FINAL

Report on Carcinogens Background Document for

Hepatitis B Virus

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FOREWORD

The Report on Carcinogens (RoC) is prepared in response to Section 301 of the Public Health Service Act as amended. The RoC contains a list of all substances (i) that either are known to be human carcinogens or may reasonably be anticipated to be human carcinogens; and (ii) to which a significant number of persons residing in the United States are exposed. The Secretary, Department of Health and Human Services (DHHS) has delegated responsibility for preparation of the RoC to the National Toxicology Program (NTP) who prepares the Report with assistance from other Federal health and regulatory agencies and non-government institutions.

Nominations for listing in or delisting from the RoC are reviewed by a formal process that includes a multi-phased, scientific peer review and multiple opportunities for public comment. The review groups evaluate each nomination according to specific RoC listing criteria. This Background Document was prepared to assist in the review of the nomination of Hepatitis B Virus. The scientific information in this document comes from publicly available, peer reviewed sources. Any interpretive conclusions, comments or statistical calculations, etc made by the authors of this document that are not contained in the original citation are identified in brackets [ ]. If any member(s) of the scientific peer review groups feel this Background Document does not adequately capture and present the relevant information they will be asked to write a commentary for this Background Document that will be included as an addendum to the document. In addition, a meeting summary that contains a brief discussion of the respective review group's review and recommendation for the nomination will be added to the Background Document, also as an addendum.

A detailed description of the RoC nomination review process and a list of all nominations under consideration for listing in or delisting from the RoC can be obtained by accessing the NTP Home Page at http://ntp-server.niehs.nih.gov. The most recent RoC, the 9th Edition, was published in May, 2000 and may be obtained by contacting the NIEHS Environmental Health Information Service (EHIS) at http://ehis.niehs.nih.gov (800-315-3010).

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Criteria for Listing Agents, Substances or Mixtures in the Report on Carcinogens

U.S. Department of Health and Human Services
National Toxicology Program

Known to be Human Carcinogens:

There is sufficient evidence of carcinogenicity from studies in humans, which indicates a causal relationship between exposure to the agent, substance or mixture and human cancer.

Reasonably Anticipated to be Human Carcinogens:

> There is limited evidence of carcinogenicity from studies in humans, which indicates that causal interpretation is credible but that alternative explanations such as chance, bias or confounding factors could not adequately be excluded; or
>
> There is sufficient evidence of carcinogenicity from studies in experimental animals which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors: (1) in multiple species, or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site or type of tumor or age at onset; or
>
> There is less than sufficient evidence of carcinogenicity in humans or laboratory animals, however; the agent, substance or mixture belongs to a well defined, structurally-related class of substances whose members are listed in a previous Report on Carcinogens as either a *known to be human carcinogen, or reasonably anticipated to be human carcinogen* or there is convincing relevant information that the agent acts through mechanisms indicating it would likely cause cancer in humans.

Conclusions regarding carcinogenicity in humans or experimental animals are based on scientific judgment, with consideration given to all relevant information. Relevant information includes, but is not limited to dose response, route of exposure, chemical structure, metabolism, pharmacokinetics, sensitive sub populations, genetic effects, or other data relating to mechanism of action or factors that may be unique to a given substance. For example, there may be substances for which there is evidence of carcinogenicity in laboratory animals but there are compelling data indicating that the agent acts through mechanisms which do not operate in humans and would therefore not reasonably be anticipated to cause cancer in humans.

Executive Summary

Introduction

Hepatitis B virus (HBV), an enveloped DNA virus, is a member of the Hepadnaviridae family, which comprises the genera orthohepadnavirus (infecting mammals) and avihepadnavirus (infecting birds). The major site of viral reproduction and pathogenesis is the hepatocyte. While some HBV infections are transient, chronic infection and the associated liver pathology may progress over time.

