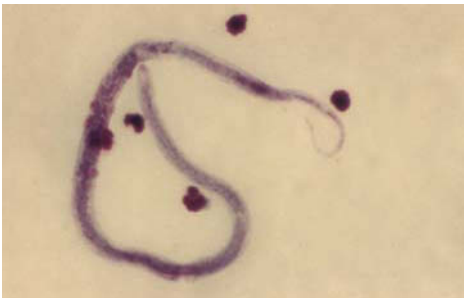

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Transmission, multiplication and incubation period

Transmission of schistosomes to humans is possible in freshwater containing the cercariae (fork-tailed larvae) excreted by the snails. The cercariae can actively penetrate the skin within a short space of time. Since the snail species that serve as intermediate hosts occur only in (sub)tropical climatic zones, transmission can only take place in waters in those regions. Construction of irrigation systems in Africa in particular has led to a dramatic increase in reproduction of the intermediate host snails, resulting in increased transmission.

Schistosomes are two-host helminths with different definitive and intermediate hosts: adult flukes reside in the definitive = final host, humans. Here, egg excretion with stools or urine occurs – depending on the species. Miracidia hatch in freshwater, penetrate an intermediate host (particular freshwater or amphibian species of snail), and larval development and multiplication occur inside the snail (sporocysts, cercariae). Thereafter, cercariae swarm into the water and actively penetrate percutaneously into humans, migrate within the definitive host, mature into adult worms and colonise the species-specific venous plexuses.

The blood flukes' principal multiplication phase takes place at the larval stage in the intermediate host snail. The number of worms harboured by the definitive host (man) is the same as the number of penetrating cercariae (one cercaria develops to one male or female adult worm according to its sex chromosomes). Multiplication in the definitive host consists solely of the production of eggs. These must find their way into water in order to develop further.

Figure 26. Light micrograph of a couple of *Schistosoma mansoni*

Figure 27. Scanning electron micrograph of a couple of *S. mansoni*

Figure 28. Light micrograph of a section through a liver granulome due to a centrally located egg of *S. mansoni*

Figure 29. Endoscopy of the inner surface of a human bladder showing many granulomas due to protruding eggs of *Schistosoma haematobium*

Figure 30, see p. 210

Figure 31. Light micrograph of a larva of hookworms

Figure 32. Giemsa stained microfilaria from blood smear

Figure 33. Adult of *Armillifer annulatus*

Figure 34. *A. annulatus*: scanning electron micrograph of the anterior end with mouth hooks

An incubation period is definable only if a host is simultaneously infested with numerous cercariae. Penetration by the cercariae may lead shortly afterwards to cercarial dermatitis, followed a few weeks later by the febrile systemic disorder known as Katayama syndrome.

Epidemiology

While *S. intercalatum* is almost exclusively a human parasite, *S. mansoni* also occurs in a number of rodent species, too. Also the form of schistosomiasis caused by *S. japonicum* is a zoonosis with cattle, buffalo, pigs, rodents, etc., as definitive hosts. In the case of *S. mekongi*, dogs and pigs act as an animal reservoir.

Anyone living in endemic areas with occupational contact with water (e.g., rice-growing, laundry work) is exposed to the risk of *Schistosoma* infection. Similarly, anyone travelling to the tropics is at risk of contracting schistosomiasis when swimming or coming into contact in some other way with infected waters.

Schistosomiasis can only occur in regions in which the vector snails live and *Schistosoma* eggs pass into waters with faeces and/or urine. This is the case in over 70 countries in hot regions. It is estimated that way over 200 million people are infected worldwide: in Africa and the Middle East with *S. haematobium* (this species does not affect the liver but the urinary tract) or *S. mansoni*, in Latin America with *S. mansoni*, in China and the Philippines with *S. japonicum*, in Laos and Cambodia with *S. mekongi* and in tropical Africa with *S. intercalatum*.

Diseases and symptoms

The signs and symptoms of schistosomiasis (= bilharziasis) vary according to the species and the duration of the disease, though all are directly or indirectly a consequence of man's immune response to the parasite's different stages.

Acute schistosomiasis
Acute schistosomiasis is characterised by a toxaemic phase (including fever, diarrhoea, exhaustion and other non-specific symptoms; 'Katayama syndrome') as a result of passage of the larvae of *S. japonicum* through the lung and their rapid growth (immune response to metabolic products) in the mesenteric veins. Formation of large granulomas (Fig. 28) is found around eggs that are deposited in the capillaries (particularly liver, intestine, bladder). Leukocytosis with marked eosinophilia. In mild infections and also in tourists, early symptoms either do not occur or remain unrecognised.

Chronic schistosomiasis
All symptoms are a direct or indirect consequence of the granulomatous reaction to the parasite eggs (Figs 28 and 29). Though the granulomas are smaller in the chronic stage than in the acute stage (immunomodulation), the quantity of eggs (i.e., of worm pairs) and the duration of the infection (many years or even lifelong without treatment) determine the course of the disease. In endemic areas, bilharziasis is relatively rarely fatal but often causes infirmity. Mild infections are not threatening. A distinction is made between urinary and intestinal schistosomiasis, depending on the site of the worms and the organs in which the eggs are deposited (Fig. 29).

Intestinal schistosomiasis
Intestinal schistosomiasis is caused by the eggs of *S. mansoni*, *S. japonicum*, *S. mekongi* and *S. intercalatum* that are deposited primarily in the intestine and the liver (Fig. 28) or are excreted with the stools. The granulomatous inflammatory reaction around the eggs leads in the large intestine in particular to mucosal hyperaemia, ulceration and later also granulomatous proliferation and bleeding. Of even greater significance with the first two of the aforementioned species, however, is that the liver is damaged by granulomas that form around eggs that are carried *via* the bloodstream to the presinusoidal bifurcations of the portal vein. Chronic phlebitis, periportal fibrosis and finally 'pipestem fibrosis' develop as a result of the granulomas in the liver. Even though the periportal fibrosis does not affect the hepatic parenchyma between areas of fibrosis and does not interfere with its functioning, it nevertheless leads to portal hypertension. Consequences of this may be as follows: formation of ascites (particularly with *S. japonicum*), oesophageal varices and hepatosplenomegaly. Worm eggs may ultimately pass *via* portosystemic collaterals into the lung and lead to pulmonary hypertension and cor pulmonale. The signs and symptoms associated with intestinal schistosomiasis range, for example, from exhaustion and abdominal pain *via* diarrhoea with loss of blood and protein through to massive variceal bleeding as a cause of death. Chronic intestinal schistosomiasis persists for years and its severity depends on the intensity and duration of the infestation. Overall, it constitutes a disease that is triggered by the immune response in humans to the worm eggs.

Immune response

Responses in the host take the form of formation of IgG, IgA, IgM and IgE, activation of various immunocompetent cell populations and circulation of immune complexes. The parasite protects itself by mimicry of host protein and by proteases that lead to inactivation of antibodies and complement factors. Immune processes at the schistosomula stage may, however, also protect against re-infection and superinfection.

Differential diagnosis and diagnosis

Differential diagnoses for both intestinal and urinary schistosomiasis are various diseases of other origin, e.g., Katayama syndrome needs to be distinguished from paratyphoid fever and typhoid fever. Furthermore, a number of parasitic and other infections need to be excluded, both in the acute and the chronic phase of the different forms of schistosomiasis.

Direct detection in smears, sediment or after concentration takes place by means of microscopic examination of stools or urine for schistosome eggs, the shape of which is species specific. A 24 h urine specimen or several grams of stool may be necessary for this purpose. Stool may contain the eggs of *S. mansoni* (114–175 × 45–68 μm, with lateral spine), *S. intercalatum* (140–240 × 50–85 μm, with terminal spine), *S. japonicum* (70–100 × 50–65 μm, with a small sized spine) and *S. mekongi* (65–67 × 56–59 μm, with a small sized spine). Quantitative methods (10 ml urine filtrate, stool smear using the Kato-Katz method) are epidemiologically important. Where very few eggs are excreted, the miracidium hatching test has proved successful. This involves placing the miracidia present in the eggs in a flask to hatch. Because of their positive phototaxis, these then migrate into an illuminated, attached side-arm of the flask, from which they can be removed.

Detection of antischistosomal antibodies in the serum by indirect immunofluorescence, indirect haemagglutination and/or ELISA. All tests are generally based on antigen preparations (worms or eggs) of *S. mansoni* and cross-react with other *Schistosoma* species. Evaluation of titre steps and possible cross-reactions with other parasitoses should be performed by institutes of parasitology or tropical medicine. Tests for circulating *Schistosoma* antigens in serum are likewise suitable for the detection of schistosomiasis.

Treatment

Praziquantel (Biltricide®) is the drug of choice against all *Schistosoma* species. The dosage is 2 x 30 mg/kg body weight (BW) (*S. japonicum*) or 3 × 20 mg/kg BW (all other species), administered orally in one day. Side effects, if they occur at all, are only minor (possible exceptions: massive infections and cerebral schistosomiasis). Metrifonate (Bilarzil®) and oxamniquine (Mansil®) are also used in some endemic areas, although these are effective only against *S. haematobium* and *S. mansoni* respectively.

Resistance

The efficacy of praziquantel against *S. mansoni* is reduced to a varying degree in different African countries as a result of development of wrong use. True resistance has not yet been shown.

Prevention

Avoidance of skin contact with contaminated waters reliably protects against infection. Other prophylactic measures (vaccination, chemoprophylaxis) are not available.

Strategies for disease prevention and control

Individual prophylaxis involves avoidance of skin contact with contaminated waters (rivers, lakes, ponds, rice fields, irrigation systems).

In highly endemic areas, mass treatment or selective treatment of infected persons with praziquantel is indicated in addition to health education measures and proper disposal of human excrement. Use of molluscicides for intermediate host control is possible only in limited areas.

Hepatitis caused by nematodes

Capillariasis: Capillaria hepatica *[5, 6]*

Other name of the agent of disease

No other specific name; disease: hepatic capillariasis

Taxonomy

Subregnum:	Animalia
Phylum:	Nematozoa
Class:	Nematodes
Subclass:	Adenophorea
Order:	Trichocephalida
Family:	Capillaridae
Genus:	*Capillaria*

Morphology

The pathogen is *Capillaria hepatica,* a nematode as slender as a human

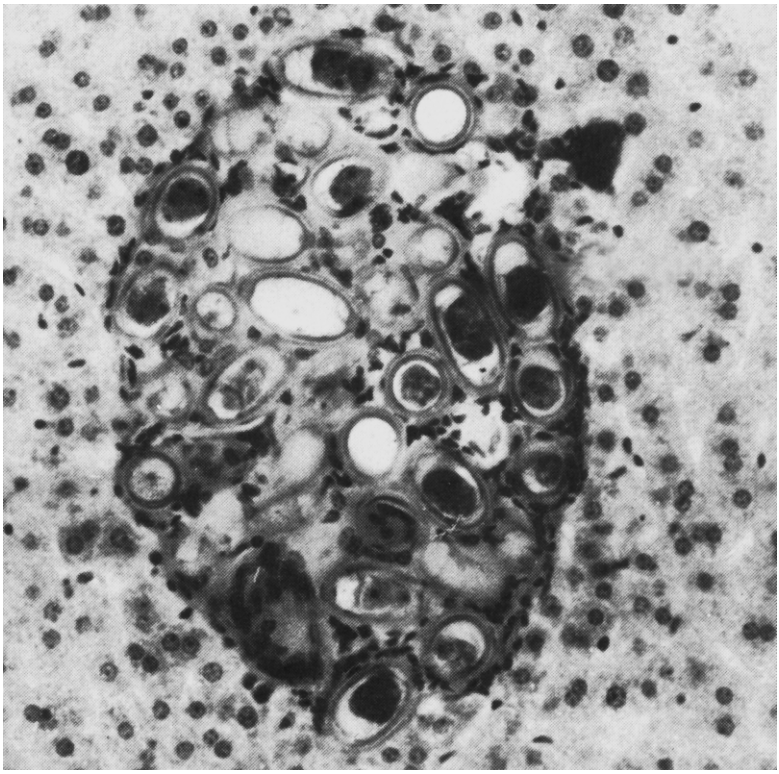

Figure 30. Light micrograph of a section showing a layer of eggs in the liver

hair, measuring up to approximately 10 cm in length in the adult stage, with marked hepatotropism.

Epidemiology

The parasite is prevalent worldwide and its principal hosts are rodents, predominantly rats, in which prevalence rates may be as high as 80% or more. Infection in humans is rare. Approximately 40 confirmed cases, in total, have been reported in North and South America, Africa, Asia and Central Europe, although it is likely that a number of cases remained unreported.

Transmission, multiplication and incubation period

Humans are infected by oral ingestion of the parasite's embryonated (larva-containing) eggs.

C. hepatica parasitises the hepatic parenchyma within which it migrates. At the same time, the adult female worms lay characteristic eggs with 2 polar plugs (Fig. 30). The eggs, which are deposited in the bore holes and are later encapsulated in tubular granulomas, reach the outside world only by means of maceration on the death of the host or, if infected animals served as prey, *via* the digestive tract of the predator (cannibalism also plays a role in the case of rats). Once in the open, an infective larval stage develops inside the egg and this is then ingested by humans with contaminated food. The larva hatches from the egg membrane in the intestine, migrates through the intestinal wall and passes *via* the bloodstream to the liver. Sexual maturity and hence commencement of egg-laying are attained after 3–4 weeks (prepatent period).

Diseases and symptoms

There are only rare cases of disease caused by single (few) liver-specific roundworms, while serious consequences occur in the event of massive infections.

The clinical presentation depends on the burden of infection. Light infection, occasionally reported as a post-mortem finding, would appear to be asymptomatic. In heavy infections, which in children may prove fatal, an acute course is to be expected at around the commencement of egg-laying, these symptoms reflecting the destruction of large areas of liver parenchyma. In such cases, unexplained extrahepatic symptoms (e.g., lung disorders) have been described in the pathogenesis. Moderate infections are associated with uncharacteristic upper abdominal pain and hepatomegaly. Leucocytosis, eosinophilia and hypergammaglobulinaemia regularly occur.

Differential diagnosis and diagnosis

Focal or disseminated damage to the liver parenchyma of diverse origin. Worm nodules with convoluted parasites and/or egg strings encapsulated in granulomas are often to be found in the subcapsular region and are laparoscopically detectable. In patent infections, the diagnosis can be confirmed by biopsy, revealing eggs measuring 30×50 μm with 2 polar plugs (Fig. 30). There are no serological methods available.

Treatment

Mebendazole is effective according to findings in animal experiments.

Prophylaxis

Avoid contaminated foods; keep children away from areas populated by rats.

Larva migrans disease: Migrating nematodes [5, 6]

Migrating larvae or adults often pass *via* the bloodstream from the intestine to the liver. These parasites have left the intestine and are *en route* to obligatory passage through the heart and lungs, during which they may be carried off *via* the bloodstream to other organs. If the larvae are unable to reach sexual maturity in humans, migration continues until their death (3–6 months). The following nematodes have been detected quite commonly in the liver (larva migrans visceralis):

- *Ascaris lumbricoides* (L2 larva and adult)
- *Strongyloides stercoralis* (larvae)
- Hookworm larvae (Fig. 31)
- *Toxocara canis* (larvae)
- Microfilariae of various filariae (Fig. 32)

Treatment

After invasion into the biliary system endoscopic extraction of *A. lumbricoides* larvae can be tried before surgical intervention. For removal of *Strongyloides stercoralis* (larvae) the benzimidazoles albendazole, thiabendazole or mebendazole can be applied. Also the macrocyclic lactone seems to be effective. Against hookworm larvae mebendazole and albendazole may be used. For infections with *Toxocara canis* (larvae) thiabendazole (2 x 25 mg/kg body weight daily over 5–7 days), albendazole (10 mg/kg body weight over 5 days) or diethylcarbamazine are recommended. Microfilariae of various filariae can be treated with diethylcarbamazine with a total oral dose of 36 mg/kg body weight given over 1–2 weeks. Also ivermectin at a single subcutaneous dose of 0.2 mg/kg body weight may be tried.

Pentastomidiasis: Pentastomida *[5, 6]*

Other names of the agents of disease

Tongue worms, Linguatulidae

Taxonomy

Phylum:	Pentastomida
Class:	Pentastomidea
Order:	Porocephalida
Family:	Armilliferidae
Genus:	*Armillifer*, *Linguatula*, *Porocephalus*

Biology/morphology

The adults of the genera *Linguatula*, *Armillifer* and *Porocephalus* live in the nasopharynx and lungs of carnivorous vertebrates (*Linguatula serrata* also in humans) (Figs 33 and 34). *L. serrata* females are up to 13 cm long, males up to 2 cm long and are found mainly in dogs. *Armillifer* species reach a similar size, *Porocephalus* species are not more than 7 cm long. The eggs, which measure about 90×70 μm (*Linguatula*) or 110 μm in length (*Armillifer*), leave the body with the nasal mucus; at the time of shedding they already contain a first stage larva. If these eggs are ingested by herbivores (or by humans), the larvae penetrate the gut wall and migrate to various organs (including liver, lung) where they undergo several moults and grow to a size of 4–5 mm (*Linguatula serrata*) or 2–3 mm (*Armillifer armillatus*). If this intermediate host is eaten by a carnivore the larvae become sexually mature in its nasopharynx after undergoing a further moult and the females begin to lay the sometimes prodigious numbers of eggs (as many as 500,000 per day).

Transmission, multiplication and incubation period

There are various routes of infection in man: Humans (like herbivores) can become infected by ingesting eggs containing larvae with their food. The larvae then hatch and migrate through the body, often invading the lung and liver. This route of infection plays a particular role in African and Asian countries as in these countries humans relatively often become infected with eggs of pentastomes of the genera *Armillifer* and *Porocephalus*, the definitive hosts of which are snakes, when handling snakes and contaminated plants or preparing them for cooking, and then become the intermediate host. By ingestion of larvae with insufficiently cooked or raw meat from infected intermediate hosts. In the case of *L. serrata* these can even become sexually mature in the nasopharynx.

Incubation period
Depends on the stage of infection. After consumption of larva-infected meat the pre-adult worms lead to local reactions within a few days. If tissue

stages occur it can be 6–7 months before the migrating larvae cause damage and symptoms.

Prepatent period
6–7 months in the case of *Linguatula*.

Patent period
15 months in the case of *Linguatula*, rarely longer.

Epidemiology

Linguatula serrata occurs worldwide, species of the genera *Armillifer* and *Porocephalus* are found in Africa and Asia.

Diseases and symptoms

The following symptoms can occur depending on whether the person is infected as an intermediate or definitive host. Most infections occur in the nasal region by adults of *L. serrata* and lead to the syndrome known as halzoun. The nasal passages can become completely blocked. In addition there is deafness and facial swelling. If there is pronounced sneezing these adults can be expelled spontaneously.

Infection of the abdominal organs, among them the liver by *Armillifer armillatus*, with the sometimes very large larvae leads to unspecific symptoms caused by the boring activity of the migrating larvae. Depending on the location of the damage this can lead to the death of the infected person, as has been observed in numerous autopsies in Africa.

Differential diagnosis and diagnosis

Microscopic detection of the eggs in nasal secretions or finding of spontaneously passed worms in the case of *Linguatula*. Histological detection of larvae located in the tissues. Differentiation from other worm eggs.

Prophylaxis

Avoiding touching snakes and dogs or their faeces, particularly in subtropical and tropical regions. Avoiding infection of oneself and of one's dog by eating only cooked meat. Training dogs not to eat rodents.

Treatment

Unknown. Worms in the nasal region can be removed by provocation of sneezing or by surgical procedures.

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Comparative Hepatitis
ed. by Olaf Weber and Ulrike Protzer

Autoimmune hepatitis in humans

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Abstract

Autoimmune hepatitis (AIH) can occur in all age groups from earliest childhood up until the 8th decade. AIH affects women more commonly than men (3:1). Clinical presentation may be an acute hepatitis up to fulminant liver failure, but can also be asymptomatic.

AIH is characterised by lympho-plasmacellular infiltrates on liver biopsy, elevated liver enzymes in serum and association with the HLA haplotypes B8, DR3 and DR4 in the absence of active viral markers. Patients characteristically present with hypergammaglobulinemia, elevated serum levels of IgG and autoantibodies, such as antinuclear antibodies (ANA), smooth muscle antibodies (SMA), antibodies to soluble liver antigen/liver pancreas (SLA/LP) or to liver-kidney microsomes (LKM).

Corticosteroids are the drug of choice for remission induction, azathioprine the drug of choice for maintenance of remission. The rapid response to immunosuppressive treatment supports the diagnosis of AIH and leads to a good long-term prognosis. Treatment duration remains controversial and can be discussed after resolution of all clinical, laboratory and histological manifestations of disease activity. Treatment should be maintained for a minimum of 3 years.

Agent

Autoimmune hepatitis (AIH) is a chronic inflammatory disorder of the liver of unknown origin associated with hypergammaglobulinaemia and the occurrence of autoantibodies [1, 2]. As in other autoimmune disorders a genetic predisposition of the host such as the presence of HLA Class I B8 and HLA Class II DR3, DR4 and DR52a has been found [3]. In addition, unknown environmental triggers such as viral or bacterial infection or drugs may then induce autoimmune hepatitis in the genetically predisposed host. The loss of immune tolerance to self-antigens may be due to the release of modified and primarily sequestered nuclear or cytosolic proteins which activate resting but naturally occurring auto-reactive T-cells. For example, hepatitis C virus has the potential to induce such auto-reactive T-cells that cross-reactively recognise the cytochrome P450 isoforms 2A6 and

2A7 which contain sequence homology to HCV [4]. This cross-reactivity between foreign and self-proteins is commonly referred to as 'molecular mimicry'. Up to 38% of patients with chronic HCV infection display autoantibodies directed against the cytochrome P450 2D6 (CYP2D6) [5]. However, autoimmune hepatitis is only extremely rarely associated with viral hepatitis. The CYP2D6 humanised mouse model of AIH expresses this human antigen in the liver and shows persistent hepatitis after infection with an Adenovirus-CYP2D6 vector [6, 7]. Most other animal models of AIH used transgenic target antigens expressed in the liver but could only demonstrate transient hepatitis [8–10].

The principal effector cells of AIH are $CD4^+$ and $CD20^+$ lymphocytes, mainly found in the portal tracts and $CD8^+$ T lymphocytes mainly found periportally [11]. The repertoire of antigens that sensitise these cells seems restricted. Infiltrating $CD8^+$ lymphocytes show defects in their apoptotic pathway which might be responsible for the perpetuation of inflammation [12]. Co-stimulatory molecules like CD86 are upregulated on liver-infiltrating monocytes in AIH patients but not in patients with chronic HCV infection [13]. Regulatory $CD4^+CD25^+$ lymphocytes have been shown to inhibit $CD8^+$ T-cell proliferation and are decreased in number and function in AIH patients [14]. It has been speculated that AIH can be triggered by certain drugs. A recent report associated statins used in the treatment of hypercholesterinemia with the onset of AIH [15]. Although the exact pathogenesis is not understood yet, diagnosing AIH and providing adequate treatment is extremely important for the patients affected by the disease.

AIH was the first liver disease in which controlled trials proved the efficacy of a therapeutic intervention (corticosteroids), and in which timely diagnosis and adequate therapy is life-saving and can reverse disease progression to liver cirrhosis resulting in a normal life expectancy in the majority of treated patients [16].

Epidemiology

The prevalence of AIH is estimated around 1:5,000–1:10,000 [6]. AIH can manifest at any age from very early childhood up until the 8th decade and like many other autoimmune diseases it is more common in women (gender ratio, 3: 1). Over 20% of the patients develop AIH after the age of 60 years [17]. As many as 34% of the patients present subclinically [18, 19] leading to delayed diagnosis. This delay explains that many patients already show cirrhosis at presentation [19, 20]. Up to one third of patients may present with a very acute disease and some even with fulminant hepatic failure, therefore AIH must be considered in all patients with a fulminant presentation [21]. Autoimmune hepatitis can be associated with concurrent immune diseases such as systemic sclerosis and polymyositis [22], multiplex neuritis [23], and polyglandular autoimmune syndrome type III [24].

Diagnosis

AIH should be considered in any patient with elevated liver enzymes. Most patients show a subacute disease presenting with lethargy and often jaundice without a risk factor for viral hepatitis. Other clinical symptoms may be arthralgias and slight right upper quadrant pain.

Diagnostic criteria

Autoantibodies are the hallmarks of autoimmune hepatitis. There is, with the possible exception of anti-SLA/LP antibodies, no single test that proves or disproves the diagnosis of AIH. Diagnosis is therefore based on the combination of history, clinical examination and a number of laboratory and serological variables.

The key features for making a diagnosis of AIH are

- hypergammmaglobulinaemia (with a preferential increase of IgG)
- demonstration of autoantibodies (ANA, SMA, SLA/LP, LKM)
- absence of viral hepatitis
- portal hepatitis (with lymphoplasmacellular infiltrates) on histology

Additional features may be further supportive:

- a personal or family history of other autoimmune disease
- a history of a spontaneously fluctuating course
- arthralgias
- presence of HLA-DR3 or DR4

The usually very prompt response to immunosuppresssion proves the diagnosis.

Screening tests

Diagnosis of AIH consists of the exclusion of liver diseases of other origin. Viral hepatitis B and C should be ruled out by testing for HBsAg and anti-HCV. Alcohol consumption, intake of hepatotoxic medication and hereditary liver diseases (Wilson disease, haemochromatosis, alpha-1 antitrypsin deficiency) should be excluded. The first hint to the presence of AIH is demonstration of elevated gamma globulins on a serum electrophoresis, which is probably the cheapest screening test for AIH. However, around 5–10% of AIH patients do not display elevated gamma globulins at initial diagnosis. Some of these have a relative elevation of their physiological gamma globulin levels, which only becomes apparent when upon immunosuppression the gamma globulin levels fall from the upper normal range to the lower normal range. Serum lipids should be tested, as fatty liver disease is an important differential diagnosis.

Quantitative IgA, IgG and IgM levels are very helpful diagnostic markers as well. In AIH there is typically a selective elevation of IgG with normal levels for IgA and IgM. Elevated IgA hints towards a toxic cause (e.g., alcohol) of the liver disease, while elevated IgM is a feature of biliary disease such as primary biliary cirrhosis or primary sclerosing cholangitis. Both these conditions can be associated with AIH (so-called Overlap-syndromes).

Autoantibodies

About 80% of all AIH patients have pathological titres of at least one autoantibody. The most common antibodies are anti-nuclear antibodies (ANA) and antibodies to smooth muscle antigen (SMA) in 40–50% of cases each, sometimes in combination. Antibodies to soluble liver antigen (SLA/LP) are present in about 20% of patients and in half of these patients the sole autoantibody is demonstrable. Antibodies to liver-kidney microsomal antigen (LKM) are uncommon (1–3%), but delineate a separate disease subgroup often called autoimmune hepatitis type 2, which is more prevalent in childhood, where it may be a very severe disease.

Antibodies to cyclic citrullinated peptides (anti-CCPs) are found in 9% of patients with autoimmune hepatitis [25]. Autoantibodies should be tested by two methods: anti-nuclear antibodies (ANA), antibodies to smooth muscle antigen (SMA) and antibodies to liver-kidney microsomal antigen (LKM) are best tested using immunofluorescence on tissue sections, which will at the same time detect anti-mitochondrial antibodies (AMA) as the characteristic test for primary biliary cirrhosis. SLA/LP-antibodies do not show up on immunofluorescence and are therefore missed by routine testing. These antibodies should be looked for by specific immunoassays such as ELISA and/or immunoblot. While ANA, SMA and LKM can also be found in some patients with other liver disease and non-hepatic autoimmune disease, SLA/LP antibodies seem to be highly specific for AIH, and thus diagnostic in the patients in which they can be demonstrated. This seems to be also true for antibodies to double-stranded DNA, which are detectable in 15% of patients, and for which the only differential diagnosis is systemic lupus erythematosus (SLE).

Histology

The diagnosis of AIH is based on liver biopsy, which is required to demonstrate typical histological AIH features of bridging or multiacinar necrosis and the presence of a lympho-/plasmacellular infiltrate, but also in order to exclude important differential diagnoses such as drug-induced liver disease or non-alcoholic steatohepatitis (NASH). As mentioned above, about a quarter of all patients with AIH already have cirrhosis at the time of diag-

nosis [16]. Cirrhosis is often macronodular, which explains why in many of these patients cirrhosis may be missed because of the sampling error (biopsy of a large regenerative nodule without fibrous septa). Some authors therefore favour laparoscopy at initial diagnosis, which can now be performed with minimal invasiveness due to the availability of mini-instruments.

Treatment

Immunosuppression dramatically improves the outcome in autoimmune hepatitis, and with individualised immunosuppression patients can look forward to a normal life expectancy and good quality of life. The prompt response to corticosteroids can be observed in nearly all AIH patients, which makes it the most reliable final diagnostic tool: non-response to immunosuppression suggests another diagnosis, provided the patient is compliant, and the dose is adequate. Corticosteroid treatment is justified even in asymptomatic patients since these patients will usually develop symptoms [18]. The preferred treatment schedule in adults is prednisolone combined with azathioprine.

Remission induction

Corticosteroids are the drugs used for the induction of remission. Exact treatment doses are controversial and need to be adjusted according to the individual severity of the disease [16, 26]. We usually recommend a starting dose of 1 mg/kg prednisolone for all patients except those with very mild disease. Severe icteric disease may cause malabsorption of corticosteroids and therefore intravenous application of prednisolone should be favoured. In patients with mild disease, half of this dose may be sufficient. Transaminase levels usually fall within days, and the prednisolone dose can soon be tapered usually in steps of 10 mg weekly dose reductions. Azathioprine should be added to the treatment as soon as the diagnosis of AIH is confirmed, because its immunosuppressive effect takes several weeks to fully develop [27]. The severity of disease activity determines the dosage: azathioprine should be added to corticosteroids at a dose of 1 mg/kg/d, patients with very active disease should be given higher doses up to 2 mg/kg/d [28]. While corticosteroids are tapered early, azathioprine dose is maintained at least until steroids are reduced below 10 mg prednisolone/day.

A typical treatment schedule for a patient with a body weight of 75 kg is given in Table 1.

Tapering of steroids can be faster in patients with a very prompt response or in milder disease. However, rapid tapering often results in early relapse, thus requiring higher overall steroid doses, and therefore the dose should not be reduced too quickly. Vitamin D (400–600 IE/day) and calcium

Table 1. Stepwise reduction of steroids and combined azathioprine immunosuppressive therapy in a 70–80 kg patient; *AZT should be added as soon as the diagnosis of AIH is considered definite

	Corticosteroid	Azathioprine*
Week 1	75 mg/day	75 mg/day
Week 2	60 mg/day	75 mg/day
Week 3	50 mg/day	75 mg/day
Week 4	40 mg/day	75 mg/day
Week 5	30 mg/day	75 mg/day
Week 6	25 mg/day	75 mg/day
Week 7	20 mg/day	75 mg/day
Week 8 + 9	15 mg/day	75 mg/day
Week 10 + 11	12.5 mg/day	75 mg/day
Week 12…	10 mg/day	75 mg/day

(1,000 mg/day) should be given to prevent development of osteoporosis, as long as steroid dose is above 5 mg/day.

Maintenance of remission

Azathioprine is the drug of first choice for the maintenance of remission. Azathioprine when started after the diagnosis of AIH has been established may then help to save steroids. In icteric patients the pharmacodynamics of azathioprine and its metabolites may be considerably altered, increasing the danger of toxicity. In these patients a fall of bilirubin below 10 times the upper limit of normal should normally be achieved before adding azathioprine, and the starting dose of azathioprine in icteric patients should be lower and gradually increased to the desired dose.

Due to the low dose of azathioprine in AIH patients, compared to higher doses in inflammatory bowel disease, tolerability is very good in the majority of patients. Toxicity may result in abdominal pain, nausea and fever. Bone marrow toxicity requires regular blood counts, initially every 1–2 weeks and slowly decreasing to once every 3 months. The superiority of azathioprine over corticosteroids in maintaining remission has been convincingly demonstrated by the King's College group [28]. Combination therapy should be given for the first 12 months, and then corticosteroids should be tapered out slowly for all those patients who are in biochemical remission (normal transaminases and normal IgG). Some groups recommend increasing the dose of azathioprine at this stage up to 2 mg/kg in order to avoid relapse upon steroid withdrawal [28], others like our group would rather

then continue low dose steroids (5 mg) and low dose azathioprine (1 mg/kg) because of worries about oncogenic effects of long-term high dose azathioprine. In each patient, transaminases and IgG levels should be monitored closely every time immunosuppression is decreased significantly as relapse upon dose reduction occurs in 20–86% of patients [29] and may often be preceded by an increase of IgG levels well before the transaminases and should lead to immediate reinstitution of the original regimen. Whenever transaminases rise there should be a short increase of steroid dosage. Exact dose and duration very much depends on how early relapse is detected as early intervention usually requires no more than a limited schedule such as 20 mg for 1 week, 15 mg for 1 week, 10 mg for 2 weeks and the original maintenance dose thereafter.

Therapy for autoimmune hepatitis is continued until remission is achieved, treatment fails or drug toxicity occurs. Complete clinical, biochemical and histological remission often takes years. Histological remission lags behind clinical and biochemical remission by 3–8 months. The majority of patients will require long-term, most patients life-long immunosuppression, many being maintained with doses between 50 and 100 mg azathioprine daily. In all patients azathioprine should be continued for a minimum of 3 years before complete treatment withdrawal is considered, because otherwise relapse rates are very high (>80% within 18 months) [30]. Treatment withdrawal should only be considered in patients who have been free of relapse for 3 years, and in whom liver biopsy has demonstrated absence of significant inflammation, because the strongest predictor of relapse after treatment withdrawal is the presence of histological signs of continued inflammation [31]. Compliance may become a problem in some long-term patients, and it may occasionally be justified to attempt treatment withdrawal despite negative predictors for stable remission, as only the proof of relapse will convince the patient to take a potentially toxic drug for many years. In these patients close observation for the first 1–2 years after treatment withdrawal is required in order to detect relapse in time. Relapse may sometimes be delayed by many months, so patients and their caregivers might be misled by a stable situation early after treatment withdrawal.

In most patients treatment monitoring can be solely based on laboratory investigations such as determination of aminotransferases and gamma globulines, and liver biopsy on follow-up is only required if aminotransferases or gamma globulines (or IgG alternatively) fail to normalise.

Patients difficult to treat

Fulminant autoimmune hepatitis

A few patients present with fulminant hepatic failure. This is more commonly observed in children and young adults. Unfortunately immunosup-

pression may come too late for some of these patients, and the standard criteria for emergency liver transplantation should be used for the patients as well, despite the presence of an underlying condition which in principle is very responsive to treatment. Some patients rapidly deteriorate on steroids in this situation, and any delay in transplantation will increase the risk of potentially fatal infection. Patients without encephalopathy, however, should undergo an intensive trial of immunosuppression, giving up to 250 mg prednisolone i.v. for the first few days.

After transplantation more intensive immmunosuppression including azathioprine is recommended as relapse of AIH is recognised in at least 17–22% of transplanted patients within 5 years [32].