Structure and biology. The hepadnaviruses have a characteristic partially double-stranded DNA genome, which is held in a circular conformation by a short, cohesive overlap between the 5' ends of the two strands. The HBV genome, characterized by four overlapping open reading frames (ORFs), codes for the viral polymerase, the core (HBcAg) and precore proteins, the X protein, and three viral envelope proteins, L (large), M (middle), and S (small). The envelope proteins may be assembled into noninfectious structures as ~22-nm rods or spheres. Virions, which constitute a minor population, have an icosahedral nucleocapsid, containing the viral genome and surrounded by an outer membrane 42 nm in diameter that contains the envelope proteins L, M, and S in decreasing order of abundance. L is thought to specify virus host range by recognizing cell surface receptors, while S is the immunodominant component of the envelope. The nucleocapsid contains only a single structural protein, C (core), which forms the nucleocapsid together with viral nucleic acids and virus-encoded reverse transcriptase, as well as host protein chaperones, including heat shock protein 90. The precore and X proteins are of uncertain function. However, some researchers have proposed that X affects multiple processes within cells that may have significant impacts on liver gene expression, cell survival, and viral replication.The precore protein is processed by posttranslational cleavage and is secreted from infected cells as a soluble protein, referred to as HBeAg, which may be detected by serological assays.

Replication. Replication mechanisms have been characterized for duck HBV (DHBV) but appear to apply to HBV as well. When the virus infects a hepatocyte, the partially double-stranded DNA is converted to a circular, fully double-stranded covalently closed circular DNA (cccDNA), from which viral RNAs are transcribed. Viral mRNAs are transported to the cytoplasm to be translated into the respective viral proteins. The virus nucleocapsid interacts with the viral envelope protein, with the enveloped protein being formed during budding into the endoplasmic reticulum; this is followed by further processing before release from the infected hepatocyte.

Host range and target cells. HBV has a limited host range, infecting only humans and the great apes. HBV infects hepatocytes and bile duct cells in the liver. Although evidence exists for infection and replication of HBV in cells of the kidney and pancreas, no evidence exists for a contribution in either a positive or negative way to the pathogenesis associated with transient or chronic HBV infections. Pathogenic effects outside the liver are most likely due either to reduced liver function or to effects of the cellular and humoral immune responses to virus antigens.

Human Exposure

Detection methods. HBV can cause acute hepatitis, chronic hepatitis, or both. HBV infection is detected by the presence of HBV-specific proteins, host antibodies against HBV viral proteins, or HBV DNA. The detection of different proteins and antibodies against these proteins are indicators of different stages of infection. The HBsAg is a marker for acute or chronic HBV infection, whereas anti-HBsAg is a marker of immunity. Strictly speaking, chronic HBV infections should be defined as two positive readings of serum HBsAg tests taken six months apart; however, this is not practical for most epidemiological studies. Since adult noncarriers of HBV are highly unlikely to show positivity for HBsAg, a single testing for HBsAg is considered a valid measure for chronic carrier status. The U.S. Food and Drug Administration (FDA) currently approves enzyme immunoassays or radioimmunoassays for HBsAg, antibody against HBsAg, and antibody against HBcAg.

Prevalence and incidence. Worldwide, the prevalence of chronic HBV infection has been reported to range from < 2% (low) in Western Europe, Australia, and North America; to 2% to 7% (intermediate) in parts of Southern and Eastern Europe, the Middle East, Japan, Western Asia through the Indian subcontinent, and parts of Central and South America; to ≥ 8% (high) in Africa, Asia (east of the Indian subcontinent but excluding Japan), the Pacific Basin, the Amazon Basin, the Arctic Rim, the Asian Republics previously part of the Soviet Union, and parts of the Middle East. In the United States the prevalence (resolved and chronic HBV infections) decreased from 5.5% in 1976 to 1980 (NHANES II) to 4.9% in 1988 to 1994 (NHANES III). The incidence rate of acute hepatitis B is decreasing in the United States largely due to a decline in incidence among homosexual men and intravenous drug users, screening of blood products, and vaccination; in 1998 the rate was 3.3 per 100,000 compared to 13.8 per 100,000 in 1987.

Risk factors and transmission. The major modes of HBV transmission are parenteral, sexual contact, maternal-neonatal, and nosocomial. In the United States, most cases result from heterosexual transmission (41%), with the next highest factors being intravenous drug use (15%) and homosexual transmission (9%). Approximately 31% of HBV infection is not associated with any known risk factors.

Prevention and treatment. HBV infections can be prevented by screening of the blood supply, by reduction of contact with potentially contaminated fluids in health-care settings, or by vaccination in the general population. The Occupational Safety and Health Administration (OSHA) has established a bloodborne pathogens standard, which uses the concept of universal precautions that require body fluids/materials to be treated as infectious. Recombinant hepatitis B vaccines, which encode HBsAg, have been available in the United States since the 1980s and are currently recommended for all infants and individuals at high risk. Therapeutic agents include immunomodulators, which are not specific for HBV, antiviral agents, and combination therapy from both classes; however, these agents have limited efficacy.

Human Cancer Studies

The cancer that is singularly associated with HBV infection is hepatocellular carcinoma, the primary histological subgroup of primary liver cancer in humans. In the United States, the incidence of liver cancer is 5.0 per 100,000 individuals. Hepatocellular carcinoma is a disease with a strong male predominance. Risk factors for hepatocellular carcinoma include HBV, HCV, dietary aflatoxin intake, excessive alcohol consumption, and exogenous use of estrogen and androgen. The latency of HBV-associated hepatocellular carcinoma is about 30 years.

In 1994, a Working Group of the International Agency for Research on Cancer (IARC) evaluated the carcinogenic risk of chronic HBV infection to humans, and reached the conclusion that "*the agent is carcinogenic to humans*." Cohort and case-control studies conducted in diverse populations by race-ethnicity and geography and published since the IARC review have further strengthened the recognized association between chronic HBV infection and the development of hepatocellular carcinoma. The recent studies generally assessed chronic HBV infection using relatively sensitive and specific serological markers of infection, and many also included information on use of alcohol and tobacco, medical history, and dietary aflatoxin (for studies in high-intake areas). These studies support a strong association between HBV and hepatocellular carcinoma that remains after adjustment for HCV and other potential confounders. A meta-analysis of 32 studies, which included research studies published prior to and after the 1994 IARC evaluation, reported a summary odds ratio for hepatocellular carcinomas and HBsAg positivity of 13.7 (95% CI = 12.2 to 15.4). Taken together, the large body of analytic epidemiological data on HBV infection and hepatocellular carcinoma risk provides some of the strongest supportive evidence linking an environmental exposure to the development of a human cancer.

Consistent and abundant data are available in support of a synergistic effect of HBV and HCV coinfection on risk of hepatocellular carcinoma. Support also exists for dietary aflatoxin and heavy alcohol intake acting as cofactors that enhance the risk of hepatocellular carcinoma in a chronic HBV carrier.

No evidence exists from any of the published studies (cohort or case-control) that chronic HBV infection increases the risk of any other cancer besides hepatocellular carcinoma.

Studies in Experimental Animals

HBV. Although a variety of experimental animal models of hepadnavirus infection are available, great apes (chimpanzees, gorillas, and orangutans), lesser apes (gibbons), and tree shrews are the only animals that can reliably be infected with human HBV. The 1994 IARC monograph on HBV concluded that there was *insufficient evidence* for the carcinogenicity of HBV in experimental animals. Despite many years of observation and significant numbers of animals infected, the risk of hepatocellular carcinoma does not appear to be increased in HBV-infected chimpanzees. Transgenic mice that express the entire HBV genome do not have an increased risk of hepatocellular carcinoma; however, some lines of transgenic mice that express high levels of the HBs gene or the HBx gene

do. The level of expression of these gene products may have an important influence on their ability to produce tumors.

Animal hepadnaviruses. Chronic hepadnavirus infection poses a considerable risk for hepatocellular carcinoma in some of the mammalian animal models of HBV infection. The risk of hepatocellular carcinoma in woodchuck hepatitis virus (WHV)-infected woodchucks is very high, approaching 100%. Ground squirrels infected with ground squirrel hepatitis virus (GSHV) also have an elevated risk of hepatocellular carcinoma, but with a longer period of latency and a lower proportion of infected animals developing tumors. Although several new avian hepadnaviruses, in addition to DHBV, have been discovered, none of them has been shown thus far to produce hepatocellular carcinomas in infected hosts. However, chronic infections have only been studied for DHBV-infected birds, and lifetime studies are needed to definitively establish the potential of chronic infection to produce hepatocellular carcinoma.