Patients intolerant of azathioprine

Intolerance to azathioprine can be observed in up to 3% of the patients, and a similar proportion of additional patients will develop side effects later on, mostly due to arthralgia (63%), lymphopenia (57%), myelosuppression (7%), hepatotoxicity, or pancreatitis requiring either dose reduction or complete drug withdrawal. While some of these patients can be managed with steroid monotherapy, the majority will require another second-line drug. Primarily because of its low toxicity, mycophenolate mofetil now has emerged as the standard second choice drug for these patients. Three small clinical studies provided evidence that about two thirds of patients fare well on MMF at a dose of 1,000 mg twice daily [33], while one third appears to not really benefit from the drug. In these few patients the choice becomes more difficult. Our personal preference (in descending order) is cyclophosphamide, methotrexate or cyclosporine. Cyclophosphamide can be given orally (100 mg/day initially) or as a bolus infusion like in SLE. These patients, however, should be treated in specialised centres, as side effects of the drug need to be closely weighed against the dangers of progressive disease.

Poor responders to standard immunosuppression

Despite using the schedule of high dose steroids in the initiation of treatment, about 9% of the patients have worsening aminotransferases or bilirubin levels indicating to fail remission [16]. In some of these higher than usual maintenance doses (10–20 mg prednisolone/day, up to 2 mg/kg/day azathioprine) may be the best choice of management. Only exceptional patients will continue to show high inflammatory activity after intensifying immunosuppression. In these patients, treatment failure warrants reassessment with regard to the accuracy of the diagnosis and exclusion of variant syndromes or other hepatic diseases, e.g., drug-induced hepatotoxicity; a

second opinion, both clinically and on the histology, may be helpful. Various experimental protocols have been used in these patients, our personal preference is cyclophosphamide. These patients should be referred and treated in specialised centres with experts in AIH to evaluate novel therapeutic approaches in patients difficult to treat.

In conclusion it has to be emphasised that individualised therapy is tailored to the individual requirements of each patient. With such careful management life expectancy of treated patients exceeds 85% at 10 years and is comparable with to those in age- and sex-matched normal population [34].

Overlap syndromes with PBC or PSC

About 10–20% of patients present at some stage of their disease with overlapping features with either primary sclerosing cholangitis (PSC) [35] or (more commonly) primary biliary cirrhosis (PBC) [36]. In these patients management should be primarily directed by the autoimmune hepatitis component of their disease, as this is the condition leading most rapidly to liver destruction and cirrhosis, and at the same time can be treated effectively. In overlap patients it appears sensible to add ursodeoxycholic acid (UDCA) to the treatment schedule, even though the evidence for this approach is weak. Patients with AIH/PSC overlap seem to run a less favourable course than patients with AIH only, while AIH/PBC overlap patients seem to have a similarly good prognosis.

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Comparative Hepatitis
ed. by Olaf Weber and Ulrike Protzer

Hepatitis in dogs

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Abstract

Liver diseases are a major focus of research in the Faculty of Veterinary Medicine. In a clinical population 1% of the dogs has a form of hepatitis. There are several aspects of canine hepatitis which make comparison with human hepatitis very useful. As a whole, liver diseases and especially different forms of hepatitis in dogs develop highly similar to human hepatitis (fulminant, acute, chronic). This makes dog diseases interesting to study important aspects in the pathogenesis of the disease. We have shown that in humans and dogs identical processes with respect to formation of fibrosis, regeneration, stem cell activation, and oxidative damage occur. In most aspects identical pathogenesis and pathophysiology of canine and human hepatitis make these spontaneous dog diseases ideal models to study the effect of new modes of intervention to stimulate regeneration and reduce or prevent cirrhosis.

Introduction

Hepatitis is a frequent liver disease in dogs. The primary inflammatory disease in dogs affects the parenchyme (hepatitis) which is different from the situation in cats where it is primarily of biliary origin (cholangitis) [1].

Following destruction of hepatic parenchyma (apoptosis or necrosis), an inflammatory reaction, regeneration of parenchyma, fibrosis, and ductular proliferation may occur. When hepatocytic destruction is limited and the reticulin network remains intact, regeneration with complete restitution of the liver structure can occur. Severe parenchymal destruction with extensive loss of hepatocytes often is followed by ductular proliferation. Many of these structures contain both liver-cell and bile-duct elements and may reflect regenerative proliferation of an hepatic stem cell population analogous to oval cells in the rat, or transformation of regenerating hepatocytes into ductular-like structures. These structures generally are most prominent in the periportal areas. Like in man, these are CK7-positive liver progenitor cells. With chronic parenchymal damage or extensive loss of hepatocytes fibrosis and postnecrotic scarring may occur with the formation of intrahe-

patic portovenous shunts; in these cases prolonged regenerative effort will result in regenerative parenchymal nodules.

The diagnosis of hepatitis and its classification (acute, chronic, etc.) is only possible with histology of the liver. The histopathologic diagnosis includes the type, pattern and extent of the necrosis and inflammation, and the possible cause, and in more chronic cases the presence, pattern and extent of fibrosis and regeneration. The activity of the inflammation is defined by the amount of hepatocellular necrosis and inflammation, and the chronicity is determined by the amount of fibrosis.

For veterinary medicine there is a liver consensus group of the World Small Animal Veterinary Association (WSAVA). This group has recently published a consensus for the nomenclature and the clinical and histological diagnostic criteria in the diagnosis of liver diseases [1]. These world standards and the accepted nomenclature has been used in this chapter.

Acute hepatitis in dogs

Etiology

Acute hepatitis can be caused by chemicals (the most familiar are organic solvents such as CCl_4, and phosphorous), viral infection [2] (infectious canine hepatitis due to the Canine Adenovirus 1 [CAV-1]), and mycotoxins (especially aflatoxin B1). Acute hepatitis may also be caused by various toxins, such as mushroom toxins (*Amanitum*) or blue-green algae toxins (*Cyanophyceae*). Drugs also may cause acute hepatitis in dogs. Drug-induced hepatitis has been reported after treatment with nalidixine acid [3, 4]. Additional forms of drug-induced hepatitis in dogs have been reported in dogs like idiosyncratic drug toxicity (sulfonamides, carprofen, and amiodarone), or dose-dependent drug toxicity (acetaminophen) [5, 6]. CAV-1 is, so far, the only known hepatitis virus in dogs, but the similarity of forms of acute and chronic hepatitis between dogs and man indicates that, like in man, there may be more hepatitis viruses in idiopathic cases of canine hepatitis. There are three forms of CAV-1-caused hepatitis, peracute, acute and chronic.

Hepatitis resulting from sepsis (non-specific reactive hepatitis), and hypoxia (haemolysis, shock) are not discussed here.

Pathogenesis and pathology

Depending on the extent of the liver cell necrosis, intracellular enzymes will be released and bile will leak back into the circulation. In acute hepatitis all of the enzymes (ALT, AST) and bile acids are usually highly elevated. Fever can, but does not always, occur as a result of pyrogens from necrotic tissue and from reduced removal of endotoxins and bacteria from the portal

blood. Diffuse intravascular coagulation is often seen. Very extensive liver cell necrosis with loss of a great amount of liver function (*fulminant hepatitis*) seems to be quite rare, but may be missed because such cases may not reach referral centres. It leads to the development of hepatic encephalopathy, disseminated intravascular coagulopathia (DIC), jaundice, and hypoglycaemia *via* loss of glycogen synthesis and glyconeogenesis. This severe form progresses rapidly to coma and death.

Acute hepatitis is characterised by liver necrosis followed by an inflammatory reaction. Depending on the severity of the hepatitis, there may be apoptosis, focal necrosis, and confluent or bridging necrosis. The inflammatory infiltrate consists of round cells and neutrophils. Infection with CAV-1 is usually characterised by confluent and bridging necrosis in the centrilobular zone and by the presence of intranuclear inclusions in hepatocytes and Kupffer cells. The virus can also be demonstrated histologically by the intranuclear inclusion bodies or immunofluorescence.

Symptoms

Symptoms comprise acute illness, apathy, sometimes fever, anorexia, vomiting, dehydration, sometimes icterus and, in severe cases, DIC. In fulminant hepatitis there is rapid worsening with hepatic encephalopathy, icterus and bleeding. The clinical picture is entirely dependent on the severity and extent of the liver damage. In most cases acute hepatitis is not a severe disease. Most dogs recover completely without treatment.

Diagnosis

If there is no jaundice, the symptoms are non-specific. Blood examination reveals increased liver enzymes, especially ALT, sometimes hyperbilirubinaemia. Ultrasonographically the liver is usually not abnormal in size, architecture or echodensity. Only in cases of very severe or fulminant hepatitis the liver may appear too small and irregular. The diagnosis is established by liver biopsy. In dogs biopsy needles of 14G may be used to obtain large enough samples to be diagnostic. Coagulation should be checked first; in case of DIC it is often abnormal).

Diagnosis of CAV-1 infection might be performed with paired serum titre tests and can be confirmed etiologically using molecular tests.

Treatment

Usually no specific treatment is needed. In more severe cases supportive treatment may extend to iv fluid infusion to correct hypovolemia,

shock, acidosis or alkalosis, hypoglycaemia, and electrolyte disturbances. Corticosteroids are contraindicated because of the suspicion of viral etiologies. In severe liver damage an antibiotic (amoxycillin) may be a helpful support to eliminate bacteraemia due to inadequate hepatic clearance of the portal circulation. Phalloidine and acetaminophen intoxication cause oxidative damage and may be treated with silymarin (50 mg/kg/day) for 3–5 days. Acetaminophen intoxication may also be treated with the combination of N-acetylcysteine (140 mg/kg PO, every 6 h during 3 days), vitamin C (25–35 mg/kg PO, every 6 h for 2 days), and cimetidine 5 mg/kg/bid for 4 days. If acetaminophen toxicity has caused hemolysis, blood transfusion may be required.

Acute hepatitis recovers spontaneously as a rule, however, in about 10% of the cases it progresses to chronic hepatitis. Chronic disease does not give clinical signs in the first months and may become clinically apparent after several months with liver dysfunction. It is therefore recommended to perform a control liver biopsy, or at least to measure plasma ALT, 4–6 weeks after the diagnosis of acute hepatitis to detect chronic hepatitis in an early phase.

Leptospirosis

Etiology

Leptospirosis is a disease resulting from infection with *Leptospiraceas*. The genus Leptospira is determined by its serovar. Due to their pathogenicity/virulence different groups of serovars have been subsumed under different 'nomen-species'. Dogs are the main host for one the major causes of leptospirosis, the *L. interrogans* serovar *Leptospira canicola*. Other serovars like *L. icterohaemorrhagiae* and *L. grippotyphosa* also cause leptospirosis. Leptospira are transmitted *via* direct contact or urine and contaminated water from dogs and rodents like rats and mice, respectively [7, 8]. It is noteworthy that leptospirosis is an anthropozoonosis.

Leptospirosis is a frequent cause of hepatitis in dogs accounting for a significant number of acute hepatitis cases in dogs with approximately 10% mortality. Prophylactic vaccines are available.

Dogs may be asymptomatic carriers which transmit the infection without being ill. Experimental infection causes disease in young dogs, but older dogs may remain non-symptomatic. In spontaneous clinical cases illness develops after an incubation of 1–3 weeks [9]. The symptoms are dominated by the uraemia. Icterus, if present, is caused by intrahepatic cholestasis. Myositis may cause painful palpation and gait. Without treatment renal insufficiency is fatal. The liver lesions are not severe and recover spontaneously. In the liver there is a non-specific reactive hepatitis. Bacterial enzymes cause detachment of the tight junctions which stimulate hepatocytes into mitosis. Therefore an increased number of mitotic figures in the hepatocytes is

characteristic. Due to intrahepatic cholestasis nearly all cases are distinctly jaundiced. Symptoms are mainly determined by acute renal failure.

Symptoms

The symptoms comprise malaise, often fever, vomiting, jaundice, sometimes diarrhoea, muscle pain, sometimes petechiae due to thrombocytopenia as a result of DIC.

Diagnosis

The disease is characterised by acute onset and icterus. However, since symptoms might be unspecific, diagnosis is supported by laboratory testing. Blood examination reveals uraemia and cholestasis with elevated bilirubin, AP, gammaGT and bile acids. CPK, reflecting myositis, is often elevated, and many cases have thrombocytopenia. Urine examination may reveal tubular epithelial cells in the sediment, and/or proteinuria.

Liver histology usually shows non-specific reactive hepatitis, which may occur in any type of sepsis. The diagnosis can be made by serology. The rapid IgM peak is maximal after 4 days, followed at least 10–14 days later by a long-lasting IgG peak. IGM stays high for 2–3 weeks, and specific measurement of IgM in serum is the only method to confirm the diagnosis in an early stage. IgG may indicate chronic infection or may be due to vaccination. Increasing IgG over time indicates active infection.

Since serology is not always a reliable method, etiological diagnosis should be confirmed by either detection of the agens in liver biopsies (Levaditi or Warthin-Starry staining) or bacterial culture (i.e., Oxoid Selective Leptospira-Broth). Diagnostic PCR is also available.

Treatment and prophylaxis

Penicillin is given until kidney function has recovered or stabilised, and then streptomycin is given on two consecutive days. Alternatively, cephalosporins are well effective [10]. This prevents the continuing excretion of Leptospira in the urine. The urinary shedding of Leptospira stops two days after the start of penicillin administration and remains absent as long as penicillin is continued. Prevention by vaccination is important for dogs at risk such as hunting dogs or dogs which like to swim.

The prognosis depends on the degree of kidney failure. Despite good therapy the infection is usually fatal. Acute disease with jaundice and uraemia should immediately be treated as if it is leptospirosis, until the diagnosis is definitely made.

Chronic hepatitis and cirrhosis

Chronic hepatitis in dogs [11–14] is characterised by periportal fibrosis, infiltration of lymphocytes and plasma cells, and periportal liver cell apoptosis or necrosis. The apoptotic hepatocytes shrink and are acidiphilic. Parenchymal expansion of the inflammation and fibrosis may cause porto-portal or porto-central bridging septa. Porto-central fibrosis causes cirrhosis, as the end-stage of chronic hepatitis. Macronodular cirrhosis is the common form in dogs, but micronodular cirrhosis is especially seen in different forms of chronic hepatitis due to copper storage. Advanced fibrosis extends to cirrhosis in a negative vicious circle, regardless of the underlying cause. Curative intervention is more successful if initiated in early stages of the disease. Therefore, fast and accurate diagnosis may be pivotal. Chronic hepatitis and cirrhosis cause intrahepatic cholestasis, but in only some 25% of the cases there is icterus.

Etiology

Chronic active hepatitis may be the result of a viral infection. Canine Adenovirus-1 is the only known canine hepatitis virus. However, it is likely that there are more different yet unknown hepatitis viruses in dogs [12, 15–17]. Although infection with CAV-1 might become chronic in vaccinated animals, CAV-1 infection results in fulminant hepatitis in all non-vaccinated animals [18, 19]. CAV-1 titres are usually low in dogs with chronic hepatitis. The lymphocytic and plasmacellular inflammation, and the good response to immunosuppressive drugs indicate that a self-perpetuating immune component of liver cell destruction is involved in the chronic progression. Chronic hepatitis in dogs may also be caused by toxins such as aflatoxicosis. Inherited copper accumulation also leads to damage of hepatocytes with secondary hepatitis and fibrosis [20–23] (see below). Copper accumulation and inflammation, however, begin in zones 3 of the liver lobules and not in zone 1 as in other types [24].

Pathogenesis

Gradually progressive liver cell necrosis may cause a continuing elevation of all liver enzymes and the bile acids. However, in not very active hepatitis and in end stages in which cirrhosis has developed, the release of enzymes into the plasma may be insignificant and then the enzymes may be normal or slightly elevated. Icterus does not always develop. Chronic hepatitis is always a diffuse process throughout the liver. Liver function is diminished by the loss of functional tissue and reduced portal blood flow. There are often low albumin and fibrinogen levels. Hepatic encephalopathy may

develop if portosystemic collaterals are formed. The end stage is usually cirrhosis. Hypoalbuminaemia and portal hypertension may cause ascites. Chronic hepatitis may occur at any age. In the breeds in which an abnormal copper metabolism causes hepatitis, the gradual accumulation of copper usually leads to clinical signs at an age of 4–6 years. Chronic hepatitis occurs in all breeds, but the most frequently affected breeds are Labrador Retrievers (Golden Retrievers to a lesser extent), Dobermann Pinschers, all Spaniel breeds, Bedlington Terriers, and West Highland White Terriers (see also hepatitis due to copper storage). The incidence of chronic hepatitis is relatively high; it is one of the most common liver diseases in companion animals and accounts for about 1% of the cases in a referral clinic.

Symptoms

The most frequent symptoms are apathy, reduced appetite, vomiting, poor endurance, polydipsia, and in about 15% of the cases icterus. In advanced cases with decompensation of the liver functions and portal hypertension there may be ascites and hepatic encephalopathy. So-called 'blue eyes' that are a result of structural changes in the sclera can frequently be observed in dogs with chronic CAV-1 infection.

Diagnosis

Physical examination usually reveals no specific findings. All of the liver enzymes are more or less increased, but ALT is the most sensitive parameter, also in early stages. In advanced cases there may be hypoalbuminaemia. The diagnosis can only be made by liver biopsy. Ultrasonography may reveal a small liver with an irregular surface and structure. In less advanced cases there are no ultrasonographic abnormalities. Regarding the very high frequency of copper storage diseases in dogs it is advisable to perform histochemical copper staining in all cases (e.g., rubeanic acid stain). Diagnosis of CAV-1 infection can only be confirmed using lab tests.

Treatment

There is no specific treatment available to date. Immunosuppression may slow the immune component of the pathogenesis. Prednisolone (1 mg/kg/day) is the usual drug given for at least 6 weeks and in more active/advanced stages for 8–12 weeks. If the side effects of steroids are intolerable 1.0 mg azathioprine/kg/-day may be used. It is important to evaluate the response to treatment by liver biopsies. Medication is aimed to achieve complete histological remission of the inflammatory process and of hepatocyte apop-

tosis, which occurs usually after 8–12 weeks. Without treatment, the disease progresses to cirrhosis.

In cases with copper accumulation the most effective drug is penicillamine, given in two doses per day (20 mg/kg/day). The usual treatment period is 12 weeks, after which a follow- up histological evaluation shows if penicillamine may be discontinued and be replaced by zinc gluconate to prevent reaccumulation of copper. There are effective hepatic support commercial diets which contain little copper and several antioxidants.

Apart from specific treatment directed at the hepatitis, advanced stage cases need supportive care to prevent or treat dehydration, ascites, and management of HE. The presence of portosystemic collateral circulation as a prerequisite for HE can be predicted with an ammonia tolerance test.

The prognosis of chronic hepatitis is guarded; the disease can be stopped completely in many dogs. However, in many cases there are recurrences which finally determine a guarded prognosis. Copper storage diseases may be treated with complete success and have a much better prognosis. Depending on the presence of collaterals dogs may require permanent support with a liver support diet and lactulose.

Chronic hepatitis due to hepatic copper storage

Etiology

Hepatitis in copper storage diseases occurs as a result of an inherited defect of hepatic copper metabolism. Food contains an excess of copper, which is absorbed in the small intestines and cleared from the portal blood by the liver. The excess is normally excreted into the bile by the hepatocytes. Copper is also an essential element and it is distributed to the body by the liver after incorporation in ceruloplasmin. The normal metabolic pathways of copper in the hepatocytes are only in part known; intracellular trafficking is only in protein-bound form. Free copper causes oxidative damage to the cells, resulting in liver cell apoptosis and an inflammatory reaction. Accumulation of copper and inflammation occur around the central veins in zone 3 of the liver lobules. Cholestasis may also cause some copper accumulation which is always localised in the portal areas (zone 1), and also is much less pronounced than the zone 3 accumulation in primary copper storage diseases [24]. Copper-related hepatitis, if untreated, progresses to chronic hepatitis and cirrhosis. The gradual accumulation leads to clinical disease typically at an age of 4–7 years in most dogs. High hepatic copper concentrations $>1{,}000$ mg/g dry tissue indicate an inherited form of copper storage disease; normal dogs have levels <400 mg/g. Histochemical staining (with rubeanic acid) shows the zonal distribution which is an essential clue to the diagnosis.

This disease occurs in different breeds [25–33]: Bedlington Terriers, Labrador Retrievers, Dobermann Pinschers, all Spaniel breeds, West Highland White Terriers, Dalmatian dogs, Anatolian Shepherds, and Skye Terriers. Only in Bedlington Terriers, which have an autosomal recessive disease, the underlying gene defect (a deletion in the COMMD1 gene) has been found. Very recently, reliable DNA tests for the deletion in the COMMD1 gene have been reported [28, 34]. For none of the other breeds the causative gene or a DNA marker is known, but ongoing research in several breeds will reveal new gene mutations within a few years.

It has recently been shown [22, 26] that Dobermann Pinschers and Labrador Retrievers have an inherited form of copper storage disease which affects about 10% of these populations. In Dobermann Pinschers this chronic hepatitis is very aggressive and nearly exclusively linked to females. Copper-associated hepatitis occurs also in different Spaniel breeds and Dalmatian dogs. In general, we have recently shown (not yet published) that 35% of all dogs with hepatitis have an underlying disorder of copper metabolism.

Pathogenesis

The increased copper concentration due to gradual accumulation is after 1 year of age. Oxidative damage causes liver cell death, activation of the Kupffer cells and an inflammatory reaction in zone 3. Chronic persistent hepatitis leads to loss of regenerative capacity and fibrosis. In the end stage, cirrhosis may develop. In copper storage diseases this is always micronodular cirrhosis, whereas in non-copper-associated forms of chronic hepatitis the regenerative nodules are macronodular.

Dogs with copper accumulation may develop an acute haemolytic anaemia, probably due to release of highly accumulated copper from necrotising hepatocytes. Because liver functions are already reduced, such dogs always have distinct jaundice.

Heterozygote carriers cannot be distinguished phenotypically from healthy animals. In Dobermanns the disease affects only females, typically at an age of 4–7 years. In all other forms of hepatitis there is a female overrepresentation, usually females represent 60–70% of all cases.

Symptoms

The symptoms are identical to those seen in non-copper-associated chronic hepatitis.

Diagnosis

On the basis of physical and blood examinations it is not possible to differentiate this disease from hepatitis/cirrhosis or haemolysis due to other causes. Early subclinical cases can be discovered by an increase of ALT in blood; other enzymes or bile acids are much less sensitive and become abnormal only in advanced cases. The diagnosis hepatitis is based on histological examination and copper staining of liver biopsies; the centrolobular copper accumulation is associated with Kupffer cell activation and inflammation and in advanced stages with fibrosis.

Treatment

Copper storage disease can be treated with a copper binding chelating drug [35]. Penicillamine is widely available, and is given in two daily doses 30 min before each meal in a daily dose of 20–25 mg/kg. Urinary excretion of the penicillamin-copper complex reduces free copper followed by the copper bound metal. The response to treatment is evaluated by follow up liver biopsies and is in most cases seen after 3 months. If the hepatitis is cured, recurrence should be prevented because the underlying gene defect cannot be treated and the disease will reoccur upon discontinuation of treatment. Long-term prevention may be achieved with zinc (30 mg/kg/day) in divided doses with each meal. Zinc induces the intestinal metallothionein production, which binds copper and prevents its absorption. Recently commercial low-copper liver support diets have become available which are very effective in preventing the disease.

Lobular dissecting hepatitis

Etiology

The etiology of this disease is unknown. The author has seen this form of hepatitis in kennels where one dog after another got ill, at different ages. We considered this suggestive for an unknown infectious origin.

Pathogenesis

This is a severe disease with pericellular fibrosis around all hepatocytes. The amount of fibrous tissue is excessive causing severe portal hypertension, ascites, portosystemic acquired collaterals and hepatic encephalopathy. The progression is usually quite fast; cirrhosis is formed within 3–5 weeks time, whereas for other forms of chronic hepatitis this is several months or

years. With specific stainings (CK7) an abundant but apparently inadequate proliferation of hepatic progenitor cells is seen; this disease shows the most prominent progenitor cell activation of all canine liver diseases and may be an excellent model to study signals involved in this process.

Symptoms

Symptoms are weight loss, vomiting, polyuria, followed by ascites and hepatic encephalopathy.

Diagnosis

Abdominal fluid is clear, colourless or yellow in case of icterus. At blood examination liver enzymes may or may not be increased, but bile acids are. Ammonia is often increased and the ammonia tolerance test is usually abnormal. Liver biopsy reveals the characteristic changes, especially with collagen stains.

Treatment

In our experience all cases die because of liver decompensation.

Comparative aspects of hepatitis in dogs and man

Liver diseases are a major focus of research in the Faculty of Veterinary Medicine (Program Tissue Repair). There are several aspects of canine hepatitis which make comparison with human hepatitis very useful. As a whole, liver diseases and especially different forms of hepatitis in dogs develop highly similar to human hepatitis (fulminant, acute, chronic). This makes dog diseases interesting to study important aspects in the pathogenesis of the disease. We have shown that in humans and dogs identical processes with respect to formation of fibrosis, regeneration, stem cell activation, and oxidative damage occur [36–38]. In most aspects identical pathogenesis and pathophysiology of canine and human hepatitis make these spontaneous dog diseases ideal models to study the effect of new modes of intervention to stimulate regeneration and reduce or prevent cirrhosis. One upcoming aspect of interest to understand new possibilities for treatment of presently untreatable stages of hepatitis is the liver stem cell. Precise understanding of the stem cell niche of the liver is essential in order to develop new therapies aimed at stimulating the proliferation and differentiation of these progenitor cells. We have shown (unpublished results) that stem cell activa-

tion in dogs and man occurs in a highly comparable way in the development of hepatitis [39]. It is also possible to draw beneficial conclusions from distinctly different forms of hepatitis, such as lobular dissecting hepatitis (LDH), which is most comparable with neonatal hepatitis in humans. The livers of dogs with LDH are loaded with activated progenitor cells, which in the face of excessive fibrosis is functionally ineffective. This disease offers chances to study stem cell activation and the relations with fibrosis and stellate cell activation using techniques such as microdissection, microarray, and immunohistochemistry.

The very frequent occurrence of copper storage diseases as a cause of hepatitis in dogs is a specific feature of canine hepatitis. Purebred dog breeds have been formed over the past 100–200 years by continuous selection of desired characteristics regarding behaviour and exterior. This stringent selection and inbreeding has caused accumulation of originally sporadic disease genes in all breeds so that nowadays each dog breed has several highly frequent breed-specific diseases. In this way defects in the function of genes involved in copper metabolism have also by chance been segregated over dog breeds. This has caused the presence of many different copper metabolism diseases in different dog breeds. Also due to the fact that traditionally dog foods contain a quite high copper concentration, the different dog breeds present an excellent opportunity to detect yet unresolved parts of the cellular pathways of copper handling. In the dog breeds mentioned before as affected by copper storage liver disease, we have excluded all presently known candidate genes involved in copper metabolism. Therefore by definition, these dogs will elucidate new elements of copper pathways. The high level of inbreeding of purebred dogs has simplified complex diseases to seemingly monogeneous diseases so that complex copper disorders of which the genetic background would not become apparent or resolvable in humans may be unravelled relatively easily in dogs.

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Comparative Hepatitis
ed. by Olaf Weber and Ulrike Protzer

Hepatitis in horses

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Abstract

The main causes of hepatitis in horses include serum hepatitis, cholangiohepatitis and chronic active hepatitis with occasional cases of haematogenous bacterial hepatitis (e.g., Tyzzer's disease in foals), abscesses, viral hepatitis, parasitism and chronic infiltrative inflammatory disease. Not all cases of hepatitis will be clinically apparent due to the large reserve capacity of the liver. However, signs of marked hepatic insufficiency and hepatic encephalopathy reduce the likelihood of a successful outcome. Serum enzymes including alkaline phosphatase, aspartate aminotransferase, gamma glutamyltranferase and glutamate dehydrogenase offer an approximate reflection of hepatic damage whereas functional indicators such as bile acids, bilirubin, albumin, globulins, urea and ammonia may provide more prognostically useful information. Nevertheless all serum biochemical parameters have significant limitations in terms of sensitivity or specificity. Liver biopsy, when guided by ultrasonographic images, offers the optimal safe and effective means of investigating suspected hepatitis in horses and will provide useful diagnostic and prognostic information in addition to aiding selection of therapeutic choice.

General and supportive care for equine hepatitis cases includes fluid therapy, antipyretics and dietary management comprising small frequent feeds of low fat, good quality protein and cereal based feeds such as grass, good grass hay, dried proprietary chaffs, alfalfa, processed wheat/maize and B vitamin supplements. Care should be taken to ensure that supplements do not contain potential hepatotoxins (e.g., iron). Specific therapy is usually selected on the basis of biopsy findings and may comprise antibacterials, anthelmintics, glucocorticoids and lactulose.

Aetiologies

There are significant geographic variations in the prevalence of hepatitis in horses. Published reviews of equine hepatic disease suggest that hepatitis is relatively common in the USA largely as a result of so-called 'serum hepatitis' [1–4]. Other common causes of equine hepatitis comprise cholangiohepatitis and chronic active hepatitis both of which have similar prevalence in USA and UK (Tab. 1). Several specific types and causes of hepatitis in

Table 1. Relative prevalence of equine hepatitides derived from four large surveys of liver disease cases

Disease type	USA studies (n = 147) [1,2]	UK studies (n = 138) [3,4]
	% of total hepatopathy cases	% of total hepatopathy cases
Serum hepatitis	29.3	0
Cholangiohepatitis	15.6	12.3
Chronic active hepatitis	5.4	6.5
Abscess	0	0.7
Total hepatitis cases	50.3	19.6

horses are discussed below and are associated with specific clinical, clinicopathological, gross pathological or histopathological diagnostic features. However, in the author's experience such clear diagnostic categorisation is impossible in many clinical cases seen in practice. This view is supported by the more general categorisation appearing in published reviews of equine liver disease cases [1–6].

Cases of acute hepatitis and necrosis in adult horses, known as serum hepatitis or Theiler's disease, are seen frequently in some geographic locations such as the USA [2, 7]. Prognosis is guarded to poor with the majority of affected horses succumbing [8]. A similar disease has also been reported from France [9] and Turkey [10]. The condition is rare in the UK although several cases have been seen by this author either as isolated cases or outbreaks. Although most cases have no obvious trigger factors, some may follow between 4–10 weeks after tetanus antitoxin administration and have also been reported following use of other equine biologicals such as plasma [11, 12]. Despite the similarities with human hepatitis B virus infection [13], and the long held suspicion of a haematogenous infection causing this condition, no infectious agent has yet been identified [2, 11]. Insect vectors have been suggested as an explanation of the summer and autumn peaks of disease [8].

Although not reported as a specific entity, the commonest form of hepatitis in horses seen by this author is associated with lymphocytic portal infiltrates *without* other hepatopathic findings such as necrosis, biliary hyperplasia and fibrosis. Such cases are seen sporadically or as outbreaks and have epidemiologic and histopathologic resemblance of viral hepatitides in other species although serologic examinations are usually unproductive. Caution should be exercised however when making a diagnosis of hepatitis on the basis of *mild* portal lymphocytic infiltrates as one study found such changes to be present in 7 of 12 (58%) liver specimens from normal horses [14]. Viral hepatitides are not well recognised in horses although equine herpesvirus 1 infection may result in severe hepatic necrosis in neonates [15] and sometimes hepatitis in older horses [16, 17]. Hepatic mononuclear phago-

cytes (Kupffer cells) are an important site of viral replication and latency in equine infectious anaemia virus infection and lymphoid hepatitis is characteristic of active infection [18, 19]. In a recent study 13% of Egyptian horses were found to be seropositive for human hepatitis E virus although there was no specific evidence of liver disease in these cases [20].

Bacterial hepatitis may arise haematogenously and is well recognised in association with neonatal septicaemia caused by several bacterial species [21]. Less commonly haematogenous bacterial hepatitis has been reported as part of a multisystemic infection with specific pathogens such as *Rhodococcus equi*, *Acinetobacter calcoaceticus*, *Ehrlichia risticii* and *Yersinia enterocolitica* [22–25]. Clostridial hepatitis is best recognised as sporadic cases or outbreaks of 'Tyzzer's disease' in foals between 1–7 weeks of age [26]. This acute bacterial hepatitis and necrosis is caused by *Clostridium piliforme* and a faecal-oral route of spread is suspected. The condition is frequently rapidly fatal but treatment can be successful [27]. A similarly severe acute clostridial hepatitis has also been reported in adult horses in association with *C. noyvi* and *C. haemolyticum* [28–32]. Liver abscesses are reported rarely in horses but may be caused by a wide variety of bacterial species including *Bacteroides fragilis*, *Corynebacterium pseudotuberculosis*, *E. coli*, *Staphylococcus aureus*, *Streptococcus equi* subsp. *equi* and subsp. *zooepidemicus* [3, 33–36].

Septic bacterial cholangiohepatitis in adult horses and foals is suspected to result from reflux of duodenal contents along the hepatic duct and is usually associated with Gram negative enteric bacteria such as *Actinobacillus equuli*, *Bacteroides vulgatus*, *Citrobacter* sp., *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella* sp. although Gram positive isolates such as *Streptococcus* sp. are also reported [8, 37–40]. Septic cholangiohepatitis has also been reported in association with hepatic neoplasia and trematode infestation [13, 41]. Biliary infection may provide favourable conditions comprising nidus formation and biliary stasis that predisposes to cholelithiasis. Occasional reports of vegetable matter found within choleliths also support this sequence of events [37]. Cholelithiasis cases appear to have geographic predisposition with more cases reported from the USA than other countries. As most equine choleliths are composed of calcium salts (bilirubinate and phosphate) [37] the practice of alfalfa feeding may have relevance to their aetiology. Stones as large as 12 cm diameter are reported and prognosis for recovery with medical or surgical management is poor [37].

Other organisms occasionally reported in association with hepatitis in horses include the protist *Pythium insidiosum* [42], protozoans including *Cycloposthium* sp., *Polymorphella ampulla* and *Sarcocystis* sp. [43, 44] and helminths such as *Echinococcus granulosus* (hydatid cysts) [45], *Fasciola hepatica* [46–48], *Fascioloides magna* [49] and *Schistosoma* sp. [50]. Other larval nematodes including *Parascaris equorum*, *Strongylus edentatus* and *S. equinus* may cause hepatitis during transit through the liver as part of their normal migratory and developmental lifecycle [51–52].

Chronic active hepatitis is a poorly understood condition characterised by persistent and progressive periportal inflammatory infiltrates, fibroplasia and biliary hyperplasia and is occasionally seen in adult horses [4, 13]. The aetiology of such cases is speculative. Some cases appear to develop from untreated or non-responsive cases of septic cholangiohepatitis or Theiler's disease [11] whereas others appear to be consistently lymphocytic without evidence of prior sepsis or necrosis. Chronic hepatotoxicity, viral infection and immune mediated disease have been proposed to explain some cases [8, 13].

Chronic hepatitis has been reported in several horses as part of a multisystemic infiltrative inflammatory disease process often additionally involving skin, lung, intestine and other organs. Multifocal granulomatous inflammation with or without evidence of Mycobacteria [53–59] and multisystemic eosinophilic epitheliotrophic disease (MEED) [57, 60–63] are the commonest forms of chronic multifocal inflammatory disease in horses. Cases usually present with signs of weight loss possibly in association with skin lesions. Hepatitis is more often discovered following serum biochemical analysis than on the basis of clinical signs.

Diagnosis

There are three fundamental aims of the investigation of cases of suspected liver disease: firstly, to differentiate subjects genuinely suffering from liver disease from those which are not; secondly, to determine the type of liver disease affecting the subject and therefore appropriate therapy; and thirdly, to differentiate those subjects which are likely to survive from those which are not.