Interaction with aflatoxin (AFB_1). Synergy between AFB_1 treatment and WHV infection in woodchucks has been demonstrated by higher tumor incidences in chronically WHV-infected animals that received AFB_1. Furthermore, AFB_1 treatment contributes to development of hepatocellular carcinoma in male transgenic HBs gene-expressing mice. In contrast, concurrent DHBV infection has not been shown to affect tumor development of ducks treated with AFB_1, although AFB_1 is a potent hepatic carcinogen in ducks.

Other Relevant Data

Pathogenesis. HBV infection of the liver may involve an acute phase, a chronic phase, or both. Acute HBV infection is characterized by histological changes including hyperplasia, inflammation, cellular proliferation, and necrosis, which appear to be mediated by an antigen-specific cellular immune response. Chronic hepatitis, as defined by circulating HBsAg of greater than six-months duration, develops in individuals who are not able to clear the virus. The risk of chronic hepatitis appears to be associated with the status of the immune system at the time of infection and is much higher in HBV-infected infants or children than in HBV-infected adults. During chronic HBV infection the host immune response results in cycles of cell death and regeneration that may progress to fibrosis of the liver and cirrhosis (replacement of liver tissue with bands of fibrosis surrounding regenerative nodules of liver tissue). Cirrhosis related to chronic HBV infection is more likely to progress to hepatocellular carcinoma than cirrhosis from other causes. The regenerative nodules that arise from cell death are considered to be precursors to hepatocellular carcinoma.

Potential mechanisms of carcinogenesis. Hepatocellular carcinoma usually emerges after 30 years of chronic HBV infection. During the decades of chronic viral infection, many changes are introduced to the cell as a consequence of the ongoing virus replication. Viral DNA becomes incorporated into cellular DNA through illegitimate recombination, and these sequences may contribute to multistep hepatocarcinogenesis by any of several mechanisms. The host immune system also may play a role in carcinogenesis, since chronic inflammation and cycles of cell death and regeneration may result in oxidative damage and DNA damage.

Viral integration. Viral integration, either by *cis*-activation or by insertional mutagenesis, can alter gene expression of growth regulatory genes. Some cellular targets of HBV integration that have been identified include ErbB-like, cyclin A2, retinoic acid receptor β, Hst-1, carboxypeptidase N-like, SERCA1, thyroid hormone receptor-associated protein, telomerase reverse transcription, minichromosome maintenance protein-related gene, and nuclear matrix protein p84 genes. Integration also may lead to a truncation in the 3' end of the *preS2* and *X* genes, leading to novel proteins that possess transactivation function. A majority of tumors contain viral proteins that have the capacity to transactivate cellular genes. Viral integration also can lead to genetic instability. HBV-positive hepatocellular carcinomas appear to contain higher levels of chromosome allele loss compared to HBV-negative hepatocellular carcinomas.

Potential oncogenic properties of HBV gene products. Experimental evidence suggests that expression of some HBV proteins may contribute to oncogenesis. The large surface antigen (preS1) can be directly cytotoxic to cells and can initiate events leading to the development of cancer; overexpression of the preS1 antigen in transgenic mice leads to permanent inflammation, oxygen radical production, and DNA damage. The truncated-form of the middle surface antigen (preS2) can transactivate cellular protein, and an HBV insert containing the truncated preS2 and the HBx gene was able to transform murine fetal hepatocytes. The HBx protein may activate viral and cellular promoters and signal transduction pathways, inhibit DNA repair, affect the cell cycle, and affect apoptosis. High levels of HBx may transform immortalized 3T3 cells. Some studies have reported that HBx can bind p53 and inhibit many of its functions; however, others studies have not been able to repeat these findings. Studies in HBx transgenic mice are conflicting, with cancer developing in some but not all strains of mice.

Interaction with other carcinogens. Human studies have suggested a possible synergism of HBV with either chronic HCV infection or aflatoxin exposure. Although the mechanisms for these interactions are not known, hepadnaviral animal models confirm the synergism between hepadnaviral infections and aflatoxins. All of these genetic changes occur on the background of immune-mediated cell death and regeneration, which facilitates the selection of cells that have a growth advantage during development of the tumor.

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1 Introduction

Hepatitis B virus (HBV) is a member of a family of highly species-specific enveloped DNA viruses, the Hepadnaviridae, which replicate by reverse transcription of a viral RNA, the pregenome (Summers and Mason, 1982). HBV, the smallest of all human DNA viruses, is characterized by a highly compact genome (Flodell *et al*., 2002). The family consists of the genus Orthohepadnavirus, which is made up of hepadnaviral species infecting mammals, and the genus Avihepadnavirus, which consists of hepadnaviruses infecting birds. All of these viruses reproduce predominately in the liver. Within the liver, the major target site of productive infection is the hepatocyte. Infection of bile duct epithelium also has been documented for duck hepatitis B virus (DHBV) (Lee *et al.*, 2001). More information on hepadnavirus structure, replication, taxonomy, and alternative animal models of infection is presented in subsequent sections. A glossary of hepatitis- or virology-related terms follows the Bibiography (Section 7).

Although they are very similar, the orthohepadnaviruses and the avihepadnaviruses differ in one key respect. Chronic infection in mammals has been associated with progressive liver damage; whereas, chronic infection in birds has not (Tennant, 2001, IARC, 1994). Thus, the remainder of this section concerns findings with the mammalian hepadnaviruses unless otherwise noted. References to the avihepadnaviruses highlight potential parallels between the two groups that may be of interest. Similar references appear in the review of hepadnavirus replication, because almost all current information on replication mechanisms (Section 1.2.4) has been derived from studies with duck HBV, an avihepadnavirus (Tennant, 2001).

The pathology of HBV infection involves cell death in the presence of inflammatory cellular infiltrate (Lewin, *et al.* 2002). Cell lysis is caused by the immune response to infected hepatocytes. There is no evidence of direct viral cytopathic effect. Infections may be either transient or chronic, depending on the ability of the immune response to clear infected hepatocytes. Liver disease in chronically infected individuals may range from mild to severe and is generally asymptomatic in early stages, with either no or only mild elevations in serum transaminases, a marker of hepatocyte injury. The primary mechanism of clearance of HBV-infected hepatocytes is regulated by the class-I major histocompatibility complex (MHC) (Lewin, *et al.* 2002). Analyses of HBV-specific cytotoxic T lymphocyte (CTL) responses following acute HBV infection have indicated the involvement of CTLs specific to various epitopes related to HBV proteins (Bertoletti and Maini, 2000). In infected patients, HBV core 18-27 (amino acids 18 to 27 of the HBV core protein)-specific CD8+ cells predominate in circulation, accounting for up to 1.3% of CD8+ circulating cells. Responses to these cells are higher in patients who successfully control HBV replication and absent in HBeAg-positive HBV chronic carriers, unless treated with lamivudine (LMV) or interferon-alpha. The recognition by antigen-specific CTL of HBV-infected hepatocytes constitutes the predominant factor relevant to the severity of liver damage (Boni *et al.*, 1998).

The risk of infection in a population can be very high because the virus is readily spread by exposure to blood or blood products; these can contain high titers of virus that has been

released by infected hepatocytes directly into the bloodstream. Thus, needle sticks, unprotected sex, and blood transfusion all are potential sources of infection. Other major risks include transmission from carrier mothers to newborns and spread to young children through family and community contacts. The age at infection is the major determinant of whether or not a person becomes a carrier (IARC, 1994). The prevalence, incidence, risk factors, and transmission of HBV infection are discussed further in Section 2.3.

In the 1960s, investigators recognized that the blood of carriers contains a characteristic antigen, then named Australia antigen (Blumberg *et al.*, 1967) and subsequently referred to as HBV surface antigen (HBsAg). Individuals who have recovered from an infection typically have antibodies to this antigen in their serum. HBsAg, initially purified from the serum of virus carriers and subsequently synthesized in yeast, served as the basis for a vaccine, which has been available for more than 20 years. Vaccination has significantly reduced the incidence of new infections (see Section 2.5.1.2). Follow-up studies of vaccinated newborns also have revealed a reduction in incidence of hepatocellular carcinoma (Chang *et al.*, 1997).

HBV was nominated for possible listing in the Report on Carcinogens by the National Institute of Environmental Health Sciences (NIEHS) on the basis of the International Agency for Research on Cancer (IARC) classification of HBV as *carcinogenic to humans* (Group 1) based on a finding of sufficient evidence of carcinogenicity in humans (hepatocellular carcinoma).