Clinical signs

Signs often associated with hepatitis cases in horses include depression, weight loss, colic, pyrexia, anorexia, photosensitisation, neurological signs (see below), diarrhoea, jaundice, oedema, pruritus, epistaxis, polydipsia, polyuria, diffuse skin disease and coronitis [8, 11, 13, 64]. The presence of severe clinical signs of hepatic insufficiency has major prognostic relevance [64]. However, hepatic disease is more common than hepatic insufficiency and many cases of liver disease in horses are subclinical [7, 64–67]. In one study of suspected equine hepatopathy cases, 61 were confirmed by biopsy to have significant liver disease but only 17 of these (28%) were showing specific clinical signs of such [67]. Therefore absence of specific clinical signs in a subject suspected to be affected by hepatitis (e.g., on the basis of laboratory results) is of limited reassurance.

Hepatitides however, with associated systemic inflammatory response, may be more likely to be clinically perceptible prior to onset of hepatic

insufficiency than non-inflammatory hepatopathies. Some specific causes of hepatitis have a suggestive clinical presentation. Serum hepatitis causes acute hepatic insufficiency and has an acute presentation of marked depression often with neurologic signs [7]. Cholangiohepatitis cases with cholelith formation are also readily diagnosed clinically with the typical signs of colic, pyrexia and jaundice [37, 38]. Multisystemic inflammatory diseases are usually suspected as a result of their intestinal or skin signs rather than from hepatitis although laboratory evidence of liver disease in a horse with protein losing enteropathy and chronic dermatitis is strongly suggestive of this condition [57].

The syndrome of hepatic encephalopathy (HE) encompasses a wide variety of neurologic signs identified in subjects with hepatic insufficiency and portal-systemic bypass [68]. Signs of HE are usually sudden in onset whether the underlying hepatic disease is acute or chronic. Diffuse cerebral and brain stem dysfunction is most commonly recognised with depression, disorientation, circling, compulsive walking, head pressing, dyspnoea (laryngeal and pharyngeal paresis), blindness, yawning and coma being observed most frequently. Occasionally excitation including aggression and seizures are seen but these signs are far less frequent than signs of depressed neurologic function. Other rarer signs attributed to HE include pruritus, foot stamping, dysphagia and gastric impaction [3, 11, 64, 69, 70].

Haematology

Reports of horses with liver disease have found highly variable and non-specific haematological findings with neutrophilia and lymphopaenia being fairly common and both anaemia and erythrocytosis also reported [6, 11, 34, 71–73]. Neutrophilia is a common finding in bacterial hepatitis cases as part of the systemic inflammatory response although one study found leucocytosis and neutrophilia to be associated with severe non-inflammatory hepatopathies also [64]. Erythrocytosis was also relatively common in those cases with a worse clinical status probably secondary to dehydration [64]. Absolute erythrocytosis is recognised as a paraneoplastic syndrome in cases of hepatoblastoma and hepatocellular carcinoma in horses [74–76] although this has not been reported in hepatitis cases.

Serum biochemistry

Biochemical substances measured in the blood of suspected equine hepatitis cases can be subdivided into intracellular enzymes reflecting damaged liver cells and substances reflecting impaired liver function. Although very useful diagnostic and prognostic information is obtained from laboratory analysis of blood samples, no biochemical tests are absolutely accurate and

the clinician will sometimes be misled if absolute reliance is placed on such information [67]. Nevertheless increased serum enzyme concentrations are a reasonably reliable indicator of hepatic disease especially when corroborated by increases in several different enzymes. Furthermore abnormal liver function tests suggest hepatic insufficiency in a subject with increased serum enzyme concentrations [64, 67].

Serum enzymes

Several intracellular enzymes found in hepatocytes and biliary epithelia can be used to infer liver disease including alanine aminotransferase (ALT), alkaline phosphatase (AP), arginase, aspartate aminotransferase (AST), gamma glutamyltranferase (γGT), glutamate dehydrogenase (GLDH), iditol dehydrogenase and lactate dehydrogenase [13, 77–78] with AP, AST, γGT and GLDH being in the most common usage.

AP has been found to become elevated within 48 h of acute hepatic insult although significant elevations have not been consistently reported in acute [79, 80] or chronic [81–82] hepatopathies. Increased serum concentrations of AP may also be derived from intestine, inflammatory cells, bone and placenta [77]. AP has been shown however, to be superior to most other enzymes for prediction of prognosis in cases of liver disease [64, 83].

Although often considered to reflect chronic hepatopathies [3, 70, 82, 84], γGT has been shown to be a sensitive indicator of acute hepatic insult also [79, 80]. γGT and AST are probably the most sensitive indicators of hepatitis but, lack specificity [67]. In contrast to a generally high regard for γGT as the most clinically useful liver-derived enzyme in common usage [3, 6], cases are frequently seen with increased γGT in which liver biopsy fails to identify significant liver disease [67]. Conversely cases of hepatopathy with normal serum γGT concentrations are less commonly reported [65–67]. γGT was found to be prognostically useful only in subjects with markedly increased levels (>399 iu/l) [64, 83]. The pancreas and renal tubules are also rich sources of γGT although renally-derived γGT is widely accepted to appear in urine and not serum [85].

It has been suggested that relative differences in serum concentrations of liver derived enzymes may infer the underlying nature of the liver disease [11, 37]. Experimental studies have suggested a primarily biliary source for γGT, hepatocellular sources for ALT, AST, GLDH and IDH and both biliary and hepatocellular sources for AP [86–89]. Although the association of enzyme patterns with subtypes of hepatitis may be an imperfect generalisation, hepatocellular diseases such as serum hepatitis will usually be associated with similarly marked increases in all enzymes although cholangiohepatitis cases, especially with biliary obstruction, will show the greatest increases in γGT and AP [7, 37]. However, elevations in serum γGT have been reported in association with acute hepatocellular necrosis in the absence of

notable biliary insult and occasional cases of liver disease are seen where increasing concentrations of serum γGT may be noted despite evidence of improvement of the hepatopathy [79, 80]. These paradoxical findings have been explained as being consequences of biliary hyperplasia [11] and one study found a highly significant association between the severity of biliary hyperplasia and the serum concentrations of AP and γGT [64].

Although GLDH is considered to be liver-specific, this author has encountered many cases in which high GLDH concentrations were not supported by liver biopsy findings [67]. This might possibly be explained by histopathologically insignificant hepatic insults resulting in increased serum GLDH concentrations. The prognostic value of GLDH is disputed [3, 64].

Biochemical indicators of hepatic function

Further serum biochemical tests may provide evidence of adequate compensation *versus* insufficiency of hepatic function in suspected hepatitis cases and generally offer greater diagnostic and prognostic value than serum enzymes [64, 67]. The monitoring of substances that are usually extracted from serum by the liver (e.g., bile acids and bilirubin) or whose serum levels are maintained by a normally functioning liver (e.g., albumin, globulins and urea) form the basis for these 'functional' tests. Such parameters are likely to be more effective than serum enzymes in the differentiation of compensated hepatitis from decompensated hepatic failure. Those in most common usage are listed above but may additionally include various amino acids, ammonia, fibrinogen, glucose, coagulation tests (activated partial thromboplastin time [APTT] and prothrombin time [PT]) and half-life of plasma bromosulphthalene, indocyanine green and radionucleides [13, 77, 78].

Failure of normal extractive processes by damaged hepatocytes often leads to increased serum bile acid concentration and this is strongly advocated as an excellent diagnostic and prognostic indicator of hepatic insufficiency in the horse [3, 6, 64, 67, 89]. The main limitation of serum bile acid measurement is its insensitivity and early or mild hepatitis cases will usually have normal results [84]. In one study only 9 of 37 (24%) biopsy confirmed liver disease cases had increased serum bile acids [67]. Serum bile acid concentrations greater than 20 μmol/l are prognostically concerning in chronic hepatopathies [64] and cases with values above 100 μmol/l are invariably fatal. However, bile acids are less prognostically concerning in acute hepatitis cases and far higher levels (>150 μmol/l) are often seen in subjects that later recover.

Unconjugated (indirect) bilirubin is also extracted from plasma by functional hepatocytes and then conjugated prior to biliary excretion. Anorexia [90] and haemolysis [91, 92] are additional causes of unconjugated (indirect) hyperbilirubinaemia with values in anorexic subjects sometimes exceeding 100 μmol/l in the absence of liver disease [93]. The majority of equine liver

disease cases have normal or only moderate increases in serum bilirubin concentration [3, 67, 83] and the unconjugated fraction usually comprises >90% of the total [11] creating interpretive problems in anorexic subjects. Occasional cases of hepatitis in the horse in which serum conjugated bilirubin represents greater than 25% of total bilirubin are very likely to be suffering from obstruction of the biliary tract [37, 38].

Marked hypoalbuminaemia (<20 g/l) is rarely seen as a result of hepatitis in horses due to the long serum half life of albumin and the large reserve capacity of the liver [1, 3, 64, 67, 83, 94] although even mild to moderate hypoalbuminaemia (e.g., 20–30 g/l) suggests a guarded prognosis [1, 64, 95]. Serum albumin concentrations below 20 g/l are very rarely encountered even in severe hepatopathies and such low values should create a suspicion of a multisystemic process (e.g., MEED) and protein-losing enteropathy [57].

Hyperglobulinaemia is a very useful diagnostic and prognostic test in equine liver disease [64, 67] and may result from the systemic inflammatory response associated with hepatitis or perhaps from systemic immunostimulation by intestinal-derived antigenic material following loss of the protective barrier of hepatic mononuclear macrophages (Kupffer cells) [95]. Hyperglobulinaemia (>45 g/l) has been demonstrated to be superior to other serum biochemical parameters in predicting non-survival of liver disease cases [64].

The liver is the main site of detoxification of ammonia (NH_3) and its biotransformation into urea and this has been used to explain the association of low serum urea and hyperammonaemia with liver failure [5,11]. Although most cases of liver disease have normal serum urea concentrations [3, 67, 83], low serum urea levels have frequently been described in equine hepatopathies [11, 38]. One study questioned the above explanation of low serum urea by finding no significant association between serum urea and NH_3 concentrations [3]. Another study also found low serum creatinine to be associated with severe liver disease and proposed polydipsia and renal washout as a causal factor in hepatopathy cases with low urea and creatinine [64]. Whatever the mechanism, it is well established that low serum urea is associated with hepatic insufficiency and has prognostic relevance [64].

Hyperammonaemia is a fairly consistent finding in equine subjects with HE [3, 64, 96] although the association between plasma NH_3 and signs of HE was only of borderline significance in one study [64]. Another investigation found that although plasma NH_3 concentration was increased in nearly all cases showing signs of HE, the concentrations varied and did not correlate with severity of the disease [3]. Furthermore, occasional cases were found to have increased plasma NH_3 without showing signs of HE [3]. Additional doubts about the pathogenic role of NH_3 in HE have been raised by the contrasting effects of experimentally induced hyperammonaemia supporting the view that other factors are often involved in the pathogenesis of HE [13, 96]. Additional and sometimes interacting pathogenic roles are proposed for tumour necrosis factor-α, aromatic amino acids, manganese,

copper, phenols, benzodiazepine-like substances, mercaptans, short chain fatty acids, monoamines, neurosteroids, bilirubin and electrolytes [96–102]. An alternative explanation of the weak association between plasma NH_3 and HE is that the ability of NH_3 to cross the blood brain barrier is increased in subjects with HE [97, 99, 103–106]. One study identified a high concentration of NH_3 in the cerebrospinal fluid of a horse with HE [12]. Astrocytic glutamine synthesis from NH_3 is greatly increased in HE cases and the osmotic effect of increased glutamine is the likely ultimate cause of astrocyte swelling, a key pathological event in HE [98, 103, 106].

Hepatitis and acute phase proteins (APPs) have an unpredictable association. Most APPs are synthesised by the liver and subsequently decreased hepatic biosynthesis of fibrinogen has often been reported in association with equine hepatitides [1, 77, 78]. However, the systemic inflammatory response frequently seen in association with hepatic insufficiency (e.g., hyperglobulinaemia, neutrophilia, tumour necrosis factor-α), and more especially hepatitides, provides a possible explanation of the association between higher plasma fibrinogen and failure to survive found in one study of equine hepatic disease [64]. Bacterial hepatitides (septic chlolangitis, abscesses, etc.) are certainly expected to provoke significant hepatic APP synthesis and higher concentrations of fibrinogen (e.g. >6 g/l) or serum amyloid A (>200 mg/l) suggest a bacterial aetiology.

Theoretically, the effect of hepatic insufficiency on blood coagulation is hard to predict as the liver is the major site of synthesis of most procoagulant, anticoagulant and fibrinolytic proteins and platelet dysfunction is also reported [107]. However, laboratory measures of haemostasis (APTT and PT) are invariably prolonged in association with hepatic failure [107]. Although clinicopathologic evidence of coagulopathy is seen relatively frequently in severe hepatitis cases, clinical signs of bleeding are quite rare [3, 64, 83].

Hepatic insufficiency is associated with increased serum concentrations of methionine and aromatic aminoacids (tyrosine, tryptophan and phenylalanine) due to reduced hepatic clearance. A similar increase is not seen in branched chain aminoacids (valine, leucine and isoleucine) that continue to be metabolised in other tissues such as muscle [108]. The altered balance of serum aminoacids may contribute to the signs of HE by leading to increases in false neurotransmitters [96]. Measurement of serum amino acids does not appear to be popular in equine practice although it has a good evidence basis [109, 110].

Hepatic gluconeogenesis is the main mechanism maintaining fasting euglycaemia and hypoglycaemia is occasionally encountered in acute and terminal hepatitis cases presumably as a result of failure of this process [6]. However, this is far more likely to be encountered in foals than in adult horses. In most clinical cases normo- to hyper-glycaemia are more common perhaps as a result of a stress response to illness with acute insulin resistance [3, 6, 64, 83].

The ability of the liver to remove exogenous agents such as bromosulphthalene, indocyanine green and radiopharmaceuticals forms the basis for dynamic tests of liver function [41, 111, 112]. Logistics of dynamic testing, and lack of availability of testing facilities in comparison to easily measured alternatives (above) have limited their use and none are popular in equine practice.

Studies have established the prognostic importance of hepatic fibroplasia in horses [14] but, unlike human medicine, no serum markers of fibrosis have been investigated in equine hepatitis cases. Hyaluronan, a parameter reflecting hepatic fibrosis in humans [113, 114], has been measured successfully in horses in experimental studies of joint and lung diseases [115] and merits further investigation in the context of equine liver disease.

Ultrasonography

Except in rare cases of marked hepatic atrophy, a proportion of the equine liver is amenable to transcutaneous ultrasonography using 3.5 to 7.5 MHz transducers [116]. The normal liver is entirely within the boundaries of the ribcage and aerated lung poses the main ultrasonographic obstruction and significantly limits the value of the technique in horses. The lung margins represent the cranio-dorsal limits of the hepatic image on both sides. In almost all cases, the right hemithorax allows imaging of a greater quantity of liver, often between the 6th and 16th ribs, although there is considerable individual variation in the number of intercostal spaces *via* which an image can be obtained. The image of the right liver lobe is approximately triangular. A hyperechoic curvilinear image of the right colon (dorsal and ventral) is consistently imaged deep to the right liver lobe and a smaller, motile duodenum is frequently imaged on the medial surface between the liver and colon in the more caudal intercostal spaces (e.g., 13–15). On the left side liver can only usually be imaged *via* 2 or 3 intercostal spaces immediately caudal to the left ventricle. Care should be taken not to confuse the hepatic image with the adjacent spleen. The latter is more caudal, more echogenic and extends from the medial aspect of the liver cranially to the lateral abdominal wall caudally over many more intercostal spaces than the relatively small and hypoechoic liver.

Studies have established significant diagnostic and prognostic clinical usefulness of hepatic ultrasonography [64, 67, 116]. Images classified as abnormal have a high specificity for the presence of significant liver disease [67] and are associated with poorer clinical outcomes [64] although low sensitivity for detecting liver disease is the main limitation and many horses with normal ultrasonographic hepatic images will be confirmed to have significant liver disease in biopsy specimens [67]. Frequently suspected ultrasonographic abnormalities such as abnormal echogenicity, size and shape are subjectively assessed although cysts, choleliths, neoplasms, abscesses,

haematoma and hepatic rupture may be more objectively defined [11, 34, 37, 64, 116]. Increased hepatic echogenicity has been reported previously in cases of hepatic fibrosis, haemosiderosis and lipidosis and rounding of hepatic margins may suggest hepatomegaly [11, 64, 116]. In this authors view, the main value of hepatic ultrasonography is as an important adjunct to biopsy. Ultrasonographic guidance allows more careful selection of a biopsy site that probably improves the safety and diagnostic success of the biopsy procedure (see below).

Laparoscopy

Rarely hepatopathy cases are seen in which no liver tissue can be imaged ultrasonographically and laparoscopy can then be used to visualise the liver and guide biopsy collection. This has proven very valuable in several cases seen by this author. The reader is referred to surgical texts for further description of the technique [117].

Liver biopsy

In the absence of non-invasive tests that can reliably distinguish horses with significant liver disease from those without evidence of significant liver disease [64], histopathological examination of liver biopsies has become established as the *ante mortem* test of greatest value in common usage [14, 67]. Histopathologic assessment of biopsy specimens is of great prognostic value and guides the choice of specific therapy in individual cases. It is also the diagnostic technique most likely to provide a specific diagnosis and aetiologic information. Additionally, given the lack of absolute reliability of serum biochemical data when monitoring response to treatment in hepatitis cases, repeat biopsy may be the best technique to establish adequate recovery and, indeed, the presence or absence of further secondary changes following initial biopsy.

Liver biopsy is an easy, safe and highly informative diagnostic procedure in cases of suspected hepatitis [14, 118]. Although adverse effects such as haemorrhage, colic, peritonitis, pleuritis and pneumothorax are all reported [118], these problems are very rarely encountered. The importance and usefulness of the information obtained by biopsy invariably outweighs the associated risks in this author's view, especially when the technique is performed under ultrasonographic guidance. Although many recommend pre-biopsy coagulation assessment [2, 6, 119], the requirement for this is questionable [11]. One study reported signs of intra-abdominal haemorrhage at autopsy following liver biopsy in three horses with prolonged clotting times although quantity and associated clinical signs were not described [6]. A further report described a single case of post-biopsy haemorrhage out of 27 liver biopsies in

horses. The case in question was known to have markedly prolonged PT but clinical signs of the haemorrhage or outcome were again not described [2]. Many further reports exist describing liver biopsies performed in horses with both subclinical and clinical coagulopathies without subsequent clinical signs of haemorrhage [3, 6, 14, 72]. No complications were reported following liver biopsies in more than 200 horses and ruminants [120] and not a single case of clinically significant haemorrhage has been seen by this author following more than 300 liver biopsy procedures. Other clinicians have also described the risks of liver biopsy in the horse as minimal [11]. The only adverse effects of liver biopsy observed by this author were two horses that showed low grade abdominal pain following biopsy that was immediately responsive to phenylbutazone. It is now this author's routine practice to administer 1–2 g phenylbutazone iv prior to the biopsy procedure.

Most ultrasonographic transducers can be fitted with a needle guide that fixes the biopsy needle in the plane of the ultrasonographic image so that progress of the biopsy needle can be observed in real time. This author usually uses a 5 MHz transducer although occasionally 3.5 or 7.5 MHz transducers may be used depending on body size and subcutaneous fat depth. A suitable site for biopsy is chosen ultrasonographically on the basis of reasonable target size and absence of large vessels. Although the right side is usually chosen, left sided biopsies are sometimes collected. Routine sedation is performed with detomidine (1 mg/100 kg iv) and butorphanol (1–2 mg/100 kg iv) and the chosen site is prepared surgically and infiltrated with local anaesthetic. A small stab incision in the skin facilitates the procedure. Sterile coupling gel or alcohol is applied to the area and a 14 gauge 16–20 cm semi-automatic spring-loaded biopsy needle is fitted to the biopsy guide and introduced through the stab incision and into the liver. Most biopsies are collected at a depth of 5–10 cm from the skin surface. It is important not to allow even mild bending of the needle as this will prevent the normal firing mechanism from cutting a biopsy specimen. At least two biopsies are usually collected. One is placed in formol saline for histopathology and the other in a sterile container for bacteriologic culture, although the latter is often unsuccessful even when a bacterial aetiology is suspected.

Determination of the presence or absence of disease is the most fundamental question that may be answered following examination of liver biopsy specimens but this requires clear histopathologic criteria with which to distinguish diseased from normal livers. Descriptions of microscopical structure of healthy and diseased livers can be found in histology and pathology texts [121, 122] although in practice the distinction may be less clear. One study examined liver specimens from 12 normal horses and found that 7/12 (58%) had up to five leucocytes (primarily lymphocytes) in representative portal tracts [14]. Significant hepatitis would thus be defined by the presence of >5 inflammatory cells per portal tract, and possibly in addition secondary changes including any degree of fibroplasia, biliary hyperplasia or necrosis; haemosiderosis affecting >50% of hepatocytes; and hydropic degeneration,

Table 2. Summary of scores assigned to the severity of five histopathological criteria contributing to a total prognostic liver biopsy score (minimum score = 0, maximum score = 14) (Irreversible cytopathology = necrosis, megalocytosis or amyloidosis) [14].

	severity		
	mild	moderate	severe
Fibrosis	–	2	4
Irreversible cytopathology	1	2	2
Inflammatory infiltrate	–	1	2
Haemosiderin accumulation	–	–	2
Biliary hyperplasia	–	2	4

cloudy change or cytoplasmic granularity of hepatocytes affecting >25% of a representative lobule [14].

A potential major cause of insensitivity of liver biopsy is the collection of an unrepresentative specimen of liver. This is illustrated, for example, in reports of eight cases of hepatic neoplasia out of which only three showed evidence of tumour deposits in liver biopsy specimens [6, 41, 75, 123], and one was initially misdiagnosed as cholangiohepatitis [41]. However, this is not a common scenario and others have found that almost all serious equine hepatic disease affects the liver diffusely and the correlation between histopathologic findings in biopsy specimens and subsequent autopsy material is excellent [2, 6].

Fibrosis is a common and non-specific response to liver injury [122] and although usually associated with chronic hepatopathies, fibrosis has been detected as soon as 7 days following acute hepatic insults in horses [124]. Marked periportal and bridging fibrosis identified in biopsy specimens have long been regarded as poor prognostic indicators [37, 125] and a more recent study confirmed fibrosis as the single most prognostically relevant histopathologic variable [14]. However, in a published series of cholangiohepatitis cases, long-term survival of three horses found to have severe periportal and bridging fibrosis was described suggesting that other factors are also prognostically important [38]. A scoring system was therefore developed in order to attempt to attribute a prognostically useful broad index of histopathologic severity comprising several histopathologic variables comprising fibrosis, biliary hyperplasia, megalocytosis, necrosis, amyloidosis, inflammatory infiltrates and haemosiderosis [14]. Application of this prognostic biopsy score (Tab. 2) indicated a very good prognosis with scores of 0–1 (96% survival), a guarded prognosis with scores of 2–6 (67% survival) and a poor prognosis with scores of 7–14 (14% survival) [14]. Biopsies scored in the highest category (>6/14) were associated with a 46 times increased risk of death within 6 months representing a far higher risk factor than any non-invasive test results [14, 64].

Differential diagnoses

Hepatitis cases rarely present with absolutely specific clinical signs and therefore differential diagnoses should always be considered. The main presenting signs of hepatitis in horses are non-specific and comprise colic, depression, neurologic signs and weight loss [8, 11, 13, 64]. Colic (and diarrhoea) is a common clinical sign of both enteritides and hepatitides and pyrexia may also be a feature of both. Differential diagnoses of acute central neurologic signs [126] primarily comprise hepatic failure, encephalitides [127], neurotoxins (e.g., moxidectin, fluphenazine) [128, 129], trauma [130] and primary intestinal hyperammonaemia [131, 132]. Clinical signs commonly observed that are more suggestive of hepatitis include photosensitisation and jaundice although in practice most horses seen with these signs will have normal livers with primary photodermatitis and anorexia respectively being the commonest causes of these signs.

In most cases serum biochemistry will provide the strongest initial evidence of hepatitis although cases of primary intestinal disease are sometimes seen with mild to moderate increases in liver derived enzymes [133–137]. This could be caused by hepatic portal transfer of noxious substances such as lipopolysaccharides from compromised intestinal mucosae or perhaps from pressure from a dilated stomach or colon. Additional confusion may arise in some cases as a result of lack of absolute sensitivity and specificity of laboratory indices of hepatitis [67] and biopsy will be required in order to make a certain diagnosis of hepatitis. This author has seen several subjects with acute neurologic signs caused by HE that were misdiagnosed as traumatic cranial injuries on the basis of evidence of external skin abrasions that actually resulted from falls caused by HE.

Treatment

General and supportive care can be supplied for any subject suspected to be suffering from hepatitis including fluid therapy and antipyretics but specific therapy cannot be logically applied in the absence of a clear specific diagnosis. Although biopsy is usually a prerequisite, certain conditions such as serum hepatitis, cholelithiasis and Tyzzer's disease may be strongly suspected on the basis of history, clinical signs, ultrasonography and serum biochemistry. In practice many hepatitis cases may not be clearly representative of the clear categorical aetiologic diagnoses described above even following biopsy [4]. Nevertheless intuitively indicated therapy can be applied such as antibacterials in cases of predominantly neutrophilic hepatitides or glucocorticoids in cases of predominantly lymphocytic, eosinophilic or plasmacytic hepatitides. Surgical management of some conditions has been reported [138] although the majority of equine hepatitides will be managed medically.

Antimicrobial drugs are indicated in all cases of bacterial hepatitis (e.g., cholangiohepatitis, cholelithiasis, hepatic abscessation and Tyzzer's disease [27, 34, 38]. Bacterial isolation and sensitivity testing is ideal although will introduce a delay in therapy and may often be unproductive or potentially misleading when biopsy specimens are submitted for culture [38]. Intuitive prediction of indicated spectra of activity may be appropriate in many cases. Clostridial hepatitis (e.g., Tyzzer's disease) should be treated aggressively with sodium benzyl penicillin (50,000 iu/kg iv *qid*) or oxytetracycline (10 mg/kg iv *bid*). Suppurative hepatitis in adult horses usually involves enteric bacteria and therefore Gram negative potency is important. However, anaerobic and Gram positive pathogens are well recognised suggesting that broad spectrum cover is advisable. Prolonged antibacterial therapy in excess of 4 weeks is usually required in cholangiohepatitis cases [38] which often precludes parenteral therapy and restricts the choice of available products. Potentiated sulphonamides (30 mg/kg combined product *per os bid*), doxycycline (10 mg/kg *per os sid-bid*) and rifampin (5 mg/kg *per os bid*) have broad spectrum activity although the latter drug should only be used in combination with another antibacterial. Erythromycin (25 mg/kg *per os tid*) has been used successfully in adult horses although may pose an unacceptable risk of colitis [139]. Enrofloxacin (7.5 mg/kg *per os Sid*) has limited Gram positive and negligible anaerobic activity, whereas the activity of metronidazole (15 mg/kg *per os* loading dose followed by 7.5 mg/kg *per os qid*) is restricted to anaerobic pathogens only.

Liver fluke (*Fasciola hepatica*) is a rare cause of hepatitis in horses and donkeys but has been treated successfully with triclabendazole at a dose of 12 mg/kg *per os* [47, 48]. *Echinococcus granulosus* (hydatid) cysts are more frequently encountered but their pathogenic relevance is often questionable unless large numbers are present or intervening hepatic parenchyma is shown to be diseased. This author has treated such cases with albendazole 10 mg/kg *per os sid* for 7 days repeated six times at 2-week intervals [140] and also by cyst aspiration and injection of sterile saturated saline into the cyst cavity.

Glucocorticoids are indicated in cases of eosinophilic or lymphoplasmacytic hepatitis and for hepatitides with significant fibroplasia (e.g., chronic active hepatitis). Prednisolone at a dose of 1–2 mg/kg *per os sid* for at least 4 weeks is usually chosen although more aggressive therapy with dexamethasone at 0.05 to 0.1 mg/kg iv *sid* or on alternate days may be indicated in more severe or non-responsive cases. Despite common anecdotally held views to the contrary, hepatic fibrosis is a potentially reversible change and exists as a dynamic balance of fibroplasia and fibrinolysis controlled by hepatic stellate cells (HSCs) [141]. The antiinflammatory effect of glucocorticoids on activated HSCs is the only commonly applied antifibrotic strategy in equine hepatitis cases. Non-steroidal antiinflammatory drugs are only usually used in pyrexic hepatitis cases such as septic cholangiohepatitis and cholelithiasis (e.g., Flunixin meglumine 1 mg/kg *sid-bid*) although benefits in cases of HE have been proposed [142].

Several previous studies have reported that HE is a poor prognostic sign in horses [3, 6, 64, 110]. However, the finding that 40% of horses showing signs of HE did survive for at least 6 months in one study [64] does justify attempts at treatment especially in cases of acute hepatitis and decompensation. Occasionally instances of excitable or aggressive HE warrants sedation with slightly reduced doses of commonly used sedatives and tranquilisers (e.g., Xylazine 0.5 mg/kg iv; Detomidine 10 μg/kg iv; Acepromazine 25 μg/kg iv). Diazepam may be contraindicated due to the proposed involvement of benzodiazepine-like substances in the pathophysiology of HE [96].

There is little evidence basis for the selection of therapeutic agents for treatment of HE and selection is largely based on personal anecdotal experience. This author generally favours lactulose (0.3 ml/kg *per os*) which can be given initially every 2–4 h in HE cases and reduced in frequency according to response. Diarrhoea is a rare complication. Failure to clinically improve within 12–24 h of lactulose treatment reflects a poor prognosis in chronic hepatitis cases although subjects with acute hepatitis and HE may improve more slowly. Lactulose is a non-absorbable, indigestible disaccharide that is metabolised by colonic bacteria to acetic and lactic acids, thus reducing luminal pH. This colonic acidification is associated with reduced absorption of ionised ammonium, suppression of faecal urease and proteases, increased bacterial NH_3 utilisation and death of many proteolytic bacteria [99, 143]. The authors of one study suggested that oral antibiotics had a subjectively better influence on HE than lactulose although long-term therapy is less suitable with antibacterials than lactulose [3]. Neomycin (15 mg/kg *per os qid*) or metronidazole (15 mg/kg *per os* loading dose followed by 7.5 mg/kg *per os qid*) have both been widely used in equine HE cases either alone or in combination with lactulose. Other products such as L-ornithine-L-aspartate may be worthy of investigation in equine HE cases. This product reduces plasma NH_3 concentration by promoting biotransformation into urea and glutamine in other tissues such as intestine, muscle and kidney and has a good evidence basis in human medicine [106, 144].

Dietary management

Subjects suffering from hepatitis and hepatic insufficiency may benefit from specific nutritional advice. Weight loss is a common feature of hepatic insufficiency and affected subjects tend to catabolise bodily protein more readily than healthy subjects [145, 146]. Adequate dietary digestible energy intake (>125 kJ/kg/day [147]) is essential but may be hard to maintain in anorectic subjects. Balanced parenteral nutrition has been successfully supplied by this author to horses with acute hepatic failure comprising equal volumes of 50% glucose, 22% aminoacids (Intrafusin 22, Fresenius Kabi) and 20% lipid emulsion (Intralipid 20, Fresenius Kabi) at a rate of 0.5 ml/kg/hour. Excess dietary protein might increase ammoniagenesis and precipitate HE whereas

inadequate provision of dietary protein potentiates further catabolism [148]. Probable optimal protein content will approximate 1–1.5 g/kg/day but will depend considerably on protein quality [147]. Grass or good quality grass hay should form the basis of hepatitis diets. Supplementary feeds can be offered in at least 4–6 daily meals [11]. A low AAA:BCAA ratio in the diet or a BCAA supplement may be offered [143, 145]. Wheat, soya, beet pulp and oats are all relatively rich in AAAs whereas alfalfa, wheat bran, maize and milo (sorghum) have a relatively high BCAA content [11, 149]. Adequate provision of dietary glucose may compensate for reduced hepatic glycogen synthesis and storage [145, 150] and limit gluconeogenesis from endogenous proteins. Processed maize and perhaps molasses may provide good readily digestible carbohydrate for this purpose [11].

Despite the popularity of high fat diets for the safe provision of additional dietary calories in equine diets, this may be inadvisable in subjects with hepatic insufficiency due to the central role of the liver in triaglycerol processing and bile synthesis. Vitamin supplementation including B vitamins, D, E and K [149–151] is widely advocated for subjects with hepatic insufficiency and is of anecdotal benefit especially in subjects with poor appetite. Zinc supplementation has also been advocated [145, 150]. The almost universal inclusion of iron in equine multivitamin supplements could be harmful to a failing liver as haemosiderosis is a common finding in diseased livers in horses and may present a significant oxidative challenge [14]. Copper, manganese and vitamin A are also potentially hepatotoxic [145] and this author has recognised increased manganese accumulation in horses with hepatic insufficiency. Lack of safety studies of many proprietary feed supplements and herbal remedies is of potential concern with several known to have hepatotoxic properties including germander, gentian, *Asafoetida*, mistletoe, senna, chaparral, valerian, comfrey, pennyroyal, *Dictamnus* and *Paeonia* [152, 153].

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The woodchuck model of hepadnavirus infection

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Abstract

Since discovery of the hepatitis B virus (HBV), closely related viruses have been described in several animal species. The first of these was the woodchuck hepatitis virus (WHV), identified in woodchucks (*Marmota monax*) that were maintained at the Philadelphia Zoological Garden and which had experienced a high prevalence of chronic hepatitis and hepatocellular carcinoma (HCC). On the basis of morphological and molecular analyses, it was concluded that WHV was closely related to HBV. Since description of WHV, infection with viruses that belong to the family Hepadnaviridae have been described in the California ground squirrel (*Spermophilus beecheyi*) and the Arctic ground squirrel (*Spermophilus parryi*), two species closely related phylogenetically to woodchucks, and in six avian species. A total of four well characterized, mammalian hepadnaviruses now have been associated with development of HCC. These observations on naturally acquired hepadnavirus infections combined with the development of HCC in woodchucks following experimental infection with WHV or with the California ground squirrel virus (GSHV) provides, by analogy, convincing comparative medical evidence for the hepatocarcinogenicity of HBV. The woodchuck has become useful as an experimental animal model for research on the pathogenesis of HBV infection and for investigation of the molecular mechanisms of hepatocarcinogenesis. The woodchuck also has been useful in the discovery and preclinical development of antiviral drugs for treatment of HBV infection and for testing new forms of immunotherapy using cytokines and therapeutic vaccination. In particular, the woodchuck has been valuable for determining the impact of long-term antiviral treatment on the outcome of chronic hepadnavirus infection in placebo controlled, lifetime survival studies which have been predictive of the results of subsequent clinical trials.

Introduction

Identification of the virus of hepatitis B (HBV) was one the most important medical advances of the 20th Century and has led to significant advances in the diagnosis, treatment, control and prevention of one of the world's most widespread and devastating infectious diseases. Since discovery of HBV,

closely related viruses have been described in several mammalian and avian species. Prof. Heinz Schaller, to whom this volume is dedicated, has utilized naturally occurring animal models to investigate the pathogenesis of HBV infection and the mechanisms of HBV replication. In so doing, he has in significant ways enhanced our understanding of the pathogenesis of HBV infection, has improved the care of patients infected with HBV, and has trained some of the most distinguished members of the current generation of hepatitis virologists. His research with animal model systems represents an impressive contribution to modern comparative medicine.