1.1 Taxonomy

Four species of Orthohepadnavirus are currently recognized by the International Committee for the Taxonomy of Viruses (ICTV): HBV, woodchuck hepatitis virus (WHV) (Summers *et al.*, 1978), ground squirrel hepatitis virus (GSHV) (Marion *et al.*, 1980), and woolly monkey HBV (Lanford *et al.*, 1998) (Figure 1-1). The sequence of a fifth isolate (arctic ground squirrel virus), more closely related to WHV and GSHV than to HBV, has been reported (Testut *et al.*, 1996) but not yet assigned within the Orthohepadnavirus genus. HBV-like viruses also have been detected in chimpanzees, gibbon apes, and orangutans. Though they differ somewhat in sequence from HBV, these differences are not particularly striking when assessed against the variation between HBV isolates. HBVs infecting humans are currently classified into several genotypes (A-G) based on their gene sequence information (Simmonds, 2001). This first-tier classification is subdivided further into second-tier subtypes. The various genotypes have > 8% sequence divergence and are distinctive based on replication, clinical features, and geographical origin (Flodell *et al.*, 2002). A two-nucleotide change, at positions 1850 and 1858 within the highly conserved bulged stem-loop region (see Figure 1-5), differentiates genotype A from the other genotypes. However, epidemiological studies have not considered individual HBV genotypes. Two species of avihepadnaviruses are currently accepted, duck HBV (DHBV) (Mason *et al.*, 1980, Wang *et al.*, 1980) and heron HBV (HHBV) (Sprengel *et al.*, 1988). A stork virus, more closely related to the heron virus than to other DHBV isolates, has been reported (Pult *et al.*, 2001), and a Ross goose virus also has been described (Pult *et al.*, 2001) (Figure 1-2). A consensus has not yet been reached on assignment of these viruses as a genotype of DHBV or HHBV, or as separate species. There is almost no sequence relatedness between the orthohepadnaviruses and the avihepadnaviruses.