Natural history of woodchuck hepatitis virus infection

The woodchuck hepatitis virus (WHV) was described originally by Summers and colleagues in a colony of woodchucks (*Marmota monax*) maintained at the Philadelphia Zoological Garden and that for some years had experienced an unusually high rate of chronic hepatitis and hepatocellular carcinoma (HCC) [1]. On the basis of morphological and molecular analyses, it was concluded that WHV was closely related to the hepatitis B virus (HBV) family of viruses and the woodchuck hepatitis virus (WHV) now is classified as a member of the family Hepadnaviridae, genus *Orthohepadnavirus* of which the prototype member is HBV (Tab. 1).

The natural habitat of the woodchuck extends from north Georgia, Alabama, and Mississippi in the southern United States, west to Oklahoma, Kansas, Nebraska, and Iowa, north to Quebec and Labrador, across Canada to British Columbia and the Yukon Territory, and includes an area of southeastern Alaska. A comprehensive, seroepidemiologic study of WHV infection throughout this range has never been performed. WHV infection is known to be hyperendemic in the mid-Atlantic region of the United States and the woodchucks studied originally by Summers et al. were from Pennsylvania [1]. In subsequent studies reported by Tyler et al. [2], 23% of the woodchucks from Pennsylvania, New Jersey, and Maryland tested positive for the WHV surface antigen (WHsAg), and 36% were positive for antibody to the WHV surface antigen (anti-WHs) for a 59% overall rate of infection. Similar high rates of WHV infection have been confirmed by others in woodchucks from the Mid-Atlantic States [3]. In contrast, the prevalence of WHV infection in central New York State is approximately 2% based on the detection of anti-WHs antibody [3] and the rate of persistent WHs antigenemia is estimated to be less than 0.2%. Although the number of woodchucks tested is small, no serologic evidence of WHV infection has been found in Vermont and Massachusetts [4–6]. Similarly, we have found woodchucks from Iowa to be anti-WH core antibody (anti-WHc) negative. We have, however, examined hepatic tissues of woodchucks from the Province of Quebec and Ohio that died of hepatocellular carcinoma (HCC). Non-neoplastic liver tissue was examined immunohistologically for the

Table 1. Hepadnaviruses of animals

Virus	Host	Scientific Name
Genus: Orthohepadnavirus		
Hepatitis B virus (HBV)*	Human	*Homo sapiens*
Woodchuck hepatitis virus (WHV)	Woodchuck, groundhog	*Marmota monax*
California ground squirrel hepatitis virus (GSHV)	California ground squirrel	*Spermophilus beecheyi*
Arctic ground squirrel hepatitis virus (AGSHV)	Arctic ground squirrel	*Spermophilus parryii*
Woolly monkey hepatitis B virus (WMHBV)	Woolly monkey	*Lagothrix labotricha*
Genus: Avihepadnavirus		
Duck hepatitis B virus (DHBV)	Domestic duck, Pekin duck	*Anus domesticus*
Heron hepatitis B virus (HHBV)	Grey heron	*Ardea cineria*
Snow goose hepatitis B virus (SGHBV)	Snow goose	*Anser caerulescens*
Ross's goose hepatitis B virus (RGHBV)	Ross's goose	*Anser (Chen) rossii*
Stork hepatitis B virus (SHBV	White stork	*Ciconia ciconia*
Crane hepatitis B virus (CHBV)	Grey crowned crane and Demoiselle crane	*Balearica regulorum* and *Anthropoides virgo*

*Naturally acquired infection with HBV also has been detected in the chimpanzee, gorilla, gibbon, and orangutan

presence of WH core antigen (WHcAg) and both were positive indicating with reasonable certainty that at the time of death both woodchucks were infected with WHV (unpublished).

Hepatic neoplasms of woodchucks from the Philadelphia Zoological Garden were reported early in the 20th Century. Multiple hepatic adenomas ranging in size up to 1.0 cm in diameter were described [7, 8]. Later, two woodchucks, one from the Washington Zoological Park and the other trapped in Bethesda, Maryland, were reported with primary hepatic neoplasms [9]. Woodchucks that originated in New York [10], Maryland [11] and Pennsylvania [12, 13] and maintained in a laboratory environment later were reported with primary hepatic neoplasms. The woodchucks from Pennsylvania were the harbingers for discovery of WHV [12, 13].

Neoplasms of the liver associated with naturally acquired WHV infection were well-differentiated, trabecular HCCs [14–16]. Chronic hepatitis was present and characterized by abundant mononuclear cell infiltration of portal tracts, sometimes with extension beyond the limiting plate. Scattered hepatocellular necrosis of parenchymal hepatocytes and modest bile duct proliferation often were observed, and in some there was evidence of early fibrosis [16–17]. Hepatic cirrhosis, however, was not characteristic and is a notable difference between the livers of HBV infected humans and WHV infected woodchucks with advanced liver disease. Some HCCs contained

significant numbers of infiltrating hematopoietic cells and progression of neoplasia from foci of altered hepatocytes to small neoplastic nodules and to frank HCC was recognized [16]. Metastasis of HCC outside the liver, which occurs in humans and other experimental animal models with some frequency, has not been observed in woodchucks with HCC by most investigators although pulmonary metastases have been reported [17].

Other naturally occurring hepadnavirus infections

Hepadnaviruses closely related to HBV and WHV have been reported in other mammalian and avian species (Tab. 1) and the morphology and genetic organization of these hepadnaviruses are similar [18–20]. California ground squirrels (*Spermophilus beecheyi*) are the host species of the ground squirrel hepatitis virus (GSHV) [21–23]. Chronic GSHV infection is associated with chronic hepatitis and with HCC, although the frequency of HCC is remarkably less than that associated with chronic WHV infection and develops in much older animals [21, 23–26].

The arctic ground squirrel hepatitis virus (AGSHV) has been reported in the arctic ground squirrel (*Spermophilus parryi*) and infection also was associated with HCC [27]. Infection with a putative hepadnavirus was described in Eastern gray squirrels (*Spermophilus carolinensis*) from the State of Pennsylvania [28] in which lesions of hepatitis were observed but hepatic tumors were not reported. Hepadnavirus infection in Richardson's ground squirrels (*Spermophilus richardsonii*) originating in the Province of Alberta has been reported [29, 30].

The hepatic lesions including HCC in Richardson's ground squirrels were similar to those described in woodchucks, California ground squirrels, and Arctic ground squirrels [30].

Duck hepatitis B virus (DHBV) has been reported in domestic Pekin ducks (*Anas domesticus*) and has a worldwide distribution [31–33]. Similar avian hepadnaviruses also have been described in grey herons (*Ardea cinerea*, heron hepatitis B virus) [34] and in snow geese (*Anser caerulescens*) [35]. Much of the current understanding of hepadnavirus replication is based on research using DHBV *in vivo* and *in vitro* [28, 36]. HCC has been infrequently associated with DHBV infection [32] and the hepatocarcinogenicity of DHBV in ducks remains questionable. Hepatic neoplasms have not been associated with heron hepatitis B virus infection [34].

Experimental woodchuck hepatitis virus infection

Woodchucks with naturally acquired WHV infection were valuable sources of virus and hepatic tissue for histological and molecular analyses. Their use experimentally, however, was limited because it was impossible to know

when and for how long trapped woodchucks had been infected with WHV or to know the nutritional history or their exposure to other environmental factors that might influence the outcome of WHV infection. Importantly, hepatic lesions caused by nematodes such as *Ackertia marmotae* and *Capillaria* sp. were common in wild woodchucks [37] and could complicate the interpretation of experimental results.

Summers and colleagues [19] described the experimental infection of 4- to 8-month old woodchucks with serum from chronic WHV carriers. They described productive, self-limited infection, but no woodchucks became chronic WHV carriers. Attempts by others to experimentally infect juvenile or adult woodchucks also resulted in acute WHV infection [3, 38, 39] but, with one exception, did not cause chronic infection.

Morphologic and molecular virological studies of the liver of woodchucks experimentally infected with WHV have shown that virtually 100% of hepatocytes become infected following experimental WHV infection [40]. Although replicative forms were cleared rapidly during recovery, covalently closed circular WHV DNA persisted in three of 10 woodchucks after evidence of WHV replication had ceased [40]. Clearance of experimental WHV infection in adult woodchucks was associated with robust humoral and cell-mediated immune responses [41–43]. Treatment with immune suppressive doses of cyclosporine A significantly increased the rate of chronicity following experimental WHV infection of adult woodchucks [44, 45].

When adult humans are infected with HBV, less than 5% become chronic carriers [46, 47]. HBV infection early in life, however, results in characteristically high rates of chronic infection [18, 48]. High rates of chronic WHV infection have been observed in woodchucks from hyperendemic areas [2, 14, 16] and suggested that, as in humans, infection of woodchucks early in life (or possibly vertical transmission) must be necessary to account for the high rates of persistent WHV infection observed.

To fully utilize the woodchuck as an experimental animal model, it was considered essential to breed and to rear woodchucks in a laboratory animal facility. This would provide woodchucks of known genetic background, a defined environmental and nutritional history, and in which the diseases of wild woodchucks including WHV infection could be controlled and prevented. A breeding colony of woodchucks that were negative for serologic markers of WHV infection was established at Cornell University in 1979. The colony has served as the source of woodchucks for experimental studies of the pathogenesis of WHV infection and hepatocarcinogenesis and more recently for the preclinical development of antiviral drugs and immunotherapeutic strategies for HBV infection. Woodchucks born in the laboratory are inoculated at birth with diluted serum from standardized infectious pools obtained from chronic WHV carriers [49–52]. After inoculation, woodchucks are monitored using specific serological markers of WHV infection (WHV DNA, WHsAg, anti-WH core antibody, and anti-WH surface antibody) [3, 53].

The rate of chronic WHV infection after neonatal inoculation is 60% or greater [49–52]. Survival of experimentally infected chronic WHV carriers was compared to that of woodchucks that recovered from neonatal WHV infection by clearing WH viremia and developing anti-WHs antibody, and of control woodchucks not infected with WHV but that were born and reared in a similarly controlled laboratory environment. All WHV carriers were dead by 56 months of age, and the lifetime risk of HCC was 100% [51, 52, 54]. The median time to death from HCC in WHV carriers was approximately 30 months. In contrast, 42% of the woodchucks with resolved WHV infection and 62% of uninfected controls were alive at 56 months of age. Although the rate of HCC in WHV carriers was significantly higher, 17% of woodchucks that recovered from neonatal WHV infection developed HCC [51, 54]. HCC was not observed in uninfected, laboratory-reared, control woodchucks.

HCC associated with experimental WHV infection was comparable to that of woodchucks with naturally acquired chronic WHV infection [14, 16, 17]. The presence of preneoplastic foci of altered hepatocytes [55, 56] and progressive aneuploid change [57, 58] also was similar. These results provide direct experimental evidence for the carcinogenicity of WHV and, by analogy, for that of other mammalian hepadnaviruses (HBV, GSHV, and AGSHV) in which naturally acquired infection has been associated with HCC.

The prevalence of HCC in humans is higher in men than in women. In Asia, where HBV infection is hyperendemic, the ratio may be as high as 4 or 5:1. The explanation for the difference is not fully understood but men have higher rates of HBV infection, and are known to have greater use of alcohol and tobacco than women [59, 60]. In experimental rodent models, higher rates of HCC also are consistently observed in males. Hormonal factors are critical in determining the male predilection to diethylnitrosamine (DEN) induced HCC because castration or administration of estrogen to male mice diminishes the development of HCC significantly [61]. Recent studies of Karin and his associates have extended our understanding of the genetic and hormonal factors that are important in hepatocarcinogenesis and have delineated the molecular mechanism responsible for the prophylactic benefit of estrogen [62]. They observed that DEN administration caused greater increases in the serum concentration of interleukin-6 (IL-6) in males than in females and ablation of the IL-6 gene eliminated the differences between genders in hepatocarcinogenesis. DEN exposure promoted production of IL-6 in Kupffer cells *via* the Toll-like receptor adaptor protein, MyD88, and ablation of MyD88 protected male mice against DEN induced HCC. Estrogen reduced plasma IL-6 in DEN-treated male mice and inhibited production of IL-6 by Kupffer cells, suggesting that reduced IL-6 production by Kupffer cells was critical in reducing the risk of HCC in females. These

authors suggested their findings might be translated for use in the prevention of HCC in high risk individuals presumably in combination with other strategies such as antiviral drug therapy to inhibit viral replication, prevent chronic hepatitis and decrease the risk of cirrhosis.

In woodchucks, gender does not influence either the rate of chronic WHV infection or the occurrence of HCC in chronic WHV carriers. This result was unexpected because of the experience in humans infected with HBV and in laboratory rodents just described. The explanation may relate to the unusual circannual reproductive cycle of the woodchuck. For at least 8 months of the year, the testicles of male woodchucks are abdominal and produce little or no testosterone resulting in functional castration [63].

Immune-mediated glomerulonephritis has been reported in woodchucks with experimentally induced chronic WHV infection. Affected woodchucks may develop severe protein losing nephropathy and signs similar to the nephrotic syndrome of humans. Such woodchucks develop hypoalbuminemia and generalitic peripheral edema. This is the only disease of woodchucks caused by WHV that occurs outside the liver [64].

Full-length clones of the genomes of HBV [65], WHV [66], DHBV [67], and GSHV [68] have been shown to produce productive infection by direct injection of plasmid DNA into the hepatic parenchyma of the respective host species. During transfection experiments with chimeras of GSHV/WHV genomes to determine the viral gene or genes responsible for restriction of host range, it was discovered that woodchucks were fully susceptible to infection with GSHV [69]. GSHV had been shown earlier to infect the chipmunk (*Eutamias* species) [70]. As described above, California ground squirrels that are chronically infected with GSHV develop HCC less frequently than woodchucks chronically infected with WHV [21, 23–25] and HCC develops in California ground squirrels at an older age [26]. Because woodchucks could be infected with both WHV and GSHV, it was possible to determine if the apparent difference in oncogenicity of GSHV and WHV in their respective natural hosts was the result of differences in host response or were related to differences in viral genetics.

When neonatal woodchucks were experimentally infected either with WHV or GSHV, the rates of chronic infection were similar [71]. By 2 years of age, however, hepatic neoplasms of various sizes were observed at laparotomy in 13 of 16 chronic WHV carriers. At the same age, only one of 16 chronic GSHV carriers had developed a grossly identifiable hepatic neoplasm, a 5 mm diameter nodule classified histologically as a hepatic adenoma. It was concluded, based on these observations, that the differences in oncogenic capacity of GSHV and WHV that were recognized in their respective natural host species were attributable to viral genetic differences between the viruses, although differences in host responses could not be excluded.

Hepatocarcinogenesis associated with hepadnavirus infection

Three categories of evidence indicate that HBV is an etiologic factor in hepatocarcinogenesis. First, seroepidemiologic evidence has demonstrated a close relation between the prevalence of infection with HBV and the occurrence of HCC [72, 73]. Significantly higher rates of HCC are observed in individuals with chronic HBV infection than in case controls in the hyperendemic regions of Africa [74–76] and Asia [77–80]. In the United States and Great Britain where the rates of HBV infection and HCC are relatively low, a similar relationship between HBV infection and HCC was observed [81–83]. Compelling epidemiologic evidence for the role of HBV in hepatocarcinogenesis comes from the epidemiological studies of Beasley and colleagues in Taiwan who prospectively demonstrated that chronic HBV carriers had extraordinarily high relative risk of developing HCC compared to non-carriers [83–86]. A similar high relative risk of HCC in HBV carriers is observed in the United States [87].

The second form of evidence suggesting an etiologic role of hepadnaviruses in hepatocarcinogenesis is molecular. Covalent integration of truncated HBV DNA sequences in the cellular DNA of hepatic tumors of most HBV carriers [88–90] suggests that HBV has a direct role in hepatocarcinogenesis similar to that of other tumor-producing viruses in which integrated viral DNA or proviral DNA in the case of retroviruses causes neoplastic cell transformation by insertional mutagenesis [89, 90]. Finally, at least four members of the family Sciuridae have been described in which persistent hepadnavirus infection is closely associated with the development of HCC [54] and this observation combined with the experimental induction of HCC in laboratory woodchucks infected at birth with WHV [52] provides credible comparative medical evidence for the etiologic role of hepadnaviruses in hepatocarcinogenesis [54].

Molecular mechanisms of hepatocarcinogenesis associated with hepadnavirus infection

The specific mechanisms by which hepadnaviruses cause HCC are not completely understood but two general mechanisms have been proposed. In the direct molecular model [90–94], integration of hepadnaviral DNA into host cell DNA results in insertional mutation(s) and altered expression of a gene or genes that regulate the cell cycle (protooncogenes and tumor suppressor genes). In this model, neoplastic transformation of hepatocytes is initiated by integration of hepadnaviral DNA in a manner analogous to that of chemical hepatocarcinogens [95, 96].

The molecular hypothesis is based on detection of hepadnaviral integrations in the cellular DNA of most HCCs from patients infected with HBV [88–92]. Characteristically, only portions of the viral genome are integrated,

and sequences often are rearranged [18, 97]. In studies of the flanking sequences, HBV integrants have been detected infrequently near a known protooncogene or tumor suppressor gene and as a consequence, integrations were considered to have been random within the genome. More recent studies of human HCCs have demonstrated recurrent patterns of HBV integration involving multiple gene families and the telomerase reverse transcriptase [91, 92]. In the study of human HCCs by Paterlini-Brechot and her colleagues, integrants were found in all chromosomes with the exception of 13, X, and Y [92].

In the HCCs of most WHV carriers, integrated WHV DNA sequences have been demonstrated [97–100]. Molecular cloning and analyses of integrated WHV DNA and of flanking cellular DNA sequences indicated that integrations occurred at multiple sites within the woodchuck genome [97, 101, 102]. Multiple integrations in a single tumor could be explained in different ways. The accumulation of multiple integrations in a single hepatocyte could occur prior to clonal propagation of the transformed hepatocyte or an initial single integration of hepadnaviral DNA could be followed by secondary rearrangements of integrated viral sequences during tumor growth [103]. A third alternative could be that two or more tumors could develop independently, fuse during growth, and then be removed and analyzed as a single tumor.

Viral integrations in the non-tumorous hepatic tissue of HBV carriers indicates that integration precedes the development of hepatic neoplasms. Using Southern blot analysis, Brechot et al. studied the state of HBV DNA in the non-neoplastic liver of groups of patients with HCC, with chronic hepatitis, and with acute hepatitis [88]. Integration of HBV DNA was detected both in the cellular DNA of HCCs and in non-neoplastic hepatic tissue and the integration patterns were different. Integration of HBV DNA also was demonstrated in some patients with chronic hepatitis but without tumor, indicating that integration may be required for hepatocyte transformation but is not the only factor responsible for the development of HCC. In the livers of two patients with acute HBV infection, restriction analysis suggested the presence of integrated HBV DNA indicating that viral DNA integration occurs very early in HBV infection.

Integrations of WHV DNA sequences have been reported in the non-neoplastic liver of WHV infected woodchucks [97, 101, 102] supporting the view that integrations of viral DNA occur early in hepatocarcinogenesis. WHV DNA integrations also have been described in small neoplastic nodules before development of frank HCC [103–105]. Summers, Mason and their colleagues have elegantly determined the frequency of hepadnaviral DNA integrations in individual hepatocytes in experimental acute and chronic WHV infection and in chronic WHV carriers after sustained antiviral drug therapy [106–109]. In experimental DHBV infection, the liver was found to contain one DHBV DNA integrant per 10(3) to 10(4) liver cells and sequence analysis suggested that linear DHBA was the predominant

substrate for integration [106]. A similar assay system and similar results were obtained in investigations of experimental WHV infection. Following resolution of acute infection, one WHV DNA integrant was found per 1,000 to 3,000 liver cells [107]. In chronic WHV infection, it was estimated that 1–2% of the total non-neoplastic liver cells contained sequences of integrated WHV DNA [108]. The frequency of integrated viral DNA in chronic WHV carriers was found to be 1 to 2 orders of magnitude greater than that observed in transient WHV infection, suggesting that integrations and other genomic alterations were accumulated over the duration of infection [109]. When chronic WHV carriers received sustained treatment with the potent nucleoside analog, clevudine (L-FMAU), WHV cccDNA was decreased by 20- to 100-fold but no detectable decrease in the frequency of WHV integrants was observed. This suggests the integrated WHV DNA sequences persisted following substantial clearance of virus, an observation the authors believed argued for early therapeutic intervention in chronic HBV infection [109].

Hepadnaviruses do not contain oncogenes similar to those found in transforming retroviruses [18, 20]. Upregulation of many of the well-characterized protooncogenes has not been demonstrated [110]. Increased expression (5- to 50-fold) of *c-myc*, however, was observed, in three of nine woodchuck HCCs [110–112] and truncation and rearrangement of the gene were demonstrated. In one tumor, there was no direct linkage between WHV DNA integration and *c-myc* activation. Activation of *c-myc* appeared to be comparable to that in Burkitt's lymphoma and in acute B- and T-cell leukemias that are associated with chromosomal translocations [110]. Activation in the other two tumors was related to insertional mutations [112] with WHV DNA insertions interrupting the *c-myc* locus. The position and orientation of the WHV integrants in relation to *c-myc* excluded involvement of the hepadnaviral promoter in *c-myc* activation (i.e., promoter insertion). WHV sequences analogous to one of the HBV enhancers were present in the viral integrants, suggesting the possibility of enhancer insertion [112]. In a large series of woodchuck HCCs, activation of *c-myc* was observed in 10% of the tumors [113].

The expression of *N-myc* has been shown to be increased in 60% of woodchuck HCCs, and this transcript is not detected in normal woodchuck liver. Two *N-myc* loci have been identified in woodchucks. One is homologous to the *N-myc* gene of other mammalian species. The other, identified as *N-myc2*, is an intronless gene with the characteristic structure of a retrotransposon [114] and *N-myc2* has been mapped to the X chromosome. Expression of *N-myc2* is highly restricted, and the brain is the only normal tissue of the woodchuck in which *N-myc2* RNA was detected [115].

The physiological function of *N-myc2*, if any, remains unknown [115]. Hepatocarcinogenesis in woodchucks with chronic WHV infection appears to be preferentially associated with alteration in expression of the *N-myc2* locus [116, 117]. Insertion of WHV enhancer sequences upstream or down-

stream of *N-myc2* was associated with increased transcription of normal *N-myc2* RNA or of a hybrid *N-myc2*/WHV transcript initiated at the *N-myc2* start site. Enhancer insertion appears to be a common mechanism [118] for increased *N-myc2* expression. A liver-specific regulatory element in the WHV genome has been identified that appears to control cis activation of *N-myc2* [119]. Downstream integration of WHV DNA also has been associated with activation of *N-myc2* [120].

Rapicetta and her colleagues have analyzed the HCCs of woodchucks trapped in their native habitat with naturally acquired, chronic WHV infection [121]. Comparison of their results with data from previous studies of woodchucks with experimental WHV demonstrated that tumors from naturally infected woodchucks had WHV integrations near *N-myc2* less frequently than tumors from experimentally infected chronic WHV carriers and *N-myc2* activation associated with WHV integrations near the *N-myc2* gene also occurred less frequently (12/28, 43% *versus* 15/20, 75%, $P = 0.0397$). Their findings were believed to be related to the less uniform infecting virus and host genetic background of the naturally infected woodchucks and suggested that viral and/or host factors could influence the site of viral insertion that was detected finally in hepatic tumors.

More than one third (11/28, 39%) of the tumors they examined with increased *N-myc2* expression showed no evidence of rearrangement either near *N-myc2* or downstream in either the *win* or *b3n* loci. These findings indicated that not all tumors with increased *N-myc2* expression were related to *b3n* or *win* integrations and the authors suggested that activation of *N-myc2* in such cases was the result of chromosomal integration of WHV DNA further down stream or to mechanisms unrelated to viral insertion [121].

Jacob et al. examined the frequency of DNA integrations and rearrangements of the *N-myc2* gene in 55 hepatic tumors from 13 woodchucks with HCC and compared the results of the molecular changes to the size and histological characteristics of the hepatic tumors [122]. Four small tumor nodules were classified histologically as adenomas and integrated sequences of WHV DNA were detected in two of the four tumor nodules and in one of the two, there was evidence of N-myc rearrangement. 51 neoplasms were classified as HCC. Seven were classified well differentiated, grade 1 HCCs. WHV DNA integrations were demonstrated in 43% but none had detectable N-myc rearrangements. 20 grade 2, moderately well differentiated HCCs were analyzed and WHV DNA integrations were detected in 80% and N-myc rearrangements were present in 38%. 24 poorly differentiated, grade 3 HCCs had WHV integrations in 79% and N-myc rearrangements were detected in 74%. In two other grade 3 HCCs, rearrangements of N-myc were detected in the absence of WHV DNA integrations. The 12 largest tumors in the series were classified as either grade 2 or grade 3 HCCs, and in 83% of the largest tumors, both WHV DNA integrations and N-myc rearrangements were demonstrable. A series of genetic alterations

were demonstrated in woodchuck HCCs that were directly related to tumor size and the degree of histological differentiation and appeared to provide progressive proliferative stimulus and/or growth advantage [122].

Buendia and her colleagues have reported that transgenic mice carrying the *N-myc2* gene under the control of WHV regulatory sequences are highly predisposed to cancer of the liver [123]. 70% developed either HCC or hepatocellular adenomas. A transgenic founder that carried the unmethylated WHV/*N-myc2* transgene sequence died at the age of 2 months with a large liver tumor, demonstrating the high oncogenic capacity of the woodchuck *N-myc* retroposon. Mutations or deletions of the β-catenin gene similar to those of HCCs from humans and mice [124] were present in 25% of the hepatic tumors of the *N-myc2* transgenic animals, and tumor latency (time-to-tumor) was significantly reduced [123]. When *N-myc2* transgenic mice were crossed with p53 null mice, the absence of one p53 allele markedly accelerated the onset of liver cancer providing direct experimental evidence for synergy in multistage hepatocarcinogenesis between activation of *N-myc2* and diminished expression of p53 [123].

Like the woodchuck, the California ground squirrel possesses a functional, transcriptionally active *N-myc2* locus in the brain [125]. However, increased *N-myc2* expression is unusual in HCCs from California ground squirrels infected with GSHV. Amplification of *C-myc* expression, however, is more frequent in ground squirrel hepatic neoplasms than in those of woodchucks [126]. HCCs from woodchucks experimentally infected either with WHV or with GSHV have been analyzed [127]. The propensity for WHV genomic DNA to integrate in or near the *N-myc2* locus in HCCs from chronic WHV carriers was confirmed, whereas in woodchuck HCCs associated with GSHV infection, such integrations were exceptional. Seven of 17 (41%) WHV-induced tumors had rearrangements of the *N-myc2* allele. Only one of 16 GSHV-associated tumors (6%), however, had such *N-myc2* rearrangements. Based on the observations in GSHV-induced HCCs from ground squirrels and from woodchucks, it was concluded that the differences in hepadnavirus insertion and in *N-myc* activation between woodchucks and ground squirrels were due primarily to viral genetic differences and not to differences in the respective host species [127]. A similar conclusion was reached by Buendia and her colleagues [125].

Transfection of a nonmalignant, SV-40 T antigen-transformed mouse hepatic cell line with HBV DNA produced cells with a malignant phenotype that grew in soft agar and were tumorigenic in nude mice. HBX transcript was expressed at a much higher level than that observed *in vivo* [128]. In subsequent studies, it was concluded that over-expression of the HBX was required for malignant transformation of the cell line [129]. HBX is known to promote hepatocarcinogenesis in *c-myc* transgenic mice [130] and may promote hepadnavirus-induced hepatocarcinogenesis [131].

The X gene appears to be essential for normal replication of hepadnaviruses *in vivo*. The WHX protein is coexpressed with WHcAg in the liver

of chronic WHV carrier woodchucks [132, 133]. Feitelson demonstrated that when WHX was co-translated *in vitro* with p53, WHX/p53 complexes developed, and similar WHX/p53 complexes were demonstrated in the livers of chronic WHV carriers [134]. Combined with observations in HBV transgenic mice [135, 136], the observations of Feitelson et al. suggest that binding of WHX to p53 may prevent entry of p53 into the nucleus, diminish tumor suppressor activity, and represent an important mechanism by which the hepadnavirus X-gene product could promote hepatocarcinogenesis [134]. Mutations of p53 also may alter its tumor suppressor activity, but such mutations were found primarily in the less differentiated hepatic tumors, suggesting the mutations altered tumor progression at a later stage of development [136, 137].

Inflammation and hepatocyte regeneration in hepadnavirus-associated hepatocarcinogenesis

Inflammation-associated infections are recognized as important risk factors for cancer. Hepatitis associated with chronic HBV infection is a well recognized example but how inflammation of the liver influences hepatocarcinogenesis is not fully understood. NF-κB can promote or inhibit carcinogenesis. NF-κB produced by inflammatory cells in response to infectious agents, to necrotic cells or to cytokines results in the production of soluble factors that enhance cell growth, survivial and vascularization of carcinoma cells. NF-κB produced by carcinoma cells increases the production of inhibitors of apoptosis and of proteases that facilitate cell invasion. (Reviewed [138, 139]).

HCC develops regularly in a setting of chronic hepatitis and evidence is accumulating that chronic liver inflammation has an important role in hepatocarcinogenesis. [59, 60, 62]. Support for a model in which inflammation is a key factor in hepatocarcinogenesis comes from the observation that chronic liver injury associated with cirrhosis frequently precedes the development of HCC in HBV-infected individuals [140, 141]. Chronic hepatitis C virus infection results in severe chronic hepatitis and cirrhosis that frequently leads to HCC but in this form of HCC, there is no evidence of viral nucleic acid integration into cellular DNA. Increased rates of HCC in humans are observed in inherited forms of chronic liver disease including hemochromatosis, alpha-1-antitrypsin deficiency, and Wilson's disease [142]. These diseases also are associated with development of progressively severe hepatocellular injury, hepatocellular regeneration, and cirrhosis that precede development of HCC [143].

Experimental support for the role of hepatic inflammation and hepatocellular injury in hepatocarcinogenesis comes from studies reported by Chisari and his colleagues using transgenic mice [144–147]. A direct relation was found between the expression and retention of HBsAg in hepatocytes

and the severity of hepatitis and the severity of hepatitis was correlated with the development of HCC [145, 146, 148]. Increased free radical production within the liver was associated with oxidative DNA damage in woodchucks [149] a finding also reported previously in mice [150].

Hepatic injury associated with WHV infection increases the rate of hepatocyte death and regeneration and provides an environment that enhances fixation of spontaneous mutations, rearrangements, or chromosomal translocations that may be responsible for malignant transformation of hepatocytes. Such a role for hepadnaviruses in hepatocarcinogenesis would be comparable to the processes of promotion and/or progression recognized in multistage chemical hepatocarcinogenesis [95, 96, 138, 139].

Another possible hepatocarcinogenic mechanism related directly to hepatic inflammation has been suggested by studies of nitric oxide (NO) production in chronic viral hepatitis. In the liver, NO biosynthesis from arginine is catalyzed by inducible NO synthetase. Endotoxin, γ-interferon, and other cytokines [151–158] can increase the activity of this enzyme significantly. Under some circumstances, NO may be protective against experimental hepatic injury [154]. Under other conditions, hepatic production of NO may contribute to development of hypotension in septic shock [159].

Woodchucks chronically infected with WHV excrete more nitrate in the urine and more NDMA than uninfected control woodchucks [160]. Similar increased NO production has been observed in HBV transgenic mice [144]. Nitrate and NDMA are derived from NO produced from L-arginine. Urinary nitrate excretion also is increased in a human patient with chronic HBV infection [161].

In primary hepatocyte cultures from normal woodchucks, NO synthesis can be induced by endotoxin, and hepatocytes cultured from chronic WHV carriers produce significantly more NO and nitrosamine than hepatocytes from uninfected controls [162]. SV-40 T antigen-transformed woodchuck hepatocyte cell lines have the capacity to produce NO in response to endotoxin [163] utilizing the L-arginine-NO pathway and to produce NDMA. WHsAg purified from woodchuck serum can induce NO production in woodchuck hepatocyte cultures [164]. NO reacts with O_2 to produce NO_2, which exists in equilibrium with N_2O_3 and N_2O_4. N_2O_3 or N_2O_4 are potent nitrosating agents capable of reacting with water to form nitrite and nitrate [165] or, in neutral solution, of nitrosating dialkylamines to form nitrosamines. NO could act as a carcinogen indirectly by causing formation of hepatocarcinogenic nitrosamines such as NDMA, which is a strong alkylating agent. Point mutations can be induced by NDMA by methylation of guanine to 7-methyl and to 0.6-methylguanine, or by methylation of adenine to 3-methyladenine [166, 167].

NO also has the capacity to act as a direct mutagen [168]. *In vitro*, NO can deaminate deoxynucleosides, deoxynucleotides, or intact DNA and contribute to deamination-related mutations. It has been predicted based on studies of the rate of deamination of guanine to xanthine *in vitro* that

guanine/cytosine γ adenine/thymine transitions could be a frequent form of mutation induced by NO. Adenosine deamination to hypoxanthine would be expected to result in adenine/thymine to guanine/cytosine transitions, and removal of the xanthine formed by guanine deamination would result in depurination and guanine/cytosine to thymine/adenine transversions [161]. The demonstration of increased NO formation in the hepadnavirus-infected liver suggests that NO could have an important role in hepatocarcinogenesis.

Interaction of hepadnavirus and other hepatocarcinogens

Aflatoxin is among the most potent hepatocarcinogens known and is a frequent contaminant of the diets of populations with high incidence rates of HBV infection and HCC. The possible interaction between hepadnavirus infection and aflatoxin has been investigated using the woodchuck model [149, 169, 170]. When administered early in life to woodchucks experimentally infected at birth with WHV, aflatoxin B_1 did not appear to increase the rate of chronic WHV infection or the rate of development of HCC [170]. When administered to established chronic WHV carriers at one year of age, aflatoxin B_1 appeared to promote HCC and the time to tumor was moderately reduced [149]. A similarly synergistic interaction between HBV and aflatoxin has been reported in the tree shrew [171].

When transgenic mice that expressed HBsAg were exposed to DEN or aflatoxin, they developed hepatic adenomas and HCC more rapidly and more extensively than unexposed transgenic controls or normal mice receiving either hepatocarcinogens. These results demonstrated a synergy between the chemical hepatocarcinogens and the chronic hepatocellular injury caused by overexpression of HBsAg in transgenic mice [148]. A similar synergistic effect of HBX expression in transgenic mice and DEN on hepatocarcinogenesis has also been reported [172].

The woodchuck model and antiviral drug development

Chronic WHV carrier woodchucks frequently have been used in the preclinical evaluation of antiviral drugs during development for the treatment of chronic HBV infection. These studies have utilized both nucleoside analogs and immune response modifiers [173–179]. The drugs are screened for antiviral activity against HBV in the 2.2.15 cell system before testing in woodchucks [180]. Acyclovir and AZT, which had no selectivity against HBV in 2.2.15 cells, had no antiviral effect in the woodchuck model of HBV infection. Ara-AMP, which had moderate antiviral activity *in vitro*, had significant antiviral activity in woodchucks, and activity was increased by specific liver targeting [181]. Most nucleoside analogs with intermediate

antiviral activity *in vitro* against HBV had intermediate antiviral activity in woodchucks. An exception was fialuridine (D-FIAU), which had only modest tissue culture activity in 2.2.15 cells but in woodchucks had potent antiviral activity during a 1 month period of treatment. During a 12 week test, however, fialuridine was highly toxic and the clinical syndrome in woodchucks was similar to that observed in humans treated with fialuridine [182]. Another fluorinated pyrimidine, clevudine (L-FMAU) also had moderate tissue culture activity but is among the most potent nucleosides in WHV carrier woodchucks [173, 183].

Lamivudine, which had a very high selective index against HBV *in vitro*, was a potent antiviral drug in woodchucks with favorable pharmacokinetics [184] and was without toxicity at doses of 5 or 15 mg/kg/day for 28 days [176]. The absence of effect of lamivudine on hepatic cccDNA was postulated to be due to the absence of cell division [185].

Lamivudine has been shown in the woodchuck model to act synergistically both with alpha-interferon [175] and with famciclovir [176]. Antiviral potency similar to lamivudine has been demonstrated in WHV carriers with the closely related emtricitabine [186]. Extended lamivudine treatment of woodchucks with chronic WHV infections was associated with development of viral polymerase mutations [187–190].

The antiviral activity of the nucleotide analog, adefovir, has been evaluated in the woodchuck [191] and adefovir has been shown to have potent antiviral activity in woodchucks with circulating lamivudine resistant WHV [192]. Tenofovir, another nucleotide analog [193] and the purine nucleoside, 9-0-beta-D-2-aminopurine dioxolane (ADP) [194] also have been shown to have significant antiviral activity in the woodchuck model.

The guanosine nucleoside, entecavir, was used in WHV carrier woodchucks to determine the effect of sustained suppression of viral replication on hepatocarcinogenesis [195]. At 8 months of age, laboratory born and reared, WHV carriers were given entecavir orally at 0.5 mg/kg per day for 8 weeks and then given a dose of at 0.5 mg/kg per week. Treatment was stopped after a total of 14 months in six woodchucks and treatment was continued in five others for 36 months. Treatment resulted in significant reductions in hepatic expression of viral antigens and of covalently closed circular WHV DNA. Three of the six woodchucks treated with entecavir for 14 months had sustained antiviral responses following drug withdrawals and when killed at 4 years of age, had no evidence of HCC. The woodchucks treated for 36 months were also killed at 4 years of age and in four of five there was no evidence of HCC. Entecavir treatment significantly delayed development of HCC and prolonged survival compared to historical WHV carrier controls which had a 4-year survival of 4%, ($P < 0.01$).

Menne et al. [196] treated WHV carrier woodchucks beginning at 1 or 2 years of age for 32 weeks with the orally administered pyrimidine nucleoside, clevudine (10 mg/kg per day) [196]. Controls received the saline vehicle as placebo. Clevudine-treated and control woodchucks each were subdi-

vided into two groups. One subgroup of the clevudine treated woodchucks received four doses of alum-adsorbed WHsAg vaccine during the next 16 weeks and a subgroup of the placebo controls received identical vaccinations. Subgroups of the clevudine and of the placebo recipients served as unvaccinated controls.

Vaccination resulted in detectable anti-WHs responses in most WHV carriers but had no effect on serum WHV DNA, on serum WHsAg, or on the serum activities of liver enzymes. Clevudine treated carriers had marked reductions in serum WHV DNA and WHsAg and when vaccinated, developed a significantly more robust anti-WHs response than did woodchucks not receiving antiviral therapy. WHsAg-specific cell-mediated immune responses developed in both clevudine and placebo recipients that were vaccinated but the immune responses were significantly augmented in WHV carriers treated with clevudine. WHsAg vaccination of clevudine-treated woodchucks disrupted virus-specific immune tolerance and induced enhanced immune responses compared to woodchucks that received either clevudine or vaccine alone. Therapy with the clevudine-vaccine combination resulted in immune response profiles that resembled those observed during resolution of experimental WHV infection [196].

In 10 of the WHV carrier woodchucks treated with clevudine (with or without vaccination), treatment was initiated at 1 year of age. Sustained reductions in serum WHV DNA and WHs antigenemia were observed during treatment and for a period of 18 months following withdrawal of clevudine treatment. Liver biopsies were obtained at the time treatment was initiated, at the end of treatment (20 months of age), and at 6 and 12 months following drug withdrawal (24 and 32 months of age). Hepatic WHV nucleic acids and WHcAg expression were reduced significantly compared to placebo-treated controls throughout the period of treatment with clevudine. The percentage of hepatic biopsies with foci of altered hepatocytes was significantly decreased in the clevudine-treated woodchucks compared to controls and development of HCC was markedly delayed. In the 10 WHV carriers in which clevudine treatment was initiated at 1 year of age, three year survival was 50% and 4 years survival was 25%. In controls, 3 year survival was 25% and 4 year survival 5% ($P=0.035$).

In a long-term chemoprevention study, 20 8-month old WHV carriers were treated for life with lamivudine (initial dose of 5 mg/kg/day followed by 15 mg/kg per day) and 20 controls received vehicle as placebo [187]. Serum WHV DNA decreased gradually over the first few months by 4–5 logs in lamivudine treated woodchucks. The antiviral effect was sustained for more than 1 year when recrudescence of viremia was detected. Recrudescence was associated with B domain mutations of the WHV polymerase gene [190]. The development of HCC in lamivudine-treated woodchucks was significantly delayed and there was an equivalent increase in survival time. The median time to cancer death in controls was 32 months and in lamivudine-treated woodchucks 44 months ($P=0.01$). In placebo controlled clinical

trial of HBV carrier patients, a comparable beneficial effect of lamivudine treatment on the occurrence of HCC and long-term survival. During a median drug treatment period of 32 months, HCC developed in 16 of 215 (7%) placebo-treated control patients and in 17 of 436 (4%) patients that received lamivudine ($P = 0.047$) [197].

In another study, woodchucks were treated for periods of 3 to 12 months with lamivudine. In this study, no long-term benefit of lamivudine treatment on hepatocarcinogenesis or survival was observed. Treatment in this study was initiated at an older age than the life time study described above, the duration of treatment was for a limited time, and the effect of treatment on viral load was not as great [188]. High rates of polymerase gene mutations were detected in woodchuck after long-term lamivudine therapy, observations similar to those detected in HBV infected humans [189, 190].

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Hepadnaviruses have a narrow host range – do they?

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Abstract

Host range describes the range of species that a virus can infect to productively propagate itself. Productive infection requires compatibility between virus and host molecules. Thus host range may be restricted by lack of appropriate permissivity factors; alternatively, hosts may actively counteract infection using restriction factors. Incompatibility between virus and host can manifest on the level of individual cells, of tissues or organs, and of the entire organism. All hepatitis B viruses are hepatotropic, but individual viruses infect the livers of only selected mammalian (orthohepadnaviruses) and avian (avihepadnaviruses) hosts. Hence a narrow host range is thought to be a salient feature of hepadnaviruses. Here we briefly review general mechanisms of host range restriction, and summarise older as well as recent data pertaining to hepadnaviral host range. Clearly, the term species-specific is inadequate for many hepadnaviruses because they can infect different species from one genus, and even species from different genera. For a few others, only a single species, or genus, has been identified that supports efficient infection; however, this could as well relate to the restricted number of experimentally addressable test species. Together with the uncertainty about quantitative phylogenetic relationships between species, still largely based on morphological rather than molecular criteria, this leaves the term narrow open to interpretation. Finally, few if any of the host molecules enabling productive infection by a hepadnavirus have unambiguously been identified, the role of restriction factors has not yet been assessed, and even on the virus side the so-called host determining regions in the PreS domains of the large envelope proteins appear to be relevant only under specialised experimental conditions. Hence this important aspect of hepadnavirus biology is still far from being understood.

Introduction

While viruses can infect all forms of life, an individual organism cannot be infected by all types of viruses. Though not surprising given the diversity between prokaryotic, plant and animal hosts, even within one kingdom many viruses are selective with respect to what for them constitutes a suitable (susceptible) and a non-suited (non-permissive) host, or host cell. Within metazoan hosts, three types of specificity (tropism) can be distin-

guished: that for specific cells (cell tropism), that for specific organs or tissues (tissue tropism), and that for members of one, but not another species, genus, family, order and so on (host tropism). Host specificity of hepatitis B viruses is the topic of this review; however, all three are closely intertwined such that host range cannot be discussed in isolation.

The common notion is that a narrow cell and tissue specificity, namely for hepatocytes and hepatocyte-derived cells, and a narrow host range are salient features of hepatitis B viruses. Below we summarise the evidence favouring, and, at least in part, questioning this concept; we briefly discuss the viral and host factors that, in general, determine host range in better understood systems and relate them to what is known for hepatitis B viruses. Because several comprehensive reviews pertaining to hepadnavirus biology have recently been published [1–3] we will emphasise unresolved issues from both early and very recent investigations. They reveal that experimental definition of hepadnaviral host range is obscured by insufficient knowledge on the involved viral and host factors as well as host phylogenetic relations, is strongly affected by the test systems used, and thus is much less understood than implicated by prevailing models.

General importance of host range

We are constantly exposed to an enormous variety of viruses of which, fortunately, only few are able to establish a productive infection in humans because of host range restrictions. Hence this species barrier protects us from permanently being infected. The importance of this phenomenon is dramatically highlighted by the occasional crossing of this barrier by a virus and its potentially devastating consequences [4]; notorious examples include human immunodeficiency viruses (HIVs) [5], severe acute respiratory syndrome (SARS) coronavirus [6], or the imminent threat of an influenza pandemic originating from, thus far avian, H5N1 influenza viruses [7]. It should be noted that not every species crossing leads to disease: Simian virus 40 (SV40), possibly introduced into the human population by the poliovirus vaccine, may or may not be associated with cancer [8], and no indication for disease-induction exists for simian foamyvirus infections of humans [9].

A different consequence of relevance for virological and medical research is that host range restrictions can prevent the establishment of animal models for infection with a human viral pathogen, for which HBV represents an eminent example.

Molecular determinants of viral host range

Viral infection, and defense against infection, depend on interactions between viral and host components which, therefore, must be matching.

Two fundamentally distinct mechanisms restrict infectability: Either a lack of factors required for the virus to complete its infectious cycle ('permissivity factors'), or the presence of host factors that actively block that cycle ('restriction factors'); the latter may be divided into factors that are constantly present (innate), and those that are induced in response to the encounter with a specific virus (adaptive). Evidently, cells from different hosts, and from different tissues within one host, differ in their expression profiles; additional diversification comes in by changes related to the cell-cycle and differentiation status. Hence permissivity factors may either completely, or temporarily be absent, or differ between host species in their exact composition such that they cannot productively interact with the corresponding viral component; the same holds, conversely, for restriction factors.

Host range restrictions by lack of host factors required by the virus

Failure to complete any one of the individual infectious cycle steps, for physical lack of, or lack of compatibility with, a cellular factor involved will exclude a cell or organism from being a host for that virus. Related species have a related genetic outfit, and the closer the relation the higher the similarity between individual genes and gene products. Hence susceptibility to a certain virus in one host species will most easily be extendable to closely related hosts. The importance of match between viral and cellular factors is also highlighted by the impact on infectability of individual polymorphisms in some cellular genes; a famous example are humans carrying a non-functional allele of the HIV-1 coreceptor CCR5 gene, CCR5Δ32 [10, 11]. Interestingly, this allele also decreases the likelihood for hepatitis B to become chronic, though probably by an immunomodulatory rather than a direct mechanism [12]; in contrast, an intact CCR5 gene protects against West Nile virus infection [13].

Nonetheless, viruses differ greatly in their host specificities. Vesicular stomatitis virus (VSV) infects cells from a very wide range of species, including vertebrates, invertebrates and even insects; some murine retroviruses, in contrast, are so much restricted that they infect one but not another strain of mice. As outlined below, hepadnavirus host specificity seems to be intermediate between these extremes.

For any virus, including HBV, the events during productive infection can broadly be categorised into attachment, entry, uncoating of the viral genome, genome replication and expression of gene products, and assembly and egress of progeny virions. Most occur at different sites in the cell, hence the importance of intracellular trafficking in virus replication, and consequently the dependence on the involved cell factors, is increasingly recognised [14].

Attachment and entry

Each virus must bind to a suitable host cell, and then must get access to the cytoplasm by crossing either the host cell plasma membrane, or an internal – usually endosomal – membrane; for enveloped viruses this generally involves membrane fusion events. Binding and fusion activities may reside in a single protein, as with VSV G protein, or on separate polypeptides. Obviously, the initial binding requires interactions between molecules that are exposed on the surfaces of the virus and of the cell, commonly named viral attachment proteins, or viral receptors, and cellular receptors. These can be different classes of molecules, including proteins, lipids, and sugars. For many viruses, though not the hepadnaviruses, the host interaction partners of their attachment and fusion proteins are known [15], in several cases even in atomic detail [16]. Examples include HIV-1 gp120 and CD4, influenza virus haemagglutinin (HA) and sialic acid residues. For protein receptors, it is obvious that their amino acid sequences will vary between host species, and hence may act as important host range restriction elements. Notably, even less complex biological structures can exert such a restrictive function, as shown by the differential recognition of α2,3 *versus* α2,6 linkages to galactose in the sialic acid receptors used by avian *versus* human influenza viruses [17]. A second increasingly appreciated feature is that often, perhaps always, more than one host surface molecule is involved. Such secondary molecules, for instance extracellular matrix components such as glycosaminoglycans (GAGs), may generally help concentrating the virus on the cell surface, or be more specific, often sequentially, acting co-receptors that are essential for productive entry. Well known examples include, for non-enveloped viruses, the initial binding of the adenovirus fiber protein to the Coxsackie-Virus Adenovirus receptor (CAR), and the subsequent interaction of the penton base protein with cellular integrins [18], and for enveloped viruses, the HIV-1 receptors and co-receptors CD4 and CXCR4 and CCR5 [19]. Of note, relatively small alterations in a viral protein can lead to the use of alternative host receptors [15] and therefore contribute to extend the virus host range. Finally, virus host cell receptor interactions may be modulated indirectly by auxiliary proteins. For many viruses, fusion activity depends on a balanced proteolytic activation of the fusion protein. The pertinent example is the haemagglutinin (HA) of influenza viruses and its dependence on properly timed cleavage into HA1 and HA2 [17]. Hence, availability of suitable proteases at the right concentration and location can well affect host range.

Uncoating of the viral genome, replication and formation of progeny virions

The steps following entry can as well profoundly affect viral host range. Poxviruses, for instance, using ubiquitously present molecules, possibly GAGs, as receptors can enter many different cells. Their narrow host range (Variola virus infects exclusively humans) must therefore relate mostly to downstream intracellular events [20]. Some of the basic cellular machinery for transcription, translation, and protein folding [21, 22] is probably universal; however, the ongoing search for a mouse model of HIV-1 infection [23] is revealing an ever increasing number of host-specific factors [24] that lack compatibility with components of the human virus. Examples include preintegration complex nuclear import [25] and the Tat protein which together with the host factors cyclin T1 and a cyclin kinase (Cdk9) binds to the TAR element on nascent viral RNA, enhancing the rate of viral RNA transcription by RNA polymerase II; HIV-1 Tat interacts productively with human but not murine cyclin T1 [26]. Also, HIV-1 assembles and buds poorly in mouse cells, suggesting that components of, or associated with, the endosomal sorting complex required for transport (ESCRT) contribute to species tropism [24]. This machinery is probably also involved in HBV morphogenesis [27–29]; however, the single most important species-specific blocks to hepadnavirus infection appear to operate during the initial steps of infection (see below).

Presence of host restriction factors

Cells may actively counteract viral infection by innate restriction factors; adaptive responses, though crucial, are not considered here. Innate restriction factors typically act in a dominant, saturable fashion, i.e., inhibition can be overcome by high doses of virus ('abrogation'), and they can target diverse steps of viral replication, as is best known for retroviruses [30–32]. Classic examples are the endogenous retroviral gene products *Fv4* which restricts Friend murine leukaemia virus by receptor blockade, and *Fv1*, a Gag-like protein that targets the capsid proteins of some retroviruses and interferes with proper functioning of the preintegration complex. Another widely present restriction system is based on the family of tripartite sequence motif (TRIM) proteins [33] which typically contain a RING domain, found in many E3 ubiquitin ligases, one or two B boxes, and a coiled-coil region, plus different C terminal domains. The most famous of the about 70 TRIM family members [34] is TRIM5α, a species-specific restriction factor for various immunodeficiency viruses and also simple retroviruses [35]; for instance, rhesus monkey, but not human, TRIM5α restricts HIV-1 and HIV-2. TRIM5α also targets the capsid protein and prevents proper reverse transcription, probably by premature uncoating, and possibly also through

degradation of Gag polyprotein precursors [36]. This mechanism may be antagonised by the host proline isomerase cyclophilin A (CypA) which augments HIV-1 infection in human cells but inhibits replication in monkey cells [37]. Other TRIM family members are also involved in antiviral resistance [38].

Another restriction system is based on the APOBEC family of cytidine deaminases [39], named after the founding member APOBEC1 which edits the mRNA for apolipoprotein B. APOBEC3G targets instead single-stranded DNA, for instance retrovirus minus-strand DNA, and introduces various C>U mutations, resulting in G>A hypermutation in the plus-strand. The U-residues render the DNA unstable, and hypermutation can prevent formation of functional gene products. The lentiviral Vif gene products protect the viruses by inducing APOBEC3G degradation; species-specificity results mainly from the ability, or inability, of a given APOBEC protein to interact with the respective Vif protein [40]. APOBEC3G and other family members, if overexpressed in hepatic cell lines, also restrict HBV though the deaminase activity is not required [41, 42]. Hepadnaviruses do not seem to encode a Vif-like anti-APOBEC activity; hence potential host range restriction would have to act directly *via* capsid-APOBEC interactions. A role for APOBEC in avihepadnavirus infections is unlikely because APOBEC3 proteins, like APOBEC1, appear to be mammal-specific [43].

Yet another innate factor restriction factor is the zinc-finger antiviral protein (ZAP) which targets viral RNA for destruction by the exosome RNA degradation machinery, as shown for retroviruses and Sindbis virus [44, 45] and recently made likely for filoviruses [46]. Whether ZAP can affect host range is currently not known.

A further, complex innate defense system against viruses and other pathogens consists of the various Toll-like receptors (TLRs). Recognition of non-self 'pathogen-associated molecular patterns' (PAMPs) by these proteins, located in the plasma membrane (TLR4) or in endosomal membranes (most other TLRs), induces antiviral signaling pathways that activate, e.g., NFkB or interferon regulatory factor 3 (IRF3), leading to expression of antiviral cytokines and interferons [47]. The viral PAMPS may be proteins (TLR2, TLR4) or nucleic acids such as dsRNA (TLR3), ssRNA (TLR7, TLR8), or dsDNA (TLR9). Recently discovered cytoplasmic sensing systems include the RNA helicases retinoic acid induced gene I (RIG-I) which appears to recognise uncapped double-stranded viral RNA bearing 5'-triphosphates, e.g., from influenza, Sendai or flaviviruses including HCV [48], and melanoma differentiation associated gene 5 (MDA5) which senses 5'-protected RNA, e.g., from picornaviruses [49]. Both activate, through their caspase recruitment domains (CARD), a mitochondria-bound protein variously known as CARD adaptor inducing IFN-β (CARDIF), interferon-β promoter stimulator 1 (IPS-1), virus-induced signaling adaptor (VISA), or mitochondrial antiviral signaling (MAVS), also leading to NF-κB and IRF3-/IRF7-mediated interferon induction. How much these antiviral fac-

tors contribute to species-tropism is not yet clear; however, the antiviral activity RIG-I is emphasised, for instance, by the much better replication of hepatitis C virus (HCV) in a hepatoma cell line clone lacking a functional RIG-I protein, and in that HCV tries to escape from this mechanism by cleaving CARDIF (as well as the TLR3 adaptor TRIF) using its NS3-4A serine protease [50]. While HBV can counteract the downstream interferon effects [51] it is currently not known whether these or similar defence systems affect hepadnaviral host range.

Finally, RNA interference may contribute to permissiveness of cells to viral infection; while this is proven for invertebrates and plants, a role in mammals is still under debate [52].

Hence cells are equipped with a large variety of defence systems that could also affect hepadnavirus infection and host range; however, currently few if any data are available.

Common features of hepadnaviruses and their infectious cycles

As outlined above, each step of the viral infectious cycle may be subject to negative regulation by lack of positive or presence of negative factors in a specific host cell; this certainly holds also for hepadnaviruses. We next briefly consider the hepadnaviral infectious cycle *in toto* (Fig. 1A); however, based on current knowledge, most of the later steps are controlled on the tissue rather than host species level, leaving the early steps of attachment and entry as the most likely candidates for determining host tropism. One line of evidence supporting this view is that artificial introduction of a transcriptional template equivalent to human HBV covalently closed circular DNA (cccDNA) into mouse liver, as a transgene or by hydrodynamic injection [53], yields virions that are infectious in an authentic host but do not infect the mouse.

All hepadnaviruses are small, enveloped, hepatotropic DNA-containing viruses that share a common genetic organisation and replication strategy [1]. Their genomes (Fig. 1B) are all between 3–3.3 kb in length, are produced by reverse transcription of the pregenomic RNA (pgRNA) intermediate, and are present in virions as partially double-stranded circular but not covalently closed, i.e. relaxed circular (RC) DNA with the reverse transcriptase, P protein, covalently linked to the 5' terminal nucleotide of the (–)-strand DNA. All encode, besides P protein, a single capsid or core protein which forms the icosahedral protein shell of the nucleocapsid, and two (avian HBVs) or three (mammalian HBVs) carboxy-terminally colinear surface proteins which are embedded into the lipid of the envelope. The surface proteins can form empty envelopes, termed subviral or S particles (SVPs), which are secreted in large excess over intact virions [54]. Most abundant is the small (S) protein (about 225 amino acids in ortho- and about 160 amino acids in avihepadnaviruses), constituting the majority of HBsAg in human

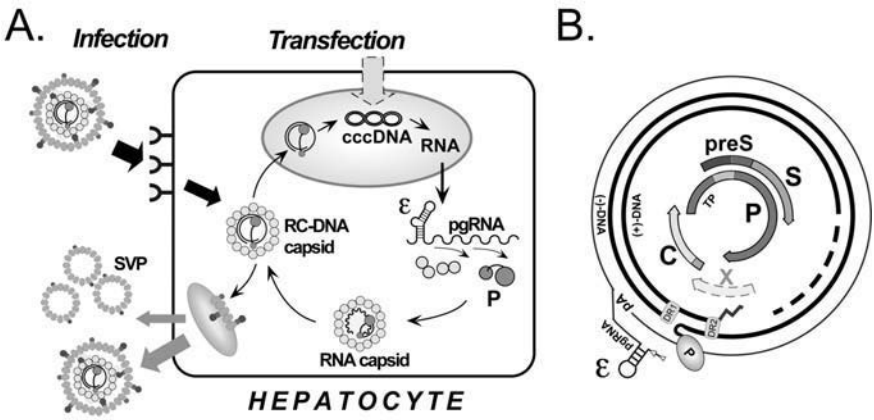


Figure 1. Common aspects of hepadnaviruses. A. Infectious cycle. Enveloped virions enter their host cells, primarily hepatocytes, *via* interaction with largely unknown receptor(s), leading to cytoplasmic release of the nucleocapsid, import of the relaxed circular (RC) DNA genome into the nucleus and conversion into covalently closed circular (ccc) DNA. From this template, subgenomic (not shown) and pregenomic (pg) RNAs are transcribed by cellular RNA polymerase II, exported from the nucleus, and translated. PgRNA acts as mRNA for the capsid and P protein, and via the encapsidation signal e, is copackaged with P into new immature nucleocapsids. Reverse transcription into the RC-DNA form occurs inside the nucleocapsid. Mature progeny capsid can repeat the intracellular amplification cycle, increasing the copy number of cccDNA molecules, or interact at compartments of the secretory pathway (lower left corner of the cell) with the envelope proteins and be secreted. Empty envelopes (subviral particles, SVPs) are secreted in large excess over virions, probably by a distinct mechanism [27, 28]. B. Genome organisation. All hepadnavirus genomes as present in virions are about 3 kb in length, with the (–)-strand DNA covalently linked to P protein and the (+)-strand being incomplete. All encode one capsid protein (C), the reverse transcriptase (P), and completely overlapping with P, a short (S) plus one (avian) or two (mammalian) longer forms (PreS/S) of envelope proteins. Intact genes for X are present in all mammalian, but only in cryptic form in the avian viruses.

HBV infection; the middle (M) protein of mammalian HBVs contains an N-terminal extension of 55 amino acids encoded by the preS2 region, and the L protein an additional extension of around 110–120 amino acids encoded by the preS1 region. Avian HBVs lack a preS2 initiation codon; their preS regions comprise about 160 codons. In all hepadnaviruses, the PreS regions of the L protein are involved in two essential functions, namely nucleocapsid envelopment and attachment to, and entry into, the host cell. This is accounted for by an unusual dual topology with about one half of the PreS domains directed towards the inner, and the other half to the outer face of the virions. An N terminal extension on the core protein, encoded by the preC region, gives rise to the secretory, non-assembling precore protein which, after N and C terminal processing events, is found in the circulation as HBeAg. Finally, mammalian, but most likely not avian, HBVs produce a regulatory protein, HBx, whose function is only poorly understood.

The hepadnaviral infectious cycle (Fig. 1A) is initiated by binding of the virion to as yet unknown cell surface receptor(s), internalisation, release of the nucleocapsids into the cytoplasm, and transport of the RC-DNA genome into the nucleus where conversion into cccDNA occurs.

None of these events is well understood. Whether fusion occurs at the plasma or at an internal membrane, and whether it requires passage through an acidic compartment is controversial, as is the nature of the fusion peptide and the requirement for its exposure, or not, by a proteolytic processing event. Some evidence suggests that artificial cleavage can facilitate entry into non-susceptible cells [55, 56] which would be compatible with the presence of a fusogenic peptide within the first trans-membrane domain (TM1) of the S domain [3]. A recent alternative proposal was that translocation motifs (TLMs), short α-helical peptide sequences predicted to be present in ortho- and avihepadnavirus PreS proteins, would mediate direct translocation [57]. This has not, however, been reproduced, for HBV, by others [58, 59].

Nuclear transport [60] of the relaxed-circular (RC) DNA genome occurs in nucleocapsids along cytoskeletal components, and probably results *via* interactions of the capsid with components of the nuclear pore complex (NPC) in the release of the DNA into the nucleus. NPC components are highly conserved through evolution, and *X. laevis* oocytes appear to behave similarly with respect to hepadnaviral nucleocapsids as human hepatocyte cell lines, suggesting the absence of species-specific effects. cccDNA formation includes completion of the (+)-DNA strand, removal of the covalently bound P protein and the small terminal redundancies, and religation. The virtual absence of cccDNA in HBV-transgenic mice suggested the involvement of species-specific factors; however, in mice with a hepatocyte nuclear factor 1a (HNF1a) null background, cccDNA becomes detectable [61]. Recently initiated efforts to systematically re-evaluate cccDNA formation are certainly worthwhile and may uncover as yet unknown species-specific factors [62].

cccDNA is the pivotal intracellular intermediate of hepadnavirus replication as it serves as template for all viral transcripts. Transcription of cccDNA by RNA polymerase II appears mostly controlled on the tissue – rather than species – specificity level, in that the core promoter driving pregenomic RNA transcription and the preS1 promoter responsible for the preS mRNA require liver enriched factors for high-level activity; for instance, HBV and DHBV replication in mouse cells of nonhepatic origin can be rescued by liver-enriched factors such as hepatocyte nuclear factor 4 (HNF4) [63]. However, WHV replication is not supported in that system and the WHV enhancer I does not work properly in human hepatoma cells that fully support HBV transcription [64]). Attempts to prove species-specific contributions for WHV are hampered by the lack of suitable woodchuck hepatoma cell lines and cloned transcription factors.

pgRNA encapsidation and reverse transcription depend on the specific interaction between the hepadnaviral RT and a stem-loop structure, ε, on

the pregenomic RNA. This interaction is strictly dependent on cellular chaperones [1]; however, there is no known requirement for species-specific chaperones, which is not that surprising given their extreme conservation throughout all kingdoms.

Newly formed mature, RC-DNA containing nucleocapsids can either recycle the genome to the nucleus for cccDNA amplification, or interact with the envelope proteins, bud into the ER or a later compartment of the secretory pathway and be secreted as enveloped virions. New data suggest the involvement of the machinery including ESCRT that generates multivesicular bodies (MVBs), unexpectedly in a distinct fashion regarding virions *versus* subviral particles [27, 28]. Again, a role for species-specific factors is unlikely because DHBV produced in transfected human hepatoma cell lines is infectious in ducks [65–67]; however, *in vivo* infection with DHBV is highly efficient [68], hence even a drastic reduction in the efficiency of virion production between avian and mammalian cells could have gone undetected.

Experimentally assessing viral host range

There are several approaches for exploring viral host range, each with its own benefits and limitations. One is to screen different animal species for the presence of a common or related virus; chances for success are sometimes increased if the virus causes a typical pathology, as is the case for some [69, 70], though not all hepadnaviruses. However, given the limited range of known hepadnavirus hosts, specimen collection is not trivial and certainly cannot be comprehensive; hence some, or many, potential host species may be missed.

A second approach is experimental transmission of a natural virus into a new host; this may be extended to viruses that have genetically been modified in a defined way, yet again ethical and other constraints apply for species that are not commonly used as experimental animals. Furthermore, even if a species is principally susceptible, infection may be difficult to detect if innate and adaptive immune responses limit replication to low levels or short periods of time. For instance, infection of 3-week old *versus* 3-day old ducklings with DHBV leads to the extremely rapid (within 1–2 days) induction of neutralising antibodies which drastically reduce viral spread in the liver and prevent persistent infection [71].

Adaptive immune restrictions can be overcome by using cultured cells or cell lines as targets. For hepadnaviruses, however, only a single human hepatoma cell line, HepaRG, has been identified that, under specialised conditions, can be infected by HBV [3, 72]; hence the nearly only source for infectable cultured cells are primary hepatocytes. Notably, however, we have recently identified a chimeric DHBV which is clearly restricted in

cultured hepatocytes yet *in vivo* replicates as well as wild-type DHBV (K. Dallmeier and M. Nassal; see below). Hence *in vitro* and *in vivo* infection may lead to different conclusions on viral host range.

Natural hepadnavirus hosts

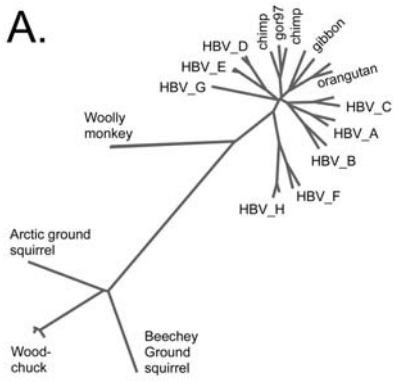
Shortly after human HBV genetically related yet distinct viruses have been found in several mammals (orthohepadnaviruses) and in selected bird species (avihepadnaviruses). Though a single cloned virus genome may not always represent an infectious clone [73], a wild-caught animal from which the corresponding virus has been isolated is a strong candidate for being a genuine host; this is less certain for viruses from captive individuals [74], yet it indicates, at any rate, that the virus can use that species as a host.

Based on this criterion, currently recognised natural orthohepadnavirus hosts are humans and hominoid primates (chimp/*Pan troglodytes*; gorilla/*Gorilla gorilla*; orangutan/*Pongo pygmaeus*; gibbons/Hylobatidae; [75]); one New World monkey (woolly monkey/*Lagothrix lagotricha*; [73, 76]); and some rodents, namely woodchuck (*Marmota monax*; [70]), California ground squirrel (*Spermophilus beecheyi*; [69]), Richardson's ground squirrel (*Spermophilus richardsoni*; [77]), and arctic ground squirrel (*Spermophilus parryi*; [78]) – but not mice or rats.

A large group of natural hosts for avian hepadnaviruses have been found in the order Anseriformes (waterfowl), but not Galliformes (landfowl), i.e., Pekin ducks (*Anas platyrhynchos* var. *domestica*; [79]) and their wild mallard ancestors; other Anatinae (duck-related birds; [74]) from the genus *Anas* (Puna teal/*A. puna*; Chiloe wigeon/*A. sibilatrix*) as well as other genera such *Aix* (Mandarin duck/*Aix galericulata*), *Neochen* (Orinoco goose/*N. jubata*), *Chloephaga* (Ashy-headed sheldgoose/*C. poliocephala*); and Anserinae (goose-related birds) including snow geese (*Anser caerulescens*; [80]) and Ross' geese (*Anser rossii*; GenBank Accession no.: M95589). Distinct hepadnaviruses have been found in two families of the order Ciconiiformes, namely storks (Ciconiidae), specifically the white stork/*Ciconia ciconia* [81], and herons (Ardeidae), specifically the grey heron/*Ardea cinerea* [82], great white heron/*Ardea alba*, great blue heron/*Ardea herodias*, and Wuerdemann's heron, a hybrid between *A. alba* and *A. herodias* [83], and finally in cranes [84] (order Gruiformes), specifically the crowned crane/*Balearica pavonia*, and demoiselle crane/*Grus virgo* (*synonym*: *Anthropoides virgo*).

Because negative results are usually not published it is unknown how many other species have been tested. Given the limited choices of established hosts, only the Pekin duck [85] and, to some extent, the woodchuck [86], have been developed into feasible animal models for hepadnavirus infection.

A.



B.

virus/ host	HBV (A-H) gen	HBV (A-H) prS	Great Apes gen	Great Apes prS	Woolly monk. gen	Woolly monk. prS	GSHV gen	GSHV prS	ASHV gen	ASHV prS	WHV gen	WHV prS	DHBV gen	DHBV prS
Humans	86	72	86	77	78	65	63	26	64	23	64	24	40	9
Great Apes	86	77	90	85	78	67	64	30	64	27	64	29	41	11
Woolly monk.	76	65	78	67			63	30	63	29	63	29	38	12
Ground sq.	63	26	64	30	63	30			85	77	84	74	38	8
Arctic sq.	63	23	64	27	63	29	85	77			85	76	38	9
Woodchuck	63	24	64	29	63	29	85	74	84	76			38	12
duck	39	9	41	11	38	12	38	8	38	9	38	12		

Figure 2. Phylogeny of orthohepadnaviruses. A. Unrooted full-length genome based tree. For the animal viruses, the host species from which the viruses were isolated are indicated. Human isolates are designated HBV plus the corresponding genotype; where applicable, sequences from two distinct subgenotypes were used. Nodes indicate common ancestors, the lengths of the connections are proportional to sequence distances. Note that divergence among individual human isolates is larger than between human and ape viruses. B. Genome and PreS domain amino acid identities between orthohepadnaviruses. Numbers indicate percent identities for the nucleotide sequences of the genomes (columns labeled gen), and protein sequences of only the PreS1/PreS2 regions (prS). Note the much lower identity scores for the PreS proteins *versus* the genomes, demonstrating the high diversity of PreS between different viruses.

Phylogenetic relationships between hepadnaviruses and between their recognised natural host species

Phylogenetic distances between different hosts are likely reflected in corresponding sequence differences between their respective viruses. The small genome size of hepadnaviruses makes derivation of sequence-based phylogenies rather straightforward; however, this is not the case for determining quantitative distances between hosts.