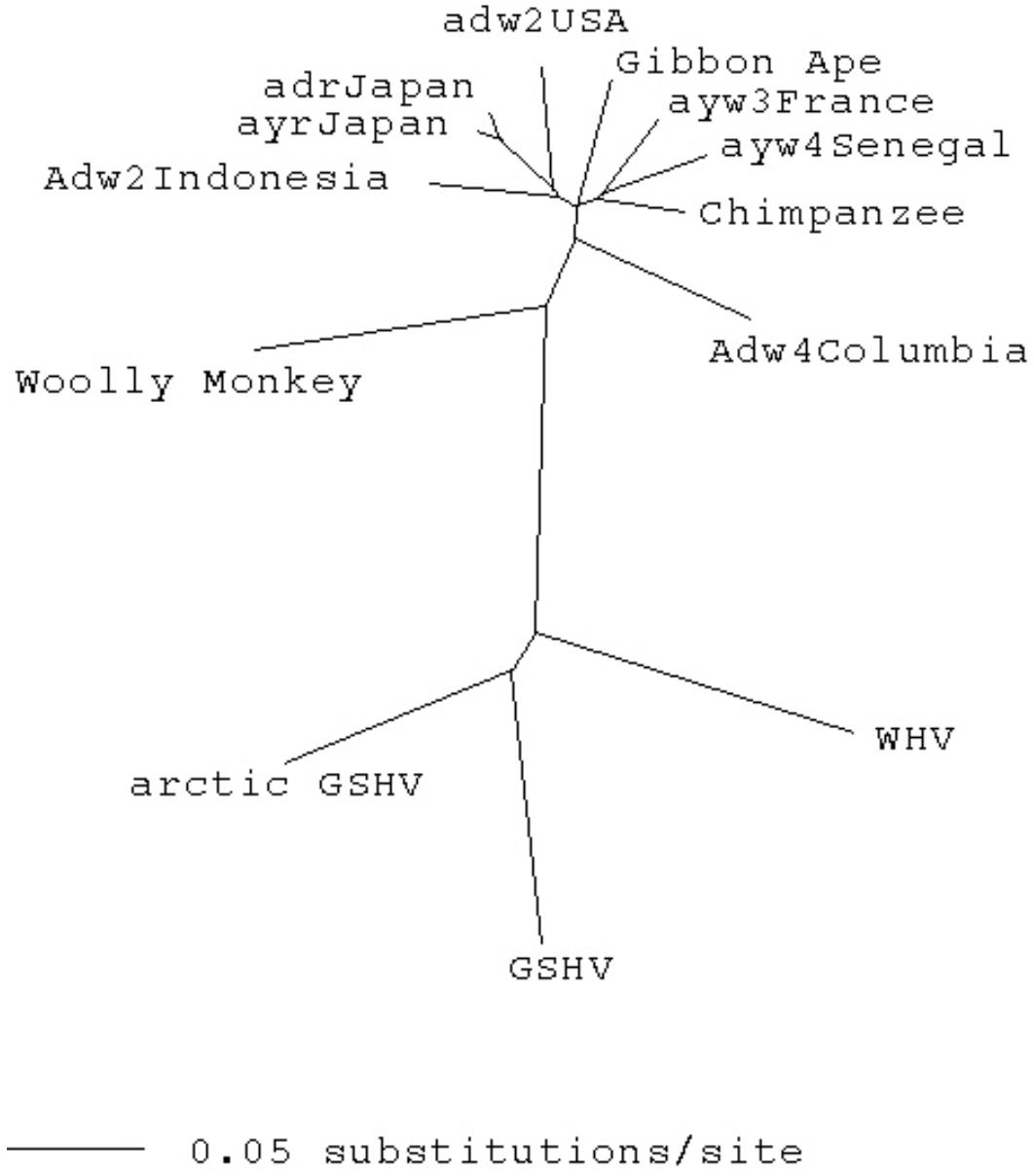

Figure 1-1. Orthohepadnaviruses: Relatedness of nucleotide sequences predicted for envelope gene using neighbor-joining method

Source: Dr. William Mason (Personal communication)

GSHV = ground squirrel hepatitis virus; WHV = woodchuck hepatitis virus; adr, adw, ayr, and adw represent four antigenic subtypes of human HBV (the viruses isolated from the gibbon ape and the chimpanzee are believed to be human HBVs); woolly monkey HBV is the fourth orthohepadnavirus.

Note: See Lanford *et al*., 1998 for accession numbers.