Due to the medical importance of HBV, thousands of genome sequences have meanwhile been determined; they can be classified into eight distinct genotypes (A to H). A genotype, by definition, must differ in sequence from another by at least 8%, subgenotypes with one genotype by at least 4% [87]. In the unrooted phylogenetic tree shown in Figure 2A, two representatives of each of the eight HBV genotypes (one from each subgenotype if applicable) have been combined with the full-genome sequences of the known animal orthohepdnaviruses; Figure 3A shows a corresponding analysis for the avihepadnaviruses, however based on the protein sequences of only the PreS regions, with three representatives of the Eastern and three of the Western clade of DHBV, including the commonly used laboratory strain DHBV16. Another parameter that illustrates relatedness is 'percent identi-

B.

host genus	Anas gen	Anas prS	Chloephaga gen	Chloephaga prS	Neochen gen	Neochen prS	Aix gen	Aix prS	Anser (Ross´) gen	Anser (Ross´) prS	Anser (snow) gen	Anser (snow) prS	Gruidae (crane) gen	Gruidae (crane) prS	Ardea (heron) gen	Ardea (heron) prS	Ciconia (stork) gen	Ciconia (stork) prS
Anas (various)	90	88	86	86	87	86	82	70	82	71	89	84	84	75	77	53	78	54
Anser (snow)	89	84					81	70	81	71			84	78	78	55	78	55
Anser (Ross´)	82	71					99	98			81	71	85	80	78	56	78	57
Gruidae (crane)	84	73					85	80	84	80	84	78			78	55	79	58
Ardea (heron)	77	53					78	55	78	56	78	55	78	55			86	78
Ciconia (stork)	78	54					78	56	79	57	78	55	79	58	86	78		

Figure 3. Phylogeny of avihepadnaviruses. A. Unrooted tree based on PreS domain protein sequences. Genera are given in italics, examples for specific species from which the viruses were isolated are indicated below. For the cranes, the family designation Gruidae is used to avoid confusion with species designation *Balearica* for the crowned crane. *Anas*, *Neochen*, *Chloephaga*, and also *Aix*, all belong to the family Anatidae. Note the near identity of the viruses from the Mandarin duck and Ross's goose, and the close relation of these viruses with the crane viruses. B. Genome and PreS domain amino acid identities between avihepadnaviruses. Divergence between isolates from different genera of the Anatinae, and even from the snow goose, is not larger than that among isolates from within the genus *Anas*; by contrast, the Mandarin duck/Ross's goose isolates are even less related to the duck isolates than the crane viruses, and their divergence from the other duck isolates is higher than that between heron *versus* the stork isolates.

ty' which, after alignment, indicates the proportion of identical nucleotides, or amino acids, at a given position (Figs 2B and 3B).

The orthohepadnaviruses cluster in three groups: hominoid (i.e., hominid plus gibbon) primate HBVs; WMHBV, i.e., New World monkey virus; and ground squirrel (GSHV) plus woodchuck (WHV), i.e., rodent viruses. For the avihepadnaviruses, three major clades are seen: one encompassing the duck and most goose viruses; one for the Ross's goose/Mandarin duck viruses which clusters with the crane viruses; and one comprising stork and heron viruses. Comparison of the genome sequences gives similar results, except that the Ross's goose/Mandarin duck and crane HBVs are not predicted to have a common ancestor; however, they remain about equidistant from the duck isolates (not shown). These distances are reflected in the percent identity values (Figs 2B and 3B), which for the entire genomes are minimally about 86% within the hominoid group, about 78% for hominoid *versus* WMHBV, 63% for hominoid *versus* rodent, and about 40% for hominoid *versus* avian; minimal identities among the rodent viruses are 84%, among the avian viruses 81–89% for duck, goose and crane viruses, about 75% for these *versus* stork and heron, and about 85% between stork and heron viruses. Percentage values are similar for the S proteins, but significantly lower for the PreS regions; this much higher than average diversity

in PreS is one of the arguments for its role in determining host range (see below). PreS identity scores drop to 72% within the hominoid group, to about 65% for hominoid *versus* WMHBV, and to below 30% for hominoid *versus* rodent viruses; among the rodent viruses, minimal identities are around 75%. Identity between ortho- and avihepadnavirus PreS regions is only around 10%.

For the avian viruses, PreS percent identity scores are around 70% within the duck and goose virus group; again a higher value is observed for these *versus* crane viruses (75–77%), but a significantly lower for all former viruses *versus* heron and stork viruses (53–55%).

There are several noteworthy points. All hominoid ape viruses cluster in between the human isolates, i.e., even the gibbon viruses are as close to the human viruses as these are among each other; WMHBV is the only true outlier. While an initial caveat was that those animals might have acquired their viruses from humans, newer sequences were derived from wild-caught animals that, *bona fide*, had not been in close contact with humans. Hence these viruses appear to be true ape viruses [75]. There is also evidence for recombination between chimp, and notably, gibbon and human HBVs [88], suggesting that primate and human HBVs can share hosts. Hence HBV is certainly not a species-specific virus.

A similar trend is seen among the avihepadnaviruses where Eastern and Western DHBV isolates are as different from each other as they are from various other duck and goose viruses, except for the Ross's goose/Mandarin duck isolates. Notably, the crane virus is not further apart from the duck virus clade than the Ross's goose/Mandarin duck viruses. Thus CHBV is much more a 'DHBV-like' virus than the heron and stork viruses.

Host phylogenies

Establishing phylogenetic relationships between host species is much more complex, and traditional morphology-based *versus* partial sequence-based analyses frequently give discordant results. Quantitative molecular relations between natural hepadnavirus hosts are firmly established only for humans and chimps (about 1% overall diversity on the genome level [89]); the about 7% diversity between the human and the Rhesus macaque genome [90] give at least an indication of the genetic distances between primates, though these monkeys are not hepadnavirus hosts.

For all other hepadnavirus hosts, classification still has to rely on a combination of morphological traits, the sequences of mitochondrial and selected nuclear genes plus for a time-scale, fossile records. Hence the phylogenetic trees shown in Figures 4 and 5 should be regarded as an only approximate indicator of relative kinship between species. This is particularly true for bird phylogeny. While traditional approaches are still continued [91] cross-hybridisation studies revolutionised the prevailing genealogies [92] but now

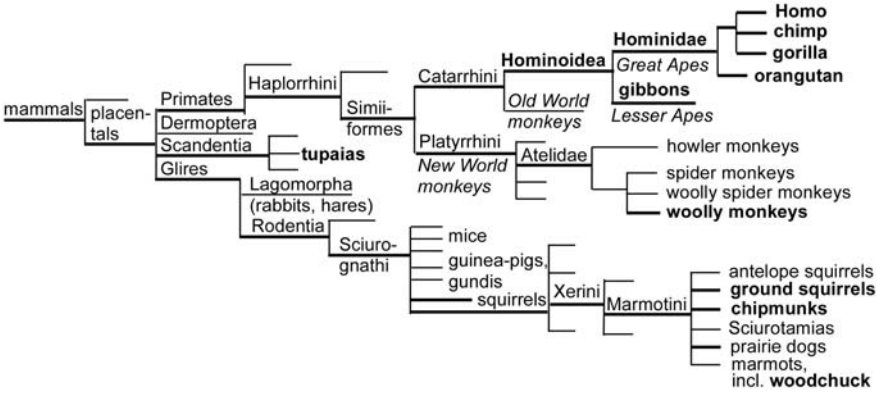


Figure 4. Phylogenetic tree of mammals with respect to recognised orthohepadnavirus host species. The tree is based on the classification used by the NCBI taxonomy data base (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser). Only those branches leading to hepadnavirus infectable host genera (bold face) are explicitly indicated; the lengths of the branches are not proportional to a specific time scale. Note that all known rodent hepadnavirus hosts belong to the tribe Xerini.

are considered more cautiously as more direct sequencing data come in; even then, the choice of DNA (mitochondrial *versus* nuclear) and of representative species can affect the results.

There is no doubt about a close relation among the Hominidae (Great Apes); however, the Hylobatidae (Lesser Apes; gibbons) are clearly distinct yet the HBVs isolated from gibbons cluster within the human isolates (above and [93]). By contrast, baboons, belonging to the Old World monkeys, are not [94], or extremely inefficiently [95], susceptible to HBV infection. Hence human HBV can not infect all primates yet all hominoid apes.

WMHBV, replicating to high levels ($>10^9$ vge/ml) in woolly monkeys, led to only inefficient infection of the supposedly closely related black-handed spider monkey (*Ateles geoffreyi*) [73]. Hence, in this sense, WMHBV appears to have a truly narrow host range. However, the picture is different with primary hepatocytes in culture (see below).

All other known mammalian HBV hosts belong to the rodents (comprising about 2,000 species), with the lagomorphs (rabbits and hares) as their apparently closest relatives. The rodentia form three major clades: mouse-related, guineapig-/gundi-related, and squirrel-/dormouse-related (Sciuridae). Within the subfamily Xerinae, both the genera *Marmota* (marmots, including woodchucks) and *Spermophilus* (groundsquirrels, rock squirrels) belong to the same tribe (Marmotini). Other genera from this tribe are chipmunks, Chinese rock squirrels, antelope-squirrels and prairie dogs [96, 97]. One special case are tupaias, commonly known as tree shrews. There is no evidence that they are a natural host for a hepadnavirus, yet they

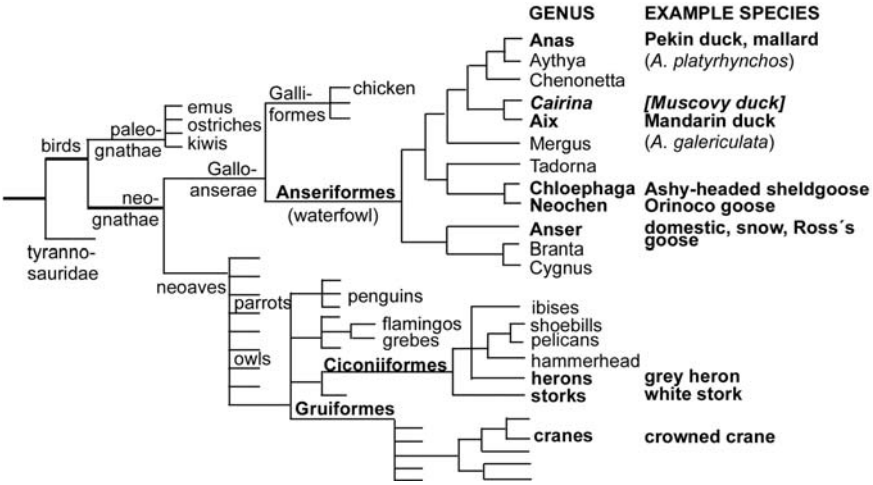


Figure 5. Phylogenetic tree of birds with respect to recognised avihepadnavirus host species. The tree is a composite based on the general Aves classification by Cracraft [105] for the Neoaves, and a mitochondrial DNA based phylogeny [104] for the Anseriformes. Note the close relation between the genera *Aix* and *Cairina*. As in Figure 4, the lengths of the branches do not imply a specific time scale.

may be infectable, at a very low level, by human HBV *in vivo* [98], and isolated tupaia hepatocytes are definitely infectable with HBV and WMHBV [99, 100] but not WHV. The evolutionary aspects of this remain obscure because tupaias are no more considered primates (Fig. 4) but as a distinct sister taxon (Scandentia) to the primates, flying lemurs (Dermoptera), and the rodents plus lagomorphs (Glires) [101].

Avian phylogeny is highly controversial but some basic concepts appear to be generally accepted (e.g., [102]; for additional information see http://animaldiversity.ummz.umich.edu). Accordingly, there are two subclasses, paleognaths (ostriches, emus, kiwis), and neognaths (Fig. 5); these are divided into two large infraclasses, Galloanserae (chicken- plus goose-/duck-related), and Neoaves, which comprise all other birds including hepadnavirus hosts such as herons, storks, and cranes (for a recent phylogenetic analysis of crane-related birds, see [103]). Galloanserae are divided into the orders Galliformes (landfowl), and Anseriformes (waterfowl) which comprise the families Anhimidae (screamers), Anseranatidae with the magpie goose as their only representative, and Anatidae; these are divided into the subclasses Anatinae (ducks), and Anserinae (geese and swans). The phylogeny of the individual genera within the Anatidae as shown in Figure 5 is based on mitochondrial DNA analyses [104], that of the Neoaves branch (see also http://tolweb.org/Neornithes/15834) on reference [105].

None of the known avihepadnavirus hosts belong to the Galliformes but many to the Anseriformes. Perhaps most striking is the wide distribution of infectable genera among the Anatidae, ranging from *Anas* (e.g., Pekin duck/*A. platyrhynchos* v. *domestica*; puna teal/*A. puna*; Chiloe wigeon/*A. sibilatrix*) and *Aix* (e.g., Mandarin duck/*A. galericulata*) to the apparently distant *Chloephaga* (e.g., Ashy-headed sheldgoose/*C. poliocephala*), and beyond to the Anserinae (e.g., snow goose/*A. caerulescens*; Ross's goose/*A. rossii*). Most surprisingly, the genus closest to *Aix* is *Cairina* yet *Cairina moschata*, the Muscovy duck, is not infectable, *in vivo*, by DHBV [106, 107].

Regarding the other bird HBV hosts, classical and molecular phylogenies agree that storks and herons (both Ciconiiformes) are relatively close to each other [102, 108] but not to cranes [103] (Gruiformes). This is consistent with the closer relation between the heron and stork *versus* the crane viruses. It should be noted, though, that even storks and herons have very long independent histories (estimated separation time about 80 Myr ago; [103]), spanning almost the same period of time as that leading to chicken and ducks after separation from their last common ancestors (about 100 Myr; [109–111]); for comparison, ducks and geese are thought to have separated about 35 Myr, rats and mice about 53 Myr ago [109].

Due to its origin from cranes, and ability to infect hepatocytes from the undoubtedly distant Pekin duck, crane HBV has been proposed to be the first, and unexpected, example of a hepadnavirus with a rather broad host range [84]. However, given the close relation of the crane virus isolates to the Ross goose/Mandarin isolates, and the ability of the latter to even infect Pekin ducks *in vivo* (see below), this result is much less surprising. Rather, the question is why the crane and Ross's goose/Mandarin duck viruses are so closely related (Fig. 3). More generally, there is no evidence that the Ross's goose/Mandarin duck virus, or DHBV, have a less broad host range than crane HBV as long no experiments pertaining to this question have been performed.

Cross-species transmission of 'natural' HBVs *in vivo*

One main benefit of experimental transmission is the ability to use a precharacterised, and possibly clonal, virus inoculum; when screening for natural infections the original source of virus is uncertain, particularly for captive animals that are kept in close to other species. Hence the near identity between the Mandarin duck virus isolates and the Ross's goose isolate may be due to that Ross's goose having contracted a hepadnavirus from other birds held in the same facility [74]; this limitation probably holds for most specimens from captive animals.

Cross-species transmission of natural hepadnaviruses, if successful, gives clear hints on host range; negative results, however, cannot be considered absolute. Essentially none of the natural hosts and their relatives is avail-

able in inbred form, and few can be kept under standardised conditions. Hence differences between strains or individuals could contribute to failure of transmission. Similarly, newborn animals which, due to their immature immune system, are more likely to be infectable than adults, are frequently not available. This holds particularly for primates, many of which, including woolly monkeys, are endangered. Even for the avihepadnaviruses, few other species than Pekin ducks are available for routine experiments. Hence, while different avihepadnaviruses have been tested in Pekin ducks, the host-range of DHBV itself has not extensively been explored.

The first cross-species transmissions of a clonal HBV isolate, by intrahepatic injection with cloned virus DNA rather than infection, were performed in chimpanzees and proved that this molecular clone of HBV was principally infectious and could use chimps as host [112, 113]; similar experiments were performed with animal HBVs [114, 115]. Probably, this is the only technique allowing establishment of infection by a single molecular clone; however, transmission is as well achievable by intraperitoneal (i.p.) or intraveneous (i.v.) injection of virions contained in serum from infected animals or supernatants from transfected cells. An alternative test system are primary hepatocytes from ducks [116], humans [117, 118], tupaias [98] and, at selected facilities, from some primates [119], and recently, the human hepatoma cell line HepaRG [3, 72]. However, *in vivo* and *in vitro* assays can give contradictory results (see below).

An additional system likely to reproduce many, though possibly not all, features of orthohepadnavirus infection is the use of hepatitis delta virus (HDV). HDV is an RNA virus that replicates autonomously but for infection and spread depends on the HBV envelope proteins [120]. Though, in contrast to HBV, PreS1 is not required for envelopment of HDV ribonucleoprotein complexes, it is essential for infectivity. Because HDV replicates to very high copy numbers (in the range of 10^5 genomes/cell), infection can very sensitively be monitored. This system was the first to raise doubts on an essential role for the TLM motifs [121], and similar negative data have been forwarded for HBV infection of human hepatocytes [59] and HepaRG cells [58]. Importantly, HDV can be enveloped with surface proteins from different orthohepadnaviruses, allowing to assess their impact on infection of primary hepatocytes from different hosts, in particular primates [119, 122].

Tables 1 to 3 summarise published data on cross-species transmission experiments with primate, rodent, and bird HBVs, indicating the inoculum virus, the recipient species, and the outcome of the experiments *in vivo*, or with cultured hepatocytes. An anecdotal report on HBV transmission into turtles [123] is not incorporated.

Neither for the mammalian nor for the bird HBVs does a uniform picture emerge from these data. HBV-like viruses can certainly infect chimpanzees, and the natural occurrence of highly similar viruses in hominid primates indicates that they can infect a spectrum of related species (Tab. 1).

Table 1. *In vivo* and *in vitro* infectivity of HBV and Woolly monkey HBV (WMHBV) for other primates, tupaias, and their isolated hepatocytes

Virus	Recipient species[a]		Infection[b] in		Remarks[c]	Reference
	Common	Systematic	vivo	vitro		
HBV	chimpanzee	*Pan troglodytes*	+		Intrahepatic HBV DNA transfection	[112, 113]
HBV	chimpanzee			+	HDV with HBV envelope; no PEG required	[119]
HBV	baboon	*Papio* sp.	-		HBV; infection also negative in immuno-suppressed animals	[94]
HBV	baboon	*Papio ursinus orientalis*	+/-		HBV; PCR positive; HBsAg and anti-HBsAg negative	[95]
HBV	baboon	*Papio* sp.		-	HDV with HBV envelope; negative even with PEG	[119]
HBV	tamarin	*Saguinus* sp.		-	HDV with HBV envelope; negative even with PEG	[119]
HBV	spider monkey	*Ateles geoffreyi*		+	HDV with HBV envelope; no PEG required	[119]
HBV	tree shrew	*Tupaia belangeri*	+/-		HBV; transient HBsAg, intrahepatic HBcAg; low HBV DNA, rapid clearance?	[98]
HBV	tree shrew		+		Adeno-HBV vector; HBsAg, HBV viremia; possibly from adenovector	[129]
HBV	tree shrew			+	Adeno-HBV vector; HBsAg, HBV replicative intermediates incl. cccDNA	[129]
HBV	tree shrew			+	HBV; HBsAg + HBeAg + HBV replicative intermediates incl. cccDNA	[99,100]
WMHBV	human			-	WMHBV; negative or below detection limit	Köck, Nassal, unpublished
WMHBV	human			(+)	HDV with WMHBV envelope; only with PEG	[119]
WMHBV	chimpanzee		+/-		WMHBV; negative or at detection limit	[76]
WMHBV	chimpanzee			(+)	HDV with WMHBV envelope; only with PEG	[119]
WMHBV	Rhesus monkey	*Macaca mulatta*	-		No details given	[73]
WMHBV	baboon		-		No details given	[73]
WMHBV	baboon			-	HDV with WMHBV envelope; negative even with PEG	[119]
WMHBV	tamarin		-		No details given	[73]
WMHBV	tamarin			-	HDV with WMHBV envelope; negative even with PEG	[119]
WMHBV	tree shrew			+	WMHBV; HBsAg + HBeAg + replicative intermediates incl. cccDNA	[100]
WMHBV	spider monkey		((+))		WMHBV; 4 log lower viremia than in woolly monkey, despite immuno-suppression	[73]
WMHBV	spider monkey			(+)	HDV with WMHBV envelope; without PEG less efficient than HDV with HBV envelope	[119]

[a]Systematic names are given only at first occurrence of the common name. [b]Infectivity scores are as follows: +, easily detectable viremia and antigenemia; ((+)), viremia and antigenemia clearly demonstrated though at low levels; +/–, some indirect evidence for infection; (+), infection detectable only in the presence of polyethylenglykol (PEG) as facilitator; –, no detectable infection. [c]Comments on specific experimental set-up

WMHBV, by contrast, appears specialised for woolly monkeys because transmission to even spider monkeys, their presumed closest relatives, was very inefficient [73]. Similarly, GSHV (Tab. 2) did not infect mice, guinea-

Table 2. *In vivo* infectivity of ground squirrel (GSHV) and Woodchuck hepatitis virus (WHV) for different rodent species

Virus	Recipient species name		Infection	Remarks	Reference
	Common	Systematic	in vivo		
GSHV	Golden-mantled ground squirrel	*Spermophilus lateralis*	-	GSHV; inoculum virus not sequenced but infectious for *S. beecheyi*	[125]
GSHV	13-lined ground squirrel	*Spermophilus tridecemlineatus*	-	as above	[125]
GSHV	Rat (Sprague-Dawley)	*Rattus norvegicus*	-	as above	[124]
GSHV	Mouse (Balb/c, C57/BL)	*Mus musculus*	-	as above	[124]
GSHV	Syrian hamster	*Mesocricetus auratus*	-	as above	[124]
GSHV	Guinea-pig	*Cavia porcellus*	-	as above	[124]
GSHV	woodchuck	*Marmota monax*	+	GSHV; high level viremia; less oncogenic than WHV in woodchuck	[126,130]
GSHV	chipmunk	*Eutamias* sp.	+	No details on chipmunk species	[125]
WHV	Beechey ground squirrel	*Spermophilus beecheyi*	-	Intrahepatic WHV DNA transfection; DNA infectious for woodchucks	[126]

pigs, and hamsters [124] and, more surprisingly, not two other squirrel species (golden-mantled ground squirrel/*Spermophilus lateralis*; thirteen-lined ground squirrel/*Spermophilus tridecemlineatus*) but could establish infection in the more distant chipmunk (*Eutamias* sp.) [125] and woodchuck [126]; conversely, WHV was not infectious for Beechey ground squirrels [126]. Thus GSHV appears to have a broader host range than WHV but there is currently no rational explanation. One problem is certainly that the true phylogenetic relationships between these species are insufficiently established.

Nearly all of the avihepadnaviruses isolated from Anatidae (Fig. 5) are infectious (Tab. 3) for Pekin ducks *in vivo*, and DHBV is transmittable to domestic geese, though not chicken [106] and Muscovy ducks (*Cairina moscata*; [106, 107]). The Orinoco sheldgoose (genus *Neochen*) isolate was infectious for Paradise shelduck (genus *Tadorna*) and Pekin duck (genus *Anas*), and even the Ross' goose virus-like Mandarin duck isolate efficiently infected Pekin ducks [74], clearly demonstrating cross-genera transmission. Thus many of these viruses have an expanded host range. However, there is also an exception in that the ashy-headed sheldgoose isolate, though closely related to DHBV (Fig. 5), did not infect Pekin ducklings (six out of six inoculated animals remained negative; [74]); whether this relates to a clone-specific problem (the full-length genome had to be restored using a separately amplified core gene) or a genuine restriction is unclear. Less surprisingly, the heron virus was not detectably infectious for Pekin ducks [82, 127].

The *in vitro* infection experiments with cultured hepatocytes gave often, though not always, consistent results; in general, *in vitro* infection was less

Table 3. *In vivo* and *in vitro* infectivity of natural avihepadnaviruses for other bird species and their hepatocytes

Virus from	Recipient species		Infection in		Remarks	Reference
	Common	Systematic	vivo	vitro		
Pekin duck	Wild mallard	*Anas platy-rhynchos*	+	n.d.	I.v. injection of DHBV-positive serum	[131]
Wild mallard	Pekin duck	*A.p. var. domestica*	+	n.d.	Possibly higher viremia than with DHBV from Pekin duck	[131]
Pekin duck	Domestic goose	*Anser anser domesticus*	+	n.d	High level viremia; increased pathogenicity (?)	[106]
Pekin duck	chicken	*Gallus gallus*	-	-	Same inoculum infectious in Pekin ducks	[106,107]
Pekin duck	Muscovy duck	*Cairina moscata*	-	n.d.	Same inoculum infectious in Pekin ducks	[106]
Pekin duck	Muscovy duck		n.d.	((+))	At most 1% as efficient as DHBV in Pekin duck hepatocytes	[107]
Puna teal	Mallard + Pekin duck		+	+	Apparently similarly efficient as DHBV	[74]
Orinoco goose	Paradise shelduck	*Tadorna variegata*	+	n.d.	Used to expand inoculum for cloning and Pekin duck infections	[74]
Orinoco goose	Mallard + Pekin duck		+	+	Similarly efficient as DHBV in vivo; reduced in Pekin duck hepatocytes	[74]
Chiloe wigeon	Pekin duck		+	+	Similarly efficient as DHBV in vivo; reduced in Pekin duck hepatocytes	[74]
Mandarin duck	Pekin duck		+	+	Efficient infection although virus most similar to Ross´ goose virus	[74]
Ashy-headed sheldgoose	Pekin duck		-	+	No in vivo infection detected in 6/6 birds, reduced in duck hepatocytes	[74]
Ross´ goose	Pekin duck		n.d.	+	~ 15% as efficient as DHBV	K. Dallmeier, unpublished
Snow goose	Pekin duck		n.d.	+	Efficient infection	[80]
White stork	Pekin duck		n.d.	((+))	Very inefficient infection	[81]
Crowned crane	Pekin duck		n.d.	+	Efficient infection	[84]
Grey heron	Pekin duck		-	n.d.	Negative with virus, and intra-hepatic HHBV DNA injection	[82,127]
Grey heron	Pekin duck			((+))	At most 1% as efficient as DHBV in Pekin duck hepatocytes	[127]

See footnotes to Table 1 for explanations; n.d., not determined.

restrictive. Examples include the ability of both HBV and WMHBV to infect primary tupaia hepatocytes [100, 128], or the infectivity, though at low efficiency, of the ashy-headed sheldgoose isolate to infect duck hepatocytes [74]. They also revealed in several cases that the apparent block to *in vivo* infection was not absolute; Muscovy duck hepatocytes were susceptible to DHBV infection [107], and Pekin duck hepatocytes to heron [127] and stork virus [81] infection, though all at 100- to 1,000-fold reduced efficiency. The crane virus infected Pekin duck hepatocytes with DHBV-like efficiency [84]; however, its sequence is, as mentioned, highly similar to the Ross's goose/ Mandarin duck isolates that are able to infect Pekin duck hepatocytes ([74] and K. Dallmeier, unpublished results) and Pekin ducks *in vivo* [74]. There are, however, also examples which are not easily reconciled with the *in vivo* data. HDV with an HBV envelope efficiently infected, in the absence of the facilitating agent polyethylenglykol (PEG), hepatocytes from humans

and chimps, but also from spider monkey yet not baboons. HDV with a WMHBV envelope was, as expected, poorly infectious for hepatocytes from humans, chimps, and baboons but the efficiency of spider monkey hepatocyte infection was not higher [119].

Hence no simple unifying concept on hepadnaviral host range can be inferred from these data.

Molecular basis of hepadnaviral host range

The genome-wide distribution of nucleotide exchanges between isolates in cross-species transmission of natural hepadnaviruses prevents an accurate assignment of infectivity phenotypes to specific genes or gene products. This problem can be overcome by using one characterised virus with defined mutations.

Attachment and entry represent major barriers for successful hepadnavirus infection (see above) yet the responsible cellular factors are only partially known for the avian HBVs, and not at all for the mammalian HBVs; hence identifying the host receptor(s) remains one of the holy grails in hepatitis B virology. For the human virus, many different candidate receptors have been proposed [3], including annexin V, IgA receptor, squamous cell carcinoma antigen 1, different forms of the asialoglycoprotein receptor, transferrin receptor, apolipoprotein H, fibronectin, or most recently, lipoprotein lipase as one of several proteins containing a peptide motif that binds to HBV PreS [132]. None, however, confers susceptibility to HBV infection of cells that, like Huh7 or HepG2, support downstream events in the HBV replication cycle. For DHBV, a 180 kDa glycoprotein (gp180, now known as carboxypeptidase D [133, 134]) and p120, a subunit of mitochondrial glycine decarboxylase [135, 136], have been implicated in the early steps of infection, yet again, neither of the two fulfils all criteria for a functional virus receptor; also, carboxypeptidase D is highly conserved between different bird species and not liver-specifically expressed, hence the PreS–carboxypeptidase D interaction can explain neither host nor tissue tropism of avihepadnaviruses [3].

In contrast, there is no doubt that the PreS domains of the hepadnaviral large envelope proteins (Fig. 6) are crucial for binding, and probably entry into, host cells [3], and thus are prime candidates for being involved in host specificity. This view is supported by the much higher sequence divergence of the PreS regions compared to other parts of the genome (Figs 2B and 3B). Hence most efforts on understanding the molecular basis of hepadnaviral host range have concentrated on mutating PreS and monitoring the consequences in one of the infection systems. While such data led to a seemingly precise definition of rather simple ‘host range determining regions’ within PreS (Fig. 6), the outcome of these experiments can drastically be affected by the specific test system used (see below).

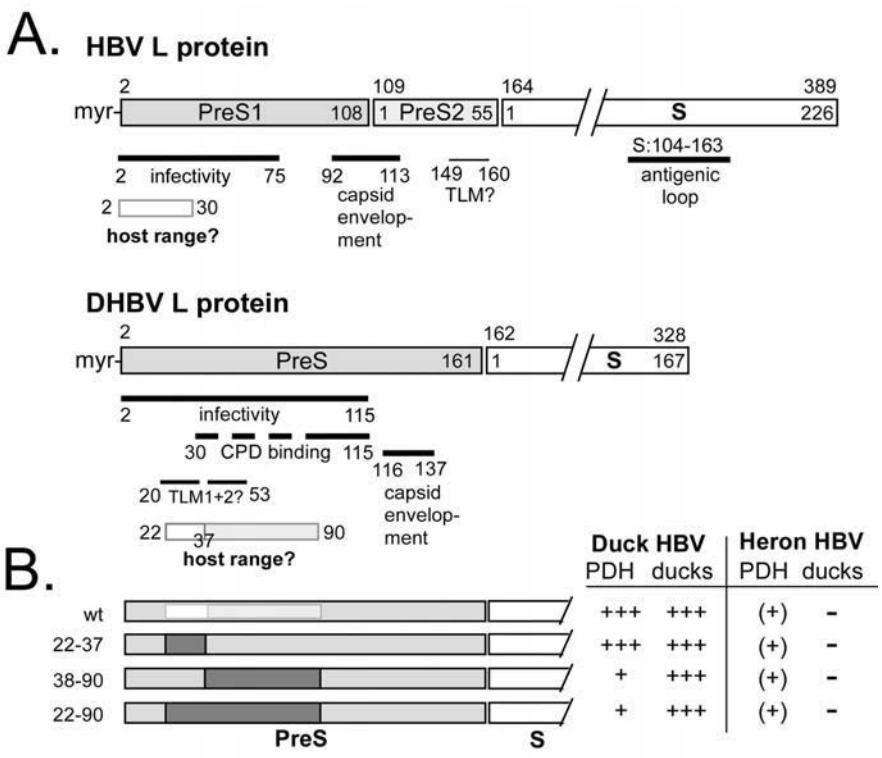


Figure 6. A. Hepadnaviral large envelope proteins and supposed functions of individual PreS regions. Numbers represent amino acid positions, numbers inside the bars indicate the lengths of the individual domains; myr refers to the myristoylation at the Gly-2 residues of all L proteins. Functional subregions are indicated similarly as described in reference [3], except that question marks have been added to the translocation motifs (TLM) and the so-called host range determining regions to indicate that their roles are, at the least, debatable. For the DHBV L protein, the carboxypeptidase D (CPD) binding region (essential, amino acids 86-115; stabilising, amino acids 30-86) and the positions of the short (PreS amino acids 22-37) and long (amino acids 22-90) forms of the proposed host determining region [127, 146] are indicated. B. Infectivities of chimeric DHBV and HHBV viruses for primary duck hepatocytes (PDH) and ducklings *in vivo.* Duck HBV refers to DHBV genomes carrying, in *cis*, the indicated regions of the heron HBV preS gene, and HHBV to heron HBV genomes carrying the corresponding regions of the DHBV preS gene. Infectivities are scored as follows: +++, wild-type DHBV-like; +, at least 10-fold reduced; (+), at least 100-fold reduced; –, no detectable infection.

Infection experiments with hepadnaviruses carrying defined envelope protein mutations

The two major experimental approaches to study the effects of PreS mutations on infectivity and host range in use are pseudotyping [127, 137] and the creation of chimeric viruses [126]; a third alternative, not discussed here in

detail, is infection inhibition by PreS-derived peptides [3]. Pseudotypes are viruses that carry an envelope which they do not encode themselves, usually provided in *trans* from a suitable expression vector; pseudotyped as well as chimeric virions can be generated by transfection of suitable cell lines, such as Huh7 or HepG2 for the primate, and LMH for the bird HBVs. An advantage is that only minimal changes in the genome are required to knockout the open reading frames for the large and, if desired, also small envelope proteins; this prevents problems potentially arising from more complex manipulations that might have unforeseen consequences on the overlapping P gene (Fig. 1B) and the numerous cis-elements distributed over the hepadnaviral genome [138]. However, because pseudotyped viruses do not encode their envelope proteins, infection is restricted to a single round and therefore can only be monitored in cultured cells, not *in vivo*. The reverse is true for chimeric viruses; however, principal replication competence and proper envelopment [54] can be tested beforehand by transfection.

Because of the lack of suitable host animals for the primate HBVs all studies so far have used the pseudotyping approach, sometimes with HDV instead HBV [119, 139–143], and primary human or primate hepatocytes or recently, the HepaRG cell line [58]. Mutations in the envelope proteins included systematic deletions in PreS, or in S [143, 144], or the replacement of segments with the homologous segments from another hepadnavirus, usually WMHBV [119, 145]. Collectively, the most recent, comprehensive data indicate that all infectivity determinants within HBV PreS1 are confined to the first 75 amino acids [58], with a possible role for the antigenic loop in S [143] but not the TLM motif in PreS2. HBV pseudotyped with a WMHBV-derived envelope displayed reduced infectivity for primary human hepatocytes, yet infectivity was restored when the N terminal 30 amino acids were derived from HBV PreS1 [145]; conversely, replacement of this segment by that of WMHBV in the context of the HBV L protein reduced infectivity. Partly congruent data have been obtained using the HDV pseudotype system [119] in that the first 40 WMHBV PreS1 amino acids implanted into the HBV L protein reduced infectivity for human hepatocytes; however, in the reverse setting the first 40 HBV PreS1 amino acids in WMHBV L only partly increased infectivity for the human cells; most surprisingly, however, this chimeric L protein had a strongly enhanced infectivity for spider monkey hepatocytes, to a level higher than that provided by a complete WMHBV L protein. Other than suggesting that PreS contains separate domains that interact with distinct cell receptors, interpretation of these results remains enigmatic, and farther-reaching *in vivo* experiments are currently not feasible with the primate HBVs.

Also the rodent HBVs are not ideal for such studies because no cell lines exist that, upon transfection, support efficient production of recombinant viruses, or pseudotypes. Hence there is only one early study on chimeric GSHVs in which about 30%, or about 80% of the S gene were replaced by WHV sequence; these viruses were infectious for ground squirrels upon

intrahepatic DNA inoculation [126], suggesting that S does not determine host range.

Due to these limitations, most studies on hepadnaviral host range have been performed with avihepadnaviruses although, until recently (see below), the potential of this system for true *in vivo* studies has not been exploited.

Most influential for the entire field has been a pseudotype study by Ishikawa and Ganem [127]. Based on the high infectivity of DHBV but about 100-fold lower infectivity of heron HBV (HHBV) for primary Pekin duck hepatocytes, a HHBV genome deficient for surface protein production was pseudotyped with chimeric HHBV envelope proteins in which various parts of the PreS region were replaced by the corresponding DHBV PreS sequences. All constructs yielded enveloped virions after transfection into LMH cells, which were then used to inoculate primary duck hepatocytes; formation of intracellular replicative intermediates indicated that the entire DHBV PreS sequence, and segments 1–108, 22–108, 1–90, but not 43–161, rescued infectivity; from this it was concluded that residues 22 to 90 determine the host range of avian HBVs. An additional chimera bearing only DHBV amino acids 22–37 was apparently also infectious for duck hepatocytes (T. Ishikawa, pers. communication), and PreS-derived peptides spanning slightly more than this region (DHBV PreS2-41), particularly if myristoylated like natural PreS, inhibited DHBV infection of duck hepatocytes [146]. Therefore, the prevailing view is that PreS amino acids 22–37, or possibly 22–90, constitute the host determining region of the avian HBVs [2, 3, 84, 146]. Notably, this region is not involved in carboxypeptidase D binding (Fig. 6), and a myristoylated peptide corresponding to the heron HBV PreS sequence 1–44 blocked DHBV infection of duck hepatocytes as efficiently as the autologous DHBV PreS peptide [146]. Evidently, this is not easily reconciled with a specific role of this PreS segment in determining host range.

In order to shed more light on the relevance of these data for a true *in vivo* infection, we generated two series of chimeric duck and heron HBVs, bearing, in cis, the short and long forms of the proposed host determining PreS regions from the other virus (Fig. 6, bottom); being able to inherit the chimeric envelope proteins to their progeny, these viruses are suitable for *in vivo* studies ([147] and Dallmeier and Nassal, submitted). Based on the published pseudotype data we expected that implantation of the heron sequence into DHBV would reduce, and conversely, implantation of the supposed DHBV PreS host determining region into heron HBV would increase infectivity for duck hepatocytes and ducklings. All the chimeric viruses, upon transfection, were fully replication-competent and formed comparable amounts of enveloped virions as wild-type DHBV. However, none of the heron HBV chimeras containing DHBV PreS segments 22–37, 22–90, 1–90 and 22–108, and or even the entire PreS region, displayed significantly increased infectivity for primary duck hepatocytes over wild-type heron HBV, or was able to establish *in vivo* infections in ducklings. Repeating the original pseudotype experiments, the complete DHBV PreS

region, as well as segments 22–108 and 22–37, though not 1–90 as reported, and 22–90 as inferred, clearly increased infectivity for hepatocytes. Thus in our hands substantial parts of, though not all, the previously published data were reproduced with the pseudotype system – but not with the chimeric viruses. There is currently no trivial explanation for this difference.

Even more disturbing are the data obtained with DHBV genomes carrying the corresponding heron HBV PreS sequences in their L proteins. As anticipated, a reduction in infectivity for duck hepatocytes was seen with chimeras in which the DHBV PreS segments 22–90, and also its subsegment 38–90, were replaced by heron HBV sequence; however, replacement of residues 22–37 had no negative impact on infectivity for duck hepatocytes. Hence in this setting, the short form of the supposed host determining region did not affect host range. Most surprising, however, were the *in vivo* infection data. All three viruses, including those with the large heterologous PreS segments and poor infectivity for cultured hepatocytes, were able to infect ducklings *in vivo*. The chimera with the entire originally proposed duck specificity determining region 22–90 replaced by heron virus sequence was further characterised and shown to establish high-titered, chronic infections in ducklings, and to be both horizontally and vertically transmittable. Quantitative monitoring during the early infection phase also showed kinetics of viral spread that were equal to, or even faster than those for wild-type DHBV. Thus, although seriously handicapped in cell culture, this virus without the proper host determining region for ducks was as infectious *in vivo* as its authentic DHBV parent. This defies the view that cultured cells are less restrictive than an intact host organisms, and it seriously questions the current concept of a short amino acid sequence in PreS as the crucial factor in host range determination of avian, and possibly also mammalian, HBVs. Unless the chimeric virus has switched to a different receptor, the data strongly suggest that, *in vivo*, the heron HBV PreS sequence interacts as well with the duck receptor(s) as the authentic DHBV PreS sequence. Notably, this is in accord with the lack of species-specific restrictions suggested by the comparably efficient inhibition of DHBV infection by both duck HBV and heron HBV PreS peptides [146], and the strong binding of the downstream PreS regions from both viruses to duck carboxypeptidase D [148]. Evidently cultured hepatocytes are not the ultimate system for definite statements on what determines hepadnaviral host range; moreover, the entire current concept of a short PreS segment as host determining region may need reevaluation.

Cellular restriction factors as determinants for hepadnaviral host range?

Another unresolved question is the suspicious non-susceptibility of Muscovy ducks for *in vivo* DHBV infection [106, 107]; in cell culture, infection is

not completely blocked, as is it with chicken hepatocytes, but is similarly inefficient as infection of Pekin duck hepatocytes with heron HBV [127]. Phylogeny provides no clue as to why the genus *Cairina* should not be infectable while Mandarin ducks can host a DHBV-like virus (see above). Hence Muscovy ducks may fortuitously lack a functional permissivity factor, or possess (a) restriction factor(s) that is/are absent from Pekin and other DHBV-infectable duck species. That non-permissiveness can, at least in cultured hepatocytes, be partially overcome by high doses of virus would be compatible with out-titration (abrogation) of such a negative factor but also with the absence of, or incompatibility with, host permissivity factors. As a first step towards distinguishing between these possibilities, we resorted to a classic genetic approach. Muscovy and Pekin ducks can interbreed, attesting to their close phylogenetic relation; such mule ducks are produced in large numbers in the food industry. A Muscovy drake was crossed with chronically DHBV infected Pekin duck females, and the offspring embryos were analysed for DHBV infection (K. Dallmeier, U. Schultz, M. Nassal; unpublished data). Four of four embryos tested showed clearly detectable, though slightly varying, levels of typical DHBV replicative intermediates in their livers. As F1-hybrids, these embryos carried one copy each of the paternal and of the maternal alleles. Hence their gain of susceptibility for infection is incompatible with the presence of a dominantly acting paternally inherited restriction factor yet compatible with the presence of a functional permissivity factor encoded by a maternal gene. The nature of this factor is currently unclear but differential cloning or similar techniques might be suited for its identification. Also, these results do not rule out that restriction factors play an important role in other hepadnavirus-host systems.

Conclusions and outlook

Many aspects of hepadnaviral replication have been elucidated in considerable detail; however our understanding of hepadnavirus host range and its molecular basis lags far behind. Certainly, hepadnaviruses do not have a broad host range yet whether their host range is truly narrow remains a matter of interpretation as long as the phylogenetic relations between the proven hepadnavirus host species are not rigidly defined. Unfortunately, hepadnaviral host range is sufficiently restricted so as to limit *in vivo* studies to few experimentally accessible species; even for DHBV the full spectrum of infectable hosts is unknown. *In vitro* infection assays allow access to cells from a wider range of hosts yet recent data both for orthohepadnaviruses, using human and Woolly monkey HBV, and for avihepadnaviruses, using duck and heron HBV, cast doubts on the transferability of *in vitro* data on statements on *in vivo* host range. One of the major challenges, as mentioned years ago [149], remains the unambiguous identification of the cellular receptor(s) mediating productive hepadnavirus infection; carboxypeptidase

D, though likely involved in DHBV infection, does not explain tissue- and host tropism, and does not at all seem to play a role for human HBV infection. Given the limited success of conventional approaches it is hoped that application of new techniques, such as expression profiling and gene knock-down by RNA interference, to permissive *versus* non-permissive cells will help answering this question; however, the potentially crucial roles of non-proteinaceous molecules as well as of host restriction factors must not be disregarded, and similarly, viral factors other than PreS could importantly contribute to hepadnaviral host range. Hence both systematic work and imaginative thinking will be required to solve this enigmatic aspect of hepadnavirus biology and realise the medically highly relevant issue of a small animal model for human HBV infection.

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Comparative Hepatitis
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The liver as immune escape site for pathogens

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Abstract

Besides its important function for protein, lipid and glucose metabolism the liver exerts scavenger function in order to clear the blood from degradation products. It is becoming increasingly clear that this scavenger function is closely linked to the liver's immune function, which favours induction of immune tolerance rather than immunity. The cell population most actively involved in scavenging of blood-borne macromolecules is an organ-resident cell population, the liver sinusoidal endothelial cells (LSEC). LSEC also have prominent immune-regulatory function as they bear the capacity to prime naive CD4 and CD8 T-cells after presentation of exogenous antigens on MHC Class II or MHC Class I molecules, respectively. The outcome of such T-cell priming by antigen-presenting LSEC is induction of T-cell tolerance. Here, we also discuss the other mechanisms and cell populations involved in mediation of hepatic immune tolerance. We describe the mechanisms of how a virus may get across cell barriers, in particular the endothelial cell barrier. Importantly, blood-borne virus is scavenged by LSEC. Here we discuss the experimental evidence in the literature that virus uptake by LSEC does not necessarily lead to lysosomal destruction but rather results in transcytotic transport of the virus to hepatocytes. Thus, LSEC may play a pivotal role in hepatocellular infection by blood-borne virus: (i) retrieval from the bloodstream and transcytotic transport for infection of the target cell, the hepatocyte, and (ii) prevention of virus-specific CD8 T-cell immunity by skewing antigen-specific T-cell responses by presentation of viral antigens at the very early stage of infection.

Introduction

The liver as an organ fulfils many different tasks that include protein and lipid metabolism, clearance function for removal of toxic waste products from the blood circulation, and a prominent immune-regulatory function. These diverse functions are performed by cooperative action of different cell populations in the liver that are either organ-resident or are derived from the bone marrow followed by functional adaptation through interaction with the unique hepatic microenvironment. Moreover, the unique microanatomy also contributes to these hepatic functions.

The hepatic microanatomy and cell populations

Hepatocytes form the most prominent hepatic cell population, which constitutes approximately 60–70% of liver cells [1]. Hepatocytes have extraordinary metabolic function for protein/lipid metabolism and for degradation/disposal of toxic material *via* the bile. Thus, hepatocytes are often regarded as the cell population that determines liver function. This assumption is supported by the clinical observations that loss of more than 90% of hepatocytes, either during acute disease such as toxic liver damage or fulminant viral hepatitis or as a consequence of chronic inflammatory disease leading to cirrhosis, is followed by the development of liver failure. However, from these observations it cannot be concluded that other cell populations do not contribute to the functional repertoire of the liver.

Hepatocytes tightly interact with neighbouring hepatocytes *via* their apical surface, which encloses the bile canaliculi. The cells are arranged in a sandwich format with their basolateral side facing the sinusoidal endothelium. Microvilli protrude from the basolateral surface of the hepatocytes to increase the surface and allow for efficient uptake and release of materials between hepatocyte and blood. Because of the metabolic function more than 30% of cardiac blood output passes through the liver in a unique mixed arterio-venous circulation. Nutrient-rich blood from the intestinal tract reaches the liver *via* the portal vein that branches into small venules entering the parenchymal area in the portal tract together with a branch of the hepatic artery. Arterial and venous vessels merge in the hepatic sinusoids that are lined by the specialised sinusoidal endothelial cells of the liver. Blood flow in the liver is slow (50–300 μm/s) as a result of the enormous cumulative sinusoidal vessel diameter and the mixed arteriovenous circulation that results in a low-pressure perfusion profile [2]. Blood percolates through the hepatic sinusoids to reach the central vein that drains into the inferior vena cava.

Kupffer cells constitute the hepatic macrophage population. They are derived from the bone marrow and show considerable half-life in the tissue. Kupffer cells are a rich source of soluble mediators such as prostanoids, cytokines and chemokines [3] that are not only operative in local immune regulation (discussed below) but also enhance hepatocellular expression of acute phase proteins through release of IL-6 and IL-1 [4, 5]. Moreover, Kupffer cells have potent phagocytic activity and eliminate opsonised microorganisms as well as cellular debris [6]. The macrophages patrol through hepatic sinusoids, even against the bloodstream and thus cause turbulence of blood flow (Fig. 1) [7]. However, most Kupffer cells are found in the periportal area, where the blood enters the liver.

Liver sinusoidal endothelial cells (LSEC) line the hepatic sinusoids from the portal tract to the central vein [8]. LSEC not only serve as a platform for migrating Kupffer cells or liver-associated lymphocytes (see below) but also mark the space of Dissé that is situated between the hepatocytes

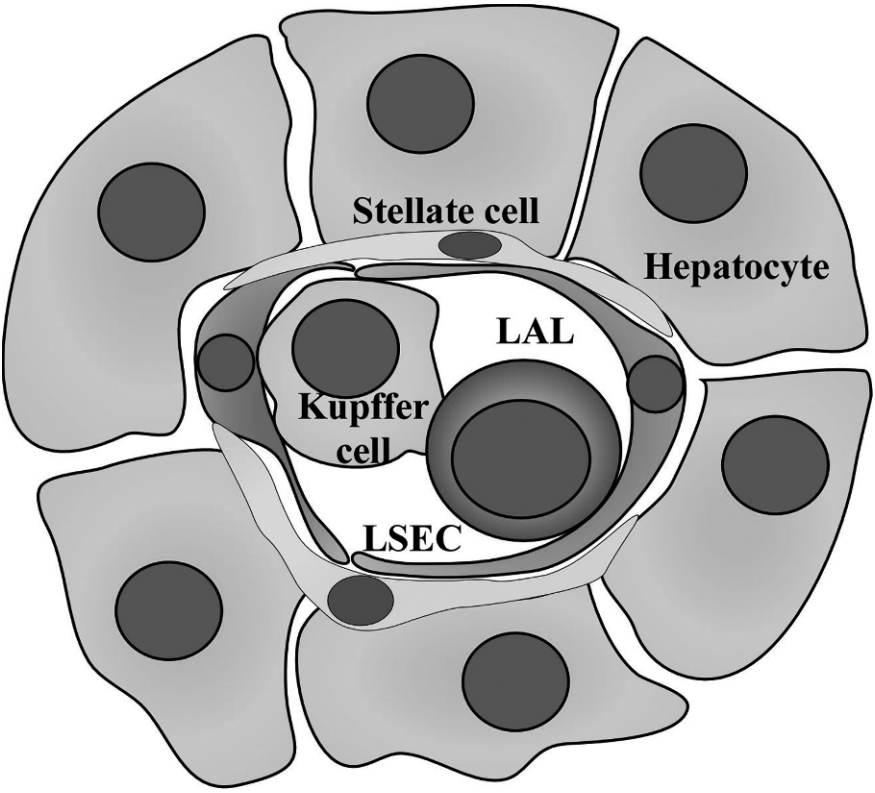

Figure 1. Schematic demonstration of a hepatic sinusoid. The lumen of the sinusoidal blood vessel is lined by liver sinusoidal endothelial cells. Liver-associated lymphocytes (LAL), a heterogeneous population of NK, NKT and T-cells, as well as bone marrow-derived liver resident macrophages, Kupffer cells, reside within the lumen of the sinusoids. LSEC physically separate cells and molecules circulating with the blood from hepatocytes. The area between hepatocytes and LSEC is called the space of Dissé. Stellate cells are located inside the space of Dissé giving them the possibility to control the diameter of the sinusoid.

and LSEC (Fig. 1). Due to the small sinusoidal diameter (7–10 μm) leukocytes and red blood cells that pass through the vessels cause a sinusoidal 'massage' or 'forced sieving' that increases fluid exchange between the sinusoidal lumen and the space of Dissé [9]. In contrast to microvascular endothelial cells in most other tissues, LSEC have fenestrations that allow for fluid exchange to occur between the blood and hepatocytes. The size of fenestrae is dynamically regulated and allows for passage of chylomicrons into the space of Dissé [10]. There is no basal membrane and tight junctions between adjacent LSEC are lacking. Although hepatocellular microvilli can protrude through endothelial fenestrae, the cumulative surface of LSEC is

much larger as compared to hepatocyte microvilli. LSEC are organ-resident cells that repopulate similar to hepatocytes from a so far ill-defined hepatic precursor cell population [11].

Stellate cells are situated between hepatocytes and LSEC within the space of Dissé (Fig. 1) [8]. These cells are the major storage site for retinoids, including vitamin A, and possess large numbers of microtubules and microfilaments. Stellate cells are involved in regulation of blood flow by actively controlling the sinusoidal diameter in response to vasodilatory or vasoconstrictory signals [12]. Moreover, stellate cells are a rich source of chemokines and cytokines. They are most important during development of hepatic fibrosis because inflammation or injury causes stellate cell activation that results in predominant deposition of extracellular matrix rather than degradation [13]. Stellate cells are in close interaction with hepatocytes and LSEC on either side and are in close association with nerve fibers [14].

Liver associated lymphocytes have originally been defined by their characteristic microscopic appearance but consist of a rather heterogeneous population of NK cells, T-cells and NKT-cells [15]. These cell populations are abundant in the liver with more than 10^{10} lymphocytes being found in human liver. Expression of certain chemokines and adhesion molecules by organ-resident liver cells and expression of appropriate homing receptors allow these cells to accumulate in the liver [16]. These cells also migrate through hepatic sinusoids [17]. The heterogeneity of hepatic lymphocytes indicates that these cells contribute in a complex fashion to local immune regulation in the liver.

In the portal tracts further cell populations are found such as bile duct cells, portal fibroblasts and dendritic cells. The portal tract appears to be a distinct functional compartment of the liver serving as entry portal for arterial and venous blood as well as exit portal for hepatic bile. The cellular and molecular mechanisms supporting the various requirements to allow these functional properties are largely unknown.

The liver as target organ for pathogens

Blood-borne pathogens that circulate with the bloodstream are eliminated mainly in two organ systems, i.e., the spleen and the liver. While macrophages in the marginal zone are responsible for elimination of bacterial pathogens in the spleen, Kupffer cells as well as platelets and neutrophils within hepatic sinusoids cooperate to achieve clearance of circulating bacteria in the liver [18, 19]. Mechanistically, intravascular neutrophil activation resulting from binding of platelets activated by bacterial TLR4-ligands leads to formation of neutrophil extracellular traps. These traps ensnare bacteria within the vasculature of the liver but also of the lung and promote bacterial trapping and phagocytic elimination [19]. Thus, the liver serves as a filter system to eliminate circulating bacteria from the blood. Central to

this antibacterial activity is the constitutive expression of innate immune receptors, such as the Toll-like receptors, on various liver cell populations that allows sensing of bacterial pathogens and appropriate cell activation [20]. Anti-bacterial defense in the liver apparently works efficiently, because there are few clinical examples for persistent bacterial infections of the liver, which are dealt with elsewhere in this book.

Viral infection of the liver, however, represents a significant problem and many hundred millions of patients persistently infected with hepatitis B or hepatitis C virus suffer from the consequences of immune-mediated chronic viral hepatitis. For distribution within a population hepatotropic viruses have to cross different barriers in order to achieve infection of a new host. The first barrier encountered is the skin or mucosal surfaces that block entry into the body. Even if this barrier is overcome, blood-borne virus again has to cross barriers such as endothelial surfaces/cell layers to reach their target cells, which are the parenchymal cells of the liver. In principle, for a virus to cross a physical barrier of epithelial or endothelial cells requires either a 'Trojan horse' strategy or utilisation of cellular transport machines with polar orientation. Both, the 'Trojan horse' strategy as well as transcytosis have been reported to be employed by viruses, such as HIV, to achieve infection of new hosts [21, 22].

Hepatotropic viruses like hepatitis A virus (HAV), hepatitis B virus (HBV) and hepatitis C virus (HCV) also have to cross epithelial barriers and the liver sinusoidal endothelial cell layer, which separates the hepatocytes from the blood, to finally infect their target cells, the hepatocytes. Very little is known on the mechanisms that allow these viruses to cross epithelial barriers in the gastrointestinal or urogenital tract [23]. Also after dissemination into the bloodstream, the mechanisms that allow efficient targeting of hepatocytes have not been clearly unravelled. Furthermore, LSEC have some unique features that complicate hepatocellular infection for a blood-borne virus.

The first obstacle is the physical barrier formed by LSEC. LSEC separate hepatocytes from leukocytes passing within the blood through the liver [8]. It has been observed that hepatocyte microvilli protrude into the sinusoidal lumen allowing for direct contact with blood-borne cells or pathogens [24]. But the overall surface area of LSEC compared to penetrating hepatocyte microvilli is much more prominent. However, LSEC have fenestrae, which are organised into sieve plates, and the mean size of these fenestrae, ranging between 100 and 200 nm, is dynamically regulated [10]. Fenestrae would provide a direct portal of entry for blood-borne viruses to the parenchyma. It has been proposed earlier for *Plasmodium berghei*, that sporozoites infect hepatocytes direcly through fenestrae [24], but this has not been confirmed by other scientists in the field; rather, sporozoite targeting of the liver involves initial infection of Kupffer cells [25] and transient infection of multiple hepatocytes by a single sporozoite before productive hepatocellular infection is finally achieved [26]. Hepatotropic viruses could also gain access

to the space of Dissé by diffusion through endothelial fenestrae. But as the space of Dissé is filled with extracellular matrix, which is not readily visible in electron microscopy, passive diffusion of particles larger than 12 nm into the space of Dissé and uptake by hepatocytes does not occur with high efficiency [27]. It is therefore reasonable to assume that hepatotropic viruses, which all exceed 12 nm in diameter, do not have direct access to hepatocellular microvilli in the space of Dissé. It is also unlikely that in the case of virus entry through fenestrae passive diffusion would suffice to bridge the distance to the hepatocyte within the extracellular matrix.

The second obstacle posed by LSEC is their enormous scavenger activity [28]. Blood-borne molecules are scavenged most efficiently and also blood-borne hepaDNA virus is found within minutes after intravenous application in the liver within LSEC, but not in other hepatic cell populations [29]. Interestingly, Kupffer cells do not contribute to the same extent as LSEC to scavenging of small molecules or pathogens from the blood stream [30]. Given the low-dose infection paradigm for hepaDNA-viruses, where one virus is sufficient to establish hepatocellular infection ([31] and S. Wiehland, personal communication). These experimental results rather support the notion that hepaDNA viruses initially taken up by LSEC still have the potential to reach and infect neighbouring hepatocytes, the only cell type where replication of this hepatotropic virus can take place.

The most likely molecular mechanism for viruses to come across this physical and functional cell barrier is transcytosis. Transcytosis is a common mechanism for macromolecules to overcome tight cell layers. It is divided into endocytosis, transport across the cell and exocytosis. The best-studied examples are transcytosis of IgA in polarised epithelial cells and the transcytosis of macromolecules through endothelium.

Receptor-mediated transcytosis

Immunglobulin A (IgA) is synthesised by plasma cells in the lamina propria of the digestive, urogenital and respiratory tract. But IgA exerts its function on the luminal side of the mucosa indicating that transport of IgA through epithelial cells is required. The trans-cellular transport of dimeric IgA (dIgA) with its receptor polymeric immunoglobulin receptor (pIgR) was extensively studied in polarised Madin-Darby canine kidney cells (MDCK), rat liver hepatocytes and hepatocyte cell lines. dIgA binds on the basolateral surface to its receptor pIgR and the resulting complex, but also the empty pIgR, and is then endocytosed *via* clathrin-coated pits [32]. On their way from the basolateral to apical cell surface the pIgR traffics through various endosomal compartments, depending on the model system. In MDCK cells the pIgR travels through a basolateral early endosome, a common endosome and an apical recycling endosome (ARE) [33–35]. In hepatocytes there are only two compartments: the basolateral early endosome and a

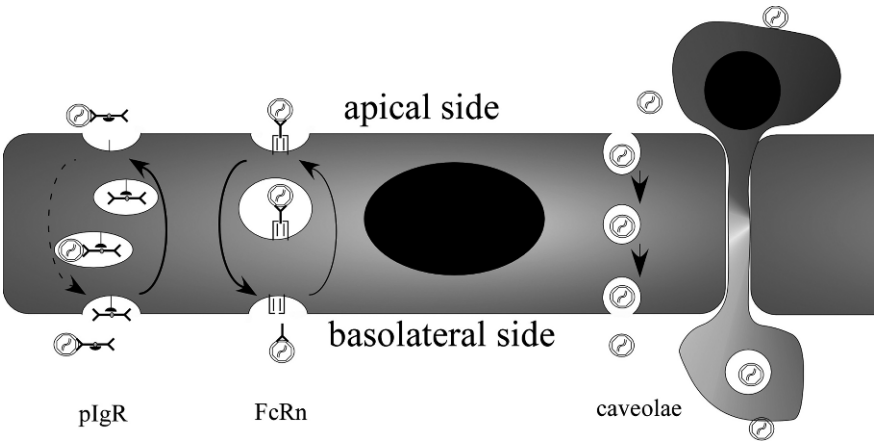

Figure 2. The different mechanisms of transcytosis. Immunglobulins or immunglobin/antigen-complexes are transported through cells after binding to specific receptors (left). The pIgR transports dIgA molecules, produced in the lamina propria, across the epithelial cell layer in a basolateral to apical direction into the lumen. The reverse mechanism, the transport of IgA-Antigen complexes from the lumen (apical) to the basolateral side of the cell, is also possible, but to a lesser extent. For IgG, the receptor involved in transcytosis is FcRn, which can transport IgGs and IgG-Antigen-complexes in both directions. In the middle, the direct, receptor-independent transport of particles through caveolae is depicted. Furthermore, transport of virus can occur independent of transcytosis through transinfection. Thereby a cell, which is not infected by the virus, transports virus bound to its surface across cell layers. After transmigration the cell mediates infection in trans of the target cells, where the virus then replicates (right).

subapical compartment (SAC) [35, 36]. After exit from the ARE/SAC, pIgR is transported to the apical plasma membrane. During this transport or at the plasma membrane pIgR is cleaved at the extracellular domain and this cleaved part is called the secretory component (SC) [37]. dIgA which has been bound to pIgR and is now in complex with cleaved SC is called secretory IgA (sIgA) (Fig. 2).

Binding of luminal IgA to virus and subsequently to pIgR on mucosal surfaces may therefore allow virus to cross the epithelial barrier. As mentioned above pIgR is endocytosed at the basolateral side irrespective if dIgA is bound or not. Transcytosis of 'empty' pIgR may thus allow for binding of luminal IgA to occur at the apical plasma membrane. This is possible because, at least in the MDCK model, pIgR molecules are not completely cleaved. IgA bound to pIgR at the apical plasma membrane has been shown to be transcytosed in a retrograde fashion and to be released at the basolateral side [38]. This empty pIgR can also bind HAV-IgA complexes on the apical side and transcytoses it back to the basolateral side where HAV-IgA complexes are released (Fig. 2). Such HAV-IgA complexes are shown to

be infectious for hepatocytes [23]. However, this retrograde transcytotic transport appears unlikely to be efficient for virus transcytosis, because the presence of virus-specific IgA leads to intracellular virus neutralisation during the process of transcytosis [39].

A second receptor involved in transcytotic transport is also specific for Ig, i.e., FcRn. FcRn is expressed in various tissues, namely the placenta where it allows selective delivery of IgG from the maternal to the fetal blood circulation, and also in the endothelium of small blood vessels and binds IgGs [40]. In intestinal epithelial cells as well as cultured intestinal T84 cells, IgGs are transcytosed in both directions by FcRn (Fig. 2) [41, 42]. This indicates a new role for the neonatal FcRn, namely transport of IgG from the basal side to apical side of the cell and subsequent secretion. After antigen-binding IgG-antigen complexes are bound by FcRn and transcytosed in the opposite direction delivering the complexes out of the lumen to the lamina propria where immune responses against luminal antigens then can take place [37]. LSEC also express FcRn and it is tempting to speculate that hepatotropic viruses complexed by IgG would bind to FcRn, which then would allow transcellular transport towards the space of Dissé. Still, it is unclear whether Ig-complexed virus would be subjected to intracellular neutralisation, similar to the observation for IgA-mediated neutralisation of HIV [39].

It has also been shown that receptor-mediated transcytosis from a non-immunglobulin receptor can lead to a transport of macromolecules/particles across a cell layer. Mannan-coated gold particles of 35 nm size, comparable in size to some viruses, injected into the tail vein of rats are rapidly taken up by endothelial cells in the liver. The mannan-coated gold particles accumulated in coated pits on the plasma membrane on endothelial cells. 1–40 h after injection, the gold particles are found near the basolateral side of LSEC next to the space of Dissé and later on they are also found in the adjacent hepatocytes [43], indicating transcytotic transport of mannan-coated particles and delivery into neighbouring hepatocytes.

Not only gold particles may be subject to transcytotic transport through LSEC. It has been suggested that blood-borne hepatotropic viruses, in particular duck hepatitis B virus (DHBV), are transcytosed through the liver sinusoidal endothelium. Breiner et al. has shown that fluorescently labelled viral particles and gold-labelled DHBV particles are rapidly taken up by LSEC [29]. Confocal laser scanning microscopy analysis revealed that fluorescently labelled viral particles colocalised with the DHBV receptor, carboxypeptidase D, in the trans-Golgi compartment, thereby rescuing virus particles from lysosomal degradation. Although rapid uptake of DHBV was observed into LSEC, there was no viral gene expression or formation of viral progeny [29]. We assumed that DHBV particles were transcytosed through LSEC following rapid initial uptake and that absence of LSEC infection would allow transcytosed DHBV to be available for subsequent infection of neighbouring hepatocytes [29]. Such a model would not only

explain the paradox that the vast majority of virus particles is taken up preferentially by scavenger LSEC and not by hepatocytes but still allows for small numbers of viruses to establish productive hepatocellular infection. It would also provide an explanation to the question how hepatotropic viruses reach the liver, because the scavenger function of LSEC would target the virus to the liver.

Receptor-independent transcytosis

Uptake/endocytosis of macromolecules can also occur in a receptor independent way. On endothelial cells negatively charged molecules are mostly rejected due to the negative charge of the apical plasma membrane. Clathrin-coated pits are also mostly negatively charged leading to the fact that cationic molecules preferentially bind to clathrin-coated pits and are subsequently degraded in lysosomes [44]. Negatively charged molecules, which are not accessible to this pathway, are taken up by pinocytosis and shuttled to the basolateral side where they are released. This transcytotic transport is mostly mediated through caveolae by caveolin 1 (Fig. 2).

It has been shown recently that caveolin 1 knockout mice cannot transport gold-labelled BSA from the luminal side of the vessel to the interstitium whereas wild-type mice are capable of transcytosing these particles. Given the small size of some viruses, which is in some cases comparable to gold particles, this is also an attractive model how viruses can cross endothelial cells to reach their target cells in the tissue.

Infection in trans

Transinfection is a common mechanism by which viruses reach their target cells. In contrast to infection in cis, where the virus directly infects its target cell, infection in trans describes a mechanism where the virus attaches first to a cell, which itself is not infected, but at later time points mediates infection of the target cell. Such a 'Trojan horse' strategy has been shown to be used by HIV, which first binds to dendritic cells in submucosal tissue and is carried by dendritic cells into the draining lymph node where infection of the CD4 T-cells occurs by close interaction of DC-surface bound HIV with CD4 T-cells [22]. Interestingly, HIV is captured by a C-type II lectin, DC-SIGN, that does not target HIV for lysosomal destruction upon receptor-mediated endocytosis but allows for re-shuttling of HIV-DC-SIGN complexes to the cell surface. This step facilitates infection of the target cells in trans. A similar mechanism for viral propagation has been reported for EBV [45]. This opens up the possibility that hepatotropic viruses bound to leukocytes that transmigrate across the endothelial barrier may achieve access to hepatocytes (Fig. 2).

However, DC-SIGN is known to bind a broad variety of different viruses [46–48] and DC-SIGN as well as its homolog DC-SIGNR (L-SIGN) are found in the liver [49]. Recent publications show that L-SIGN and also DC-SIGN are expressed on LSEC and that these lectins are capable of capturing HCV particles with high affinity [49, 50]. These molecules however do not promote entry of HCV and subsequent infection of LSEC. In contrast, it has been shown that liver sinusoidal endothelial cells promote the uptake of HCV-like particles in primary rat and human hepatocytes [51]. Highly purified hepatocytes in culture do not take up HCV-like particles, whereas in co-culture with LSEC uptake of virus particles into hepatocytes was observed. Collectively, these results suggest the notion that virus internalised by LSEC is not degraded but is available at later time points for infection of hepatocytes in trans.

The liver as an immunoregulatory organ

There is a continuous need to regulate antigen-specific immune responses in order to achieve protection from infection by microorganisms and at the same time to avoid autoimmune reactivity. This balance is maintained by regulating the activity of antigen-specific effector T-cells at various levels. Autoreactive T-cells are eliminated early on during their development in the thymus by negative selection, which assures absence of T-cells that recognise self-antigens with high affinity [52]. In the periphery, T-cell activity is further controlled by segregation of antigen thereby avoiding antigen-specific stimulation of T-cells; by clonal deletion following contact with tolerogenic antigen-presenting cells or incomplete T-cell stimulation; by induction of anergy again through tolerogenic antigen-presenting cells or by skewing immune responses either by modulating T helper cell responses (Th1–Th2) or by regulating effector cell function through regulatory T-cells. Peripheral immune tolerance entails the control of effector activity of CD4 T-cells but even more importantly of effector CD8 T-cells.

Secondary lymphatic tissue is believed to be the only location where induction of immune responses, either in the direction of immunity or peripheral tolerance, can be initiated. But numerous reports have pointed to the fact that the initiation of immune responses is not limited to secondary lymphatic tissue but may occur at many sites in the body [30, 53]. Early work in experimental transplantation surgery revealed that liver transplants were better tolerated by the recipient's immune system and that this tolerance was antigen-specific, because liver transplantation transferred protection from immune rejection provided the second transplanted organ was from the same donor, whereas third-party grafts were inevitably rejected [54–56]. Thus, cell populations within the liver contribute to the induction of peripheral immune tolerance.

Investigating the immune regulatory function of hepatic cell populations, one has to consider that the ability to present exogenous antigens on MHC Class II or MHC Class I molecules to CD4 or CD8 T-cells, respectively, is restricted to professional antigen presenting cells, i.e., macrophages, B cells and dendritic cells. Only these immune cell populations are equipped with co-signalling molecules that deliver in addition to T-cell receptor signaling further stimuli that influence the quality (immunity or tolerance) of the ensuing T-cell response.

Hepatocytes express little MHC Class I and no MHC Class II molecules and bear the ability to initiate CD8 T-cell responses. However, priming of naive CD8 T-cells by hepatocytes leads to incomplete T-cell activation and following initial proliferation, T-cells undergo apoptosis resulting in clonal deletion of T-cells recognising their antigen on hepatocytes [57]. Interestingly, location and timing during the induction of an immune response determines also the quality of an immune response. If antigen is first recognised by CD8 T-cells on hepatocytes immune tolerance ensues, even if antigen recognition occurs later on professional antigen-presenting dendritic cells in lymphatic tissue. In contrast, if antigen is first seen on activated dendritic cells in lymphatic tissue immunity develops, and subsequent antigen recognition on hepatocytes leads to development of immune-mediated hepatitis [58]. These results demonstrate that the initial antigen-specific contact of naive CD8 T-cells determines the outcome of an immune response. As virus-infected hepatocytes will present viral antigens on MHC Class I molecules to CD8 T-cells it is tempting to speculate that such tolerising mechanisms may also be operative during viral hepatitis.

There is a continuous debate as to whether there is direct contact of circulating CD8 T-cells with MHC Class I molecules on hepatocytes across stellate cells and LSEC. Using ultrastructural analysis it was reported that CD8 T-cells could penetrate with podosomes through sinusoidal fenestrations and establish direct, focal contact sites with hepatocytes [59]. The invasion of endothelial cells with podosomes initiates transcellular diapedesis of T-cells [60], but it has not been convincingly shown that T-cells recognising their antigen in the liver necessarily undergo transmigration into the hepatic parenchyma. However, recognition of a ubiquitously expressed antigen leads to preferential accumulation of CD8 T-cells in the liver, which supports the notion mentioned above that the liver initiates T-cell responses [61]. Controversial results were obtained when using different mouse strains to determine whether CD8 T-cells can recognise their antigen on hepatocytes *in vivo*. Depending on the mouse model used, naive or activated CD8 T-cells were either secluded from recognising their antigen on hepatocytes [62, 63], which indicated efficient barrier function by sinusoidal cell populations, or were incompletely stimulated [57, 59], which suggested direct T-cell-hepatocyte contact. Collectively, these results suggest that in case of infection with a hepatotropic virus, presentation of viral antigens by hepatocytes will trigger the induction of a CD8 T-cell response, which however

will be insufficient to clear infection. It is of interest to note that interaction with recently primed CD8 T-cells licenses hepatocytes to engage in further interaction with naive CD8 T-cells [64]. This would allow hepatocytes to engage in immune regulation, once the initial trigger for induction of an immune response has been given by a professional antigen-presenting cell population.

Bone marrow-derived antigen-presenting cells in the liver, such as Kupffer cells and dendritic cells, also contribute to local modulation of the immune response in the liver. Kupffer cells induce tolerance rather than immunity [65]. This tolerogenic phenotype of Kupffer cells may be related to the continuous exposure to gut-derived LPS [66, 67] that stimulates production of the immunoregulatory cytokine IL-10 in Kupffer cells [68]. In the absence of a strong proinflammatory stimulus such continuous expression of IL-10 may prevent the initiation of T-cell responses by this cell population. Furthermore, Kupffer cells are not endowed with the molecular machinery to present exogenous antigens on MHC Class I molecules to CD8 T-cells [30] and therefore cannot contribute to induction of virus-specific immunity, which requires cross-presentation of viral antigens on MHC Class I molecules to CD8 T-cells [69].

Hepatic dendritic cells are a heterogeneous population consisting of myeloid and plasmacytoid dendritic cells [70]. While dendritic cells are considered to be most potent in inducing T-cell immunity upon appropriate stimulation, various reports have shown that hepatic dendritic cells do not undergo full maturation upon contact with TLR-ligands and subsequently fail to induce strong T-cell immunity [71, 72]. It is likely that cross-presentation of viral antigens by hepatic dendritic cells also fails to induce virus-specific immunity and rather promotes induction of antigen-specific immune tolerance.

Stellate cells have been for many years in the focus of research on the molecular mechanisms promoting hepatic fibrosis [73]. Among the soluble profibrogenic mediators released by stellate cells is the potent immune-regulatory cytokine TGFb. But stellate cells do not only contribute as bystander cells to hepatic immune regulation but also have cell-autonomous immune functions. They constitutively express TLRs and are responsive to stimulation with LPS, which endows them with sentinel function [74]. Furthermore, they bear the capacity to present antigens on MHC Class I and CD1 molecules to CD8 or NKT-cells [75]. However, stellate cells do not have prominent scavenger function (Schurich and Knolle, unpublished observation) and therefore are unlikely to contribute to the control of T-cell responses directed against soluble viral antigens.

Among the different antigen-presenting cell populations in the liver, LSEC appear to have a prominent role, because they combine extraordinary scavenger activity with antigen-presenting capacity [28, 76]. Scavenger function of LSEC is related to most efficient receptor-mediated endocytosis and constitutive expression of scavenger receptors and

various pattern-recognition receptors, such as the mannose receptor, C-type lectins such as L-SIGN, the hyaluronic acid receptor (LYVE-1) and the members of the stabilin family [49, 77, 78]. This high endocytotic activity of LSEC is combined with their ability to present exogenous antigens both on MHC Class II molecules as well as on MHC Class I molecules. Antigen presentation on constitutively expressed MHC Class II molecules to CD4 T-cells is not very efficient [79] but in combination with co-stimulatory signaling through low level expression of CD80/86 allows LSEC to prime naive CD4 T-cells [80]. Importantly, CD4 T-cells primed by antigen-presenting LSEC fail to undergo functional maturation and do not develop into Th1 effector cells [80]. These cells rather express IL-4 and IL-10 upon antigen-specific restimulation and therefore likely have regulatory function for other immune responses.

More importantly, LSEC have the capacity to prime naive CD8 T-cells towards soluble antigens presented on MHC Class I molecules. In contrast to MHC Class II-restricted antigen presentation, the cross-presentation of exogenous antigens on MHC Class I molecules to CD8 T-cells is very efficient in LSEC [30]. But again, CD8 T-cells primed by antigen-presenting LSEC fail to undergo functional differentiation and rather gain a state of anergy where they do not respond to antigen-specific restimulation with T-cell effector function such as expression of IL-2/IFN-γ or cytotoxicity [30]. Tolerance induction by LSEC towards soluble antigens plays a role in two physiological situations; (i) oral tolerance: upon ingestion oral antigens rapidly spread in the entire organism within minutes to hours. Oral antigens are also distributed to the liver, where they are cross-presented by LSEC. Cross-presentation of oral antigens by LSEC leads to induction of CD8 T-cell tolerance towards oral antigens [11] and thus complements the induction of regulatory CD4 T-cells by tolerogenic dendritic cells within mesenteric lymph nodes [81]. (ii) tolerance towards apoptotic cell material: together with the spleen the liver contributes to removal of apoptotic cells from the systemic circulation [82–84]. We have reported that apoptotic cell material taken up by LSEC is processed for antigen presentation on MHC Class I molecules and that apoptotic cell material taken up *in vivo* is sufficient to drive antigen-specific T-cell stimulation [85] . However, the outcome of T-cell stimulation is again tolerance, because CD8 T-cells primed by LSEC presenting antigen from apoptotic cells failed to show antigen-specific effector functions [85]. Mechanistically, tolerance induced by LSEC requires the delivery of co-inhibitory signals *via* B7H1-PD1 receptor-ligand interaction to T-cells during the priming process [86]. Despite initial stimulation and proliferation, tolerance eventually develops in LSEC-stimulated T-cells, which express antiapoptotic molecules such as bcl2 [86]. This demonstrates that tolerance induced by LSEC is an active process that does not result from insufficient stimulation but rather relies on delivery of co-inhibitory signals. Furthermore, T-cells are anergised and not clonally eliminated after

contact with antigen-presenting LSEC [86], which is in contrast to clonal elimination of T-cells observed after T-cell priming by tolerogenic dendritic cells [87]. These results suggest that LSEC continuously skew CD8 T-cell responses towards circulating antigens and thereby contribute to the control of self-reactivity by preventing autoimmune reaction against apoptotic cell material or development of immunity against innocuous antigens such as food antigens.

Tolerance is not only achieved by skewing the functional repertoire of naive CD8 T-cells but also results from attenuation of effector CD8 T-cell function. Kupffer cells and LSEC contribute to antigen-specific retention and elimination of activated CD8 T-cells in the liver [88–90]. The co-inhibitory molecule B7H1 that is expressed on LSEC and Kupffer cells also contributes to elimination of activated CD8 T-cells in the liver [91]. Interestingly, B7H1-mediated elimination of CD8 T-cells contributed to the development of chronicity of viral infection in the liver [91].

The liver as an immune escape site for pathogens

Given the tolerogenic nature of the hepatic cell populations and the hepatic microenvironment it is not surprising that the liver functions as an immune escape site for pathogens. The sequence of antigen encounter in liver or lymphatic tissue and the nature of the cell that presents antigen first to CD8 T-cells determine the outcome of the antigen-specific immune response. This means that infection of the liver with hepatotropic viruses, which are rapidly taken up by LSEC and which infect hepatocytes, gives rise to a situation where antigen is presented by two prominent tolerogenic cell populations in the liver. Initial presentation of viral antigens in the liver could skew subsequent virus-specific immune responses for a certain time period so that additional mechanisms of viral persistence such as widespread hepatocellular infection or other immune escape mechanisms could develop. Thus, LSEC may contribute at two stages to viral infection of the liver: first, in acute infection where scavenging and possibly transcytosis may support liver-specific targeting of hepatotropic viruses and facilitate hepatocyte infection; and second, during the initial and later phases of antiviral immune responses where LSEC may contribute to attenuation of virus-specific CD8 T-cell responses by inducing virus-specific CD8 T-cell tolerance.

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Comparative Hepatitis
ed. by Olaf Weber and Ulrike Protzer

Drug candidates for the treatment of viral hepatitis

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Abstract

While progress has been made in treating viral hepatitis some problems are not adequately addressed with current therapies. This includes the challenge of treating both chronic as well as fulminant infections. This chapter provides an overview about current activities in the development of novel therapies for viral hepatitis. The activities in this area are very competitive and dynamic and, therefore, the list of projects and compounds might not be complete or reflect the very latest status of certain projects. However, it is clear that the medical need is being addressed and that novel approaches can be expected that might hopefully strengthen the antiviral armamentarium in the future.

Drug candidates under development for the treatment of hepatitis

There are many causes of hepatitis in humans – both infectious agents as well as non-infectious etiologies are of importance. This chapter provides a short overview about some drugs under development for the treatment of hepatitis. It is important to notice that a 'one-for all' treatment of hepatitis does not exist due to the diversity of the agents causing hepatitis and the various clinical courses of hepatitis. Table 1 illustrates this diversity; the causes of hepatitis will be discussed elsewhere in this book.

This chapter is focusing on recent developments in some of the epidemiologically most important forms of viral hepatitis. Several chapters in this book describe viral hepatitis in detail. Table 2 provides an overview about types of hepatitis caused by specific hepatotropic viruses.

In addition, viruses including the following ones may cause concomitant hepatitis upon a generalised infection: Hantavirus, certain Picornaviridae like Coxsackie viruses, Parvovirus B19, Adenoviruses, Mumps virus, European and Russian Spring-Summer encephalitis virus, haemorrhagic fever viruses like Lassavirus, Krim-Kongo-virus, Rift-valley fever virus, Marburg virus, Ebola virus.

Table 1. Major causes of human hepatitis

Infections that cause hepatitis	
Viruses	Hepatitis Viruses A,B,C,D,E, Concomitant viral hepatitis caused by Herpesviruses and other viruses, Denguevirus
Bacteria	*Brucella* spp. *Leptospira* spp. *Coxiella burnetii* other bacteria
Fungi	*Candida* spp.
Parasites	*Fasciola hepatica* *Echinococcus granulosus* *Schistosoma mansoni* *Leishmania* *Leishmania*
Non-infectious causes of hepatitis	
Toxins	Alcohol Mycotoxins Drugs
Abnormal metabolism/storage	Haemochromatosis Morbus Wilson
Cholangitis	
Autoimmune disorders	Lupoid hepatitis Chronic active hepatitis

Immunomodulatory agents under development

This chapter does not include combination products and very early preclinical approaches. The development of immunomodulatory agents is increasingly reported. This includes both therapeutic agents and therapeutic and prophylactic vaccines.

Albuferon (albumin interferon alfa)

Human Genome Sciences (HGS) and Novartis are developing albinterferon alfa-2b (Albuferon; ABF-656; Albuferon-alpha) for the treatment of HCV infection. Albuferon is a long-acting injectable albumin/human IFN-alpha-2b fusion protein. HGS is currently conducting two pivotal Phase III clinical trials of Albuferon: ACHIEVE 1 in treatment-naïve patients with genotype 1 chronic hepatitis C, and ACHIEVE 2/3 in treatment-naive patients with genotype 2 or 3 hepatitis C [1]. Clinical data suggest that Albuferon could offer hepatitis C patients a therapeutic option requiring less frequent injections and a virologic response comparable to that obtained with pegylated interferon alfa-2a, with the potential for less impairment of health-related quality of life [2–6].

Table 2. Overview about types of hepatitis caused by specific hepatotropic viruses

Virus		Disease		Transmission	Prophylaxis	Treatment
Hepatitis A virus (HAV)	Family: *Picornaviridae* Genus: Hepatovirus	hepatitis A	generally acute, approx. 10% of adults show a delayed course of the disease	Faecal-oral, food contamination (seafood)	vaccination, hygiene, education	symptomatic
Hepatitis B virus (HBV)	Family: *Hepadnaviridae* Genus: Orthohepadna-virus	Acute and chronic hepatitis B	Infection may become chronic (5–10% of infections), some age-dependency (*post partum*)	Parenteral transmission (blood, sexual transmission)	Vaccination, hygiene, education	Variety of agents including interferon alpha, pegylated interferon alpha, lamivudine, adefovir dipivoxil
Hepatitis C virus (HCV)	Family: *Flaviviridae* Genus: Hepacivirus	Acute and chronic hepatitis C	Infection may become chronic (50–80% of infections)	Parenteral transmission (Blood, blood products, sexual transmission, surgical instruments, iv. drug abuse)	Hygiene, education	Variety of agents including interferon alpha, pegylated interferon alpha, ribavirin
Hepatitis D virus (HDV)	Family: not classified Genus: Deltavirus	co-infection with HBV	up to 90% of infections become chronic, sometimes fulminant hepatitis	Parenteral transmission (blood, sexual transmission)	Hygiene, education	Symptomatic, treatment of HBV co-infection
Hepatitis E virus (HEV)[1]	Family: not classified Genus: Hepevirus	Acute hepatitis E	No chronic infections	Faecal-oral, food contamination (seafood)	Hygiene, education	Symptomatic
GB Virus Type C (GBV-C)		Not pathogenic	–	Vertical transmission, parenteral transmission (blood, sexual transmission)	Hygiene, education	–

[1]According to the International Committee on Taxonomy of Viruses (ICTV) HEV is still officially unassigned. The genus is named 'Hepevirus' and the family '*Hepeviridae*' in the NCBI taxonomy database

Table 3. Other (concomitant) viral hepatitis

Virus		Disease		Transmission	Prophylaxis	Treatment
Denguefever virus	Family: Flaviviridae Genus: Flavivirus Dengue type 1-4, yellow fever virus	Acute hepatitis with icterus ("yellow-fever"), fulminant hepatitis possible	Acute	Transmission by *Aedes aegypptii*	Reduction of *Aedes aegypptii* No vaccination available yet (under development)	Supportive treatment
Herpes simplex virus (HSV)	Family: Herpesviridae Subfamily: Alphaherpesvirinae Genus: Herpes simplex virus Herpes simplex virus type 1 and 2	Hepatitis in immuno-compromised patients, newborns	Acute	Contact infection (herpes)	-	Symptomatic or treatment of herpesvirus infection
Varicella zoster virus (VZV)	Family: Herpesviridae Subfamily: Alphaherpesvirinae Genus: Varicellovirus	Hepatitis	Acute	Contact infection (chicken pox)	Vaccination	Symptomatic or treatment of herpesvirus infection
Epstein Barr virus (EBV)	Family: Herpesviridae Subfamily: Gammaherpesvirinae Genus: Varicellovirus	Hepatitis in up to 20% of acute EBV infections	Acute	Contact infection	–	Symptomatic or treatment of herpesvirus infection
Cytomegalovirus (CMV)	Family: Herpesviridae Subfamily: Betaherpesvirinae Genus: Cytomegalovirus	Hepatitis in immuno-compromised patients, newborns	Acute	Contact infection	–	Symptomatic or treatment of herpesvirus infection

Pegasys® (Peginterferon Alfa-2a)

Pegasys® is a pegylated interferon alfa-2a that utilises a 40 kDa polyethylene glycol (PEG) strand to allow for stable and sustained therapeutic serum levels of interferon alfa-2a for up to a week with a single dose. Roche launched Pegasys® in 2001 for the treatment of chronic HCV and in 2003 for the treatment of chronic HBV infection and is currently investigating the drug in a variety of clinical settings including patients with various HCV genotypes, in subsets of HBV patients or in combinations. These combinations include ribavirin (Copegus®).

Pegylated interferon is also currently assessed for the treatment of chronic hepatitis D in a Phase II trial sponsored by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) [7]. This study will evaluate the effects of pegylated interferon on hepatitis D and hepatitis B. It will determine whether long-term therapy with this drug improves inflammation and scarring of the liver, thereby delaying or reversing cirrhosis, and whether the improvement can be maintained.

Human leukocyte interferon alfa

Human leukocyte interferon alfa, a formulation of natural interferon alfa, was launched in 1989 by Hemispherx Biopharma for the treatment of refractory or recurring external genital and perianal exophytic warts caused by human papillomavirus (HPV). It is currently investigated in various additional indications such as HIV, West Nile virus infection, severe acute respiratory syndrome (SARS) and HCV.

Hepatitis B immune globulin

A highly purified human hepatitis B immune globulin (gamma globulin) containing antibodies to hepatitis B surface antigen was approved in the US in 2006 for use after acute exposure to blood containing HBsAg including perinatal exposures of infants born to HBsAg-positive mothers and distributed as HepaGam B by Apotex.

Hepatitis C – ED004 – HCV neutralising monoclonal antibodies

One HCV disease-related area of special concern is liver transplantation. Chronic hepatitis C is the most common indication for liver transplants in the US and Europe (>10,000/year), but there is no satisfactory strategy at present to manage the post-transplantation recurrence of HCV. The need to prevent HCV re-infection is given since morbidity and mortality in these patients are

especially high. Innogenetics has an active preclinical program using monoclonal antibodies (mAbs), targeting the E1 and E2 HCV envelope regions. These human mAbs may have potential to cross-neutralise HCV genotypes as recently identified in collaboration with the NIH (NIAID, USA) [8].

Civacir® (Hepatitis C Immune Globulin)

Civacir® [Hepatitis C Immune Globulin (Human)] is an investigational human polyclonal antibody product that contains antibodies to hepatitis C virus (HCV). Preclinical studies indicate that Civacir contains antibodies that are neutralising to HCV. Nabi Biopharmaceuticals is developing Civacir to prevent re-infection with hepatitis C disease in HCV-positive liver transplant patients [9].

INNO 101

Evidence has been reported that the humoral and cellular immune responses to the HCV genotype 1b envelope protein E1 are largely impaired in patients with chronic active hepatitis C and may be important for clearance of HCV [10]. A candidate therapeutic vaccine was developed by Innogenetics consisting of a proprietary viral E1 protein of HCV together with an alum adjuvant to enhance the immune response.

Innogenetics is developing INNO-101 (InnoVac-C, Innovax-C), an injectable recombinant protein as a therapeutic vaccine for the treatment of chronic hepatitis C virus (HCV) infection. In June 2005, the company extended its ongoing Phase IIb trial.

INNO 102

INNO-102 is a polyepitope recombinant plasmid-DNA vaccine currently being investigated in a Phase I program for the prevention of hepatitis B infection.

Interferon Omega

On 21 May 2007 Intarcia Therapeutics announced final results from a Phase II study comparing the combination of injectable omega interferon and ribavirin to omega interferon alone in treatment-naïve patients with genotype 1 chronic hepatitis C. The results demonstrated that omega interferon in combination with ribavirin was well tolerated and showed robust antiviral activity comparable to published data on the use of alpha interferon plus ribavirin in similar patient populations [11]. However, omega interferon was administered by daily injections.

EHT 899

EnzoTherapeutics is developing EHT 899, a formulation of HBV viral protein designed to eliminate the undesirable immune response elicited by the HBV infection. According to the company, EHT 899 also enhances a secondary immune response to clear the viral infection, resulting in reduction in liver damage and decrease in viral load. In a clinical study a formulation of EHT 899 was administered orally to a total of 42 patients with chronic active hepatitis. Patients received the medication three times a week for 20–30 weeks and were followed for an additional 20 weeks. Results of the trial have shown that the drug was well tolerated in all subjects; 46% of subjects showed a decrease in HBV viral load and improvement in liver function tests; and 33% of subjects showed a decrease in inflammation seen on liver biopsy [12].

EHC 18

Enzo is also developing EHC 18 as a therapy for HCV. The Phase I clinical trial conducted by physicians at the Liver Unit of Hadassah University Medical Center in Jerusalem, Israel has met its safety endpoints. Enzo is currently looking to the next level of study [12].

Bavituximab

Bavituximab is a chimeric anti-phosphatidylserine monoclonal antibody developed by Peregrine Pharmaceuticals. A Phase Ib open-label, escalating repeat-dose trial of bavituximab in patients with chronic hepatitis C has completed enrolment [13].

R-7025

In collaboration with Maxygen, Roche is developing R-7025, a novel pegylated interferon alpha variant created through the use of Maxygen's proprietary MolecularBreeding® against HCV [14].

Resiquimod

Resiquimod is an immunomodulating agent, basically a Toll-like receptor 7 and 8 agonist that induces endogenous interferon-alpha. The agent is currently being developed for enhancing the protective response to hepatitis B vaccine. A clinical study was recently conducted to explore safety, pharmacokinetics, and pharmacodynamics of oral administration of resiquimod, in subjects with

chronic HCV infection. Two randomised, double-blind Phase IIa studies of resiquimod administered two times per week for 4 weeks. In a multicentre study in the US 12 subjects received resiquimod 0.01 mg/kg and four received placebo. In another single centre study six subjects received resiquimod 0.01 mg/kg, 11 received 0.02 mg/kg and six received placebo. Resiquimod 0.01 mg/kg was tolerated; two 0.2 mg/kg subjects discontinued treatment. More subjects reported severe grade adverse events at 0.02 mg/kg; events were consistent with systemic cytokine induction, including fever, headache, shivering, and lymphopenia. Mean maximum serum resiquimod concentrations were 3.82 ± 1.47 and 7.55 ± 4.17 ng/mL for 0.01 mg/kg and 0.02 mg/kg, respectively. At 0.02 mg/kg, two, three and one subjects had maximal reductions in viral levels of at least 1-, 2- and 3-logs, respectively; reductions were generally transient. Interferon-alpha levels appeared to correlate with decreases in viral titre and lymphocyte counts, as well as increase in neutrophil counts. Oral administration of resiquimod 0.02 mg/kg transiently reduced viral levels but was associated with adverse effects similar to interferon-alpha [15].

Small molecules under development

A-831

A-831 is developed by Arrow Therapeutics (recently acquired by AstraZeneca) for the treatment of hepatitis C. Arrow's HCV program is focused on NS5a which is essential for virus RNA production. NS5a lacks any homologue in the human genome and is well conserved across HCV genotypes. A-831 has shown potent activity in the replicon assay. Phase I trials on the compound were initiated in Q4 2006 [16].

ANA-380

ANA-380 (LB-80380) is developed by Anadys and LG Life Sciences for the oral, once daily treatment of chronic and acute lamivudine-resistant HBV infection. LB-80380 is a prodrug of the guanosine phosphonate nucleoside LB-80317 and was developed to improve oral bioavailability.

AVI-4065

AVI-4065 is a NeuGene antisense compound that is currently in Phase II trials at AVI BioPharma for the treatment of chronic HCV infection. However, AVI BioPharma announced on 8 August 2007 to discontinue their current hepatitis C clinical development program as a consequence of a focus effort [17].

BIVN-401

Bioenvision is developing BIVN-401 (methylene blue, Suvus®) for the treatment of chronic HCV infection. Suvus®, especially when photo-sensitised by light, acts by preventing replication of nucleic acid (DNA and RNA) in pathogens. Investigator sponsored Phase II clinical trials have been conducted. Bioenvision announced interim results at the UBS Global Life Sciences Conference in New York in September 2005. Suvus® was given to 25 patients with genotype 4 hepatitis C who had failed a prior treatment, including interferon in many of the patients. Sixteen (64%) of the patients had cirrhosis. Suvus® was given orally for 100 days and measurement of the viral load was performed at day 50. At 50 days, 22 (88%) patients had shown a reduction in viral load of greater than 70%. Of these responders, 14 (64%) had a clearance of greater than 90%, with four responders having shown complete viral clearance. Seven of the 25 patients had viral load measured at 100 days. Six of these patients show continued reduction in viral load and the seventh patient, who had been one of the three non-responders at 50 days, had a greater than 90% reduction in viral load. No major adverse events were noted. According to the company's website, an NDA was filed in Egypt [18].

Celgosivir

Celgosivir (MX-3253) is a alpha-glucosidase I inhibitor currently in Phase II clinical trials at Migenix as a monotherapy in first-line and interferon-intolerant genotype 1 HCV patients in Canada and in combination with peginterferon alfa-2b with or without ribavirin for the treatment of chronic HCV genotype I infections in Canada and the US. Celgosivir added to peginterferon alpha-2b and ribavirin results in improved viral load reductions and clinically significant benefit in genotype-1 non-responders [19]. Celgosivir is an oral prodrug of castanospermine, a natural product derived from the Australian black bean chestnut tree *Castanospermum australe*. Migenix acquired worldwide rights for the drug from Virogen [20].

Clevudine

Clevudine is an oral, once daily pyrimidine nucleoside analogue that has been approved for the treatment of hepatitis B in South Korea in 2006. It is marketed by Bukwang. Clevudine is currently being investigated in Phase III clinical trials in Asia by Eisai and in the US and in Europe by Pharmasset. Clevudine has demonstrated efficacy in both HBe-Ag-negative chronic hepatitis B with durable off-therapy viral suppression and in HBeAg-positive chronic hepatitis B [21, 22].

DDB-S

DDB-S (Lebecel) in currently in Phase II clinical trials at Daewoo Pharmaceuticals for the treatment of acute and chronic hepatitis.

DEBIO-025

DEBIO-025 is a cyclophilin inhibitor developed by Debiopharm for the treatment of HCV infections. It is currently in Phase II clinical trials. DEBIO-025 monotherapy for 15 days induced an antiviral effect *versus* HCV of 3.6 Log10 reduction in HIV-HCV co-infected patients, a population generally responding poorly to anti-HCV treatment. The antiviral effect was consistent throughout treatment on the three genotypes identified in the study. DEBIO-025 did also show some anti-HIV-1 effect (1 Log10 reduction). Reversible and dose-dependent hyperbilirubinaemia seemed to be the dose limiting factor for DEBIO-025 [23].

GS-9190

GS-9190 is an inhibitor of RNA-directed RNA polymerase (NS5B) currently under clinical evaluation for the treatment of hepatitis C [24].

HCV-796

HCV-796 is a non-nucleosidic NS5B inhibitor in Phase II clinical development at ViroPharma and Wyeth Pharmaceuticals for an interferon-combination treatment of HCV infection. On 10 August 2007 the company announced discontinuation of dosing in this trial due to potential safety issues [25].

ITMN-191 (R-727)

The compound is an oral HCV NS3/4A protease inhibitor being developed by InterMune and Roche. The compound has entered Phase I clinical trials [26].

NIM-811 (R-727)

NIM-811 is Novartis' non-immunosuppressive cyclosporin derivative that is currently being developed against HCV infections. Analysis using cyclosporin A (CsA) as a bioprobe showed that cyclophilin (CyP) B, a cellular

target of CsA, regulates the function of HCV RNA polymerase NS5B, which is essential for efficient viral genome replication. By targeting CyP, HCV genome replication was drastically suppressed [27]. The compound is currently in Phase I clinical trials.

R-7128

R7128 is being developed by Pharmasset and Roche for the treatment of chronic HCV infections. R7128 is a prodrug of a molecule named PSI-6130, an oral cytidine nucleoside analog that inhibits the RNA-directed RNA polymerase (NS5B) [28, 29]. PSI-6130 is the active component of R7128. At low concentrations, PSI-6130 was shown to be an inhibitor of HCV replication, specifically targeting the HCV RNA polymerase. In combination with interferon, PSI-6130 was active and additive to the activity of interferon alone in these preclinical assays. In October 2006, a Phase 1 clinical trial under an IND was initiated. This trial is a multi-centre, observer-blinded, randomised and placebo-controlled study designed to assess the pharmacokinetics, pharmacodynamics, safety, tolerability and food effect of R7128 in healthy volunteers and in patients chronically infected with HCV genotype 1, as well as provide antiviral potency data over 14 days in patients chronically infected with HCV genotype 1 [30].

R-1626

Roche is developing R-1626, an oral HCV NS5B polymerase inhibitor. The compound is a prodrug of R-1479. A Phase II trial was initiated in 2006 [31].

Tenofovir disoproxil fumarate

Gilead is investigating tenofovir disoproxil fumarate (Viread®), a prodrug of tenofovir, the nucleotide reverse transcriptase inhibitor as a treatment against HBV infections. Start of a Phase II clinical study is imminent evaluating tenofovir disoproxil fumarate (DF) monotherapy *versus* the combination of emtricitabine and tenofovir DF for the treatment of chronic HBV infection [32]. In June 2006, Gilead completed enrolment of two Phase III clinical trials to evaluate Viread® for the treatment of HBV [24].

Sch-503034 (Boceprevir)

The Schering-Plough compound boceprevir is an HCV NS3 protease inhibitor currently in Phase II clinical trials for the treatment of chronic hepatitis

C genotype 1 infection in combination with pegylated interferon with and without ribavirin in patients who do not respond to pegylated interferon and ribavirin combination. Fast track designation was granted by the FDA [33].

Taribavirin hydrochloride

Taribavirin (1-b-D-ribofuranosyl-1H-1, 2, 4-triazole-3-carboxamidine) is a synthetic nucleoside (guanosine) analogue under development by Valeant for the treatment of chronic hepatitis C. After oral administration, taribavirin is rapidly absorbed, after which it is readily and extensively taken up by the liver and converted into its active metabolite, ribavirin. Valeant has begun enrolling patients in a Phase IIb clinical study combining taribavirin with a pegylated interferon. This study is subsequent to two pivotal Phase III trials for taribavirin that were completed in 2006 [34].

Telaprevir

Telaprevir (VX-950) is an oral HCV protease inhibitor developed by Vertex in combination with pegylated interferon for the treatment of hepatitis C. The compound is currently in Phase II clinical trials. In June 2006, Vertex and Janssen Pharmaceutica entered into a co-development and co-promotion agreement. The compound has been granted fast track designation by the FDA. Start of Phase III studies is imminent [35].

UT-231B

UT-231B is in Phase II clinical trials at United Therapeutics for the treatment of HCV. The 12 week study assesses the antiviral effect of oral UT-231B in non-cirrhotic HCV patients who have failed interferon-based therapy. UT-231B is an iminosugar able to alter the assembly of the virus [36].

Valopicitabine

Idenix announced in July 2007 that the development program of valopicitabine (NM283) for the treatment of hepatitis C has been placed on clinical hold in the United States based on the overall risk/benefit profile observed to date in clinical testing [37]. Valopicitabine is an RNA polymerase inhibitor that was investigated in a Phase II study to evaluate the combination of pegylated interferon alfa plus valopicitabine in patients with hepatitis C.

Valtorcitabine dihydrochloride

Idenix is conducting clinical trials of valtorcitabine, an investigational drug candidate for the treatment of chronic hepatitis B. Valtorcitabine is being developed as a fixed-dose combination with telbivudine for those patients for whom treatment with a single agent may not be adequate. In laboratory studies and animal models of the disease, telbivudine and valtorcitabine each demonstrate highly specific antiviral activity against HBV. In these preclinical studies, the combination of the two agents has demonstrated even greater antiviral activity than either drug alone. Valtorcitabine is one of several novel agents in clinical development that have shown promising clinical profiles in patients with chronic hepatitis B [38].

VCH-759

ViroChem Pharma Inc has identified several low molecular weight inhibitors of HCV polymerase which inhibited HCV replication in the cellular replicon model. One of them completed Phase I clinical trials and is progressing in Phase IIa proof-of-concept trials [39].

VGX-410C (Mifepristone)

Mifepristone is currently investigated by Viral Genomix (now named VGX Pharmaceuticals) in a randomised, open-label Phase II trial in HCV-infected patients [40].

Vaccines and other drug candidates under development

GI-5005

GI-5005 is currently developed by GlobeImmune in Phase Ib clinical trials for the treatment of HCV infections. GI-5005 belongs to a group of targeted molecular immunotherapies called Tarmogens (“targeted molecular immunogens”) for the treatment of infectious diseases and cancer. Tarmogens are whole, heat-killed recombinant *Saccharomyces cerevisiae* yeast particles genetically modified to express one or more protein targets that stimulate the immune system against diseased cells. The whole heat-killed yeast with the antigen expressed inside is administered to the patient. Cytotoxic T-cells are being discussed as effector cells. Interim results have shown that GI-5005 generated cellular immune responses in 12/29 patients (41%), elicited a statistically significant improvement in alanine amino transferase (ALT) levels from baseline and caused near 1 log10 viral load reductions in three patients [40].

HCV E1E2/MF59C.1 Vaccine

Novartis (Chiron) is developing an HCV vaccine in a Phase I randomised, observer-blinded, placebo-controlled study to evaluate the safety, tolerability and immunogenicity of HCV E1E2/MF59 Vaccine in healthy HCV-negative adults. Enrolment has been completed [41].

Hepatitis B vaccines

This section does not include combination products and very early preclinical approaches.

Various efforts to develop novel vaccines against hepatitis B are underway and include approaches like virus-like particles (VLP) as by CellDex Therapeutics' CDX-2101. CDX-2101 is a VLP comprising 240 modified HBcAg protein subunits, which have been engineered to improve stability, remove the DNA-binding C-terminus, and to inactivate a dominant epitope recognised by HBcAg-specific antibodies from infected individuals. Celldex has completed a full preclinical research program that demonstrates the effectiveness of CDX-2101 vaccine in animal models. Clinical studies are imminent according to the company [42].

Dynavax is developing HBV-ISS (Heplisav), an HBV vaccine that combines an immunostimulating sequence (ISS), ISS-1018, of HBV DNA which acts as a toll-like receptor (TLR)-9 agonist, with recombinant HBsAg for the potential treatment of HBV infection. A pivotal Phase III trial is ongoing in Singapore, Taiwan, Korea and the Philippines [43]. Dynavax is also developing another HBV vaccine that combines surface and core antigens of HBV for the potential treatment of chronic HBV. A Phase I study was initiated earlier in 2007. Henogen is developing HB-AS02V, an adjuvanted vaccine, for the potential prevention of HBV infection in immunocompromised subjects. Phase III studies were initiated in 2007.

Hepatitis C vaccines

Although numerous candidate vaccines are being investigated in very early preclinical settings there is currently only limited data on clinical programs available. A therapeutic HCV vaccine is developed by Pevion by using its PeviTER and PeviPRO virosomes containing synthetic HCV peptides (PEV2A and PEV2B). A Phase I trial was initiated in 2006 [44]. Intercell has a therapeutic HCV vaccine in Phase II clinical trials [45]. This peptide vaccine (IC41) consists of five synthetic peptides harbouring HCV T-cell epitopes and poly-L-arginine as synthetic adjuvant. In a randomised, placebo-controlled trial, 128 HLA-A2 positive healthy volunteers received four s.c. vaccinations of seven different doses IC41, HCV peptides alone, poly-

L-arginine alone or saline solution, every 4 weeks. IC 41 induced responses in all dose groups. Poly-L-arginine was required for the aimed-for Th1/Tc1-type immunity [46].

Hepatitis E vaccine

GlaxoSmithKline is investigating a prophylactic hepatitis E virus vaccine in Phase II clinical trials [47].

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