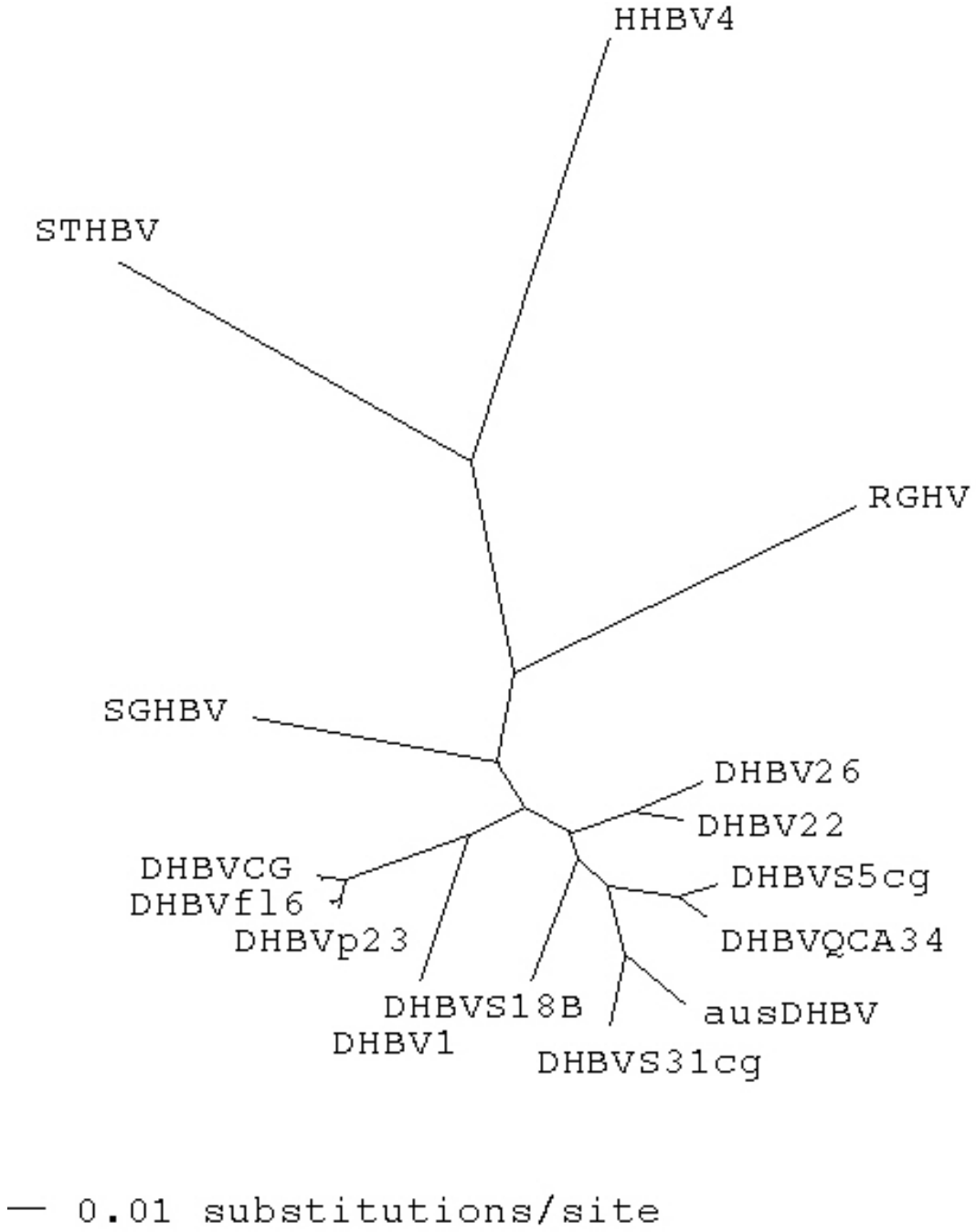

Figure 1-2. Avihepadnaviruses: Relatedness of nucleotide sequences predicted for envelope gene using neighbor-joining method

Source: Dr. William Mason (Personal communication)

DHBV = duck hepatitis B virus; ausDHBV = australian strain of DHBV; HHBV = heron hepatitis B virus; RGHV = Ross goose hepatitis virus; SGHBV = snow goose hepatitis B virus; STHBV = stork hepatitis B virus.

Note: See Triyatni *et al.*, 2001 for accession numbers.

1.2 Structure and biology

1.2.1 Virion structure

Hepadnavirus infections are characterized by production of two distinct categories of particles. The most abundant are not viruses at all, but noninfectious structures that contain the viral envelope proteins, L (large), M (middle), and S (small), as the only virus-specific constituents (Heermann *et al.*, 1984) (Figures 1-3, 1-4). For the orthohepadnaviruses, these particles are found as ~22-nm rods and spheres and are usually present in > 100-fold excess over virions. Virions, which constitute a minor population, have an icosahedral nucleocapsid that contains the viral genome; the viral genome is surrounded by an outer membrane (42 nm in diameter), formed during nucleocapsid budding into the endoplasmic reticulum. L, M, and S, in decreasing order of abundance, are also present in virion envelopes. L is believed to specify virus host range by recognition of cell surface receptors, while S is the immunodominant component of the envelope. S alone is sufficient to produce the 22-nm spheres; and such particles, produced in yeast, are the basis for the current HBV vaccine (Wynne *et al.*, 1999).

Unlike the viral envelope, the nucleocapsid contains only a single structural protein, C (core), the product of the viral core open reading frame (ORF). This protein can form into icosahedral structures by itself, but nucleocapsids normally contain both viral nucleic acids and the virus-encoded reverse transcriptase, as well as host protein chaperones, including heat shock protein 90 (Hu *et al.*, 1997). Reverse transcription of viral RNA into DNA occurs within nucleocapsids in the cytoplasm of the infected hepatocyte. Thus, immature nucleocapsids contain viral RNA, while in mature capsids, the RNA has been replaced by reverse transcription into DNA. The reverse transcriptase is retained within nucleocapsids throughout virion maturation and may have a role in completing formation of a double-stranded, viral DNA during initiation of new rounds of infection (see below).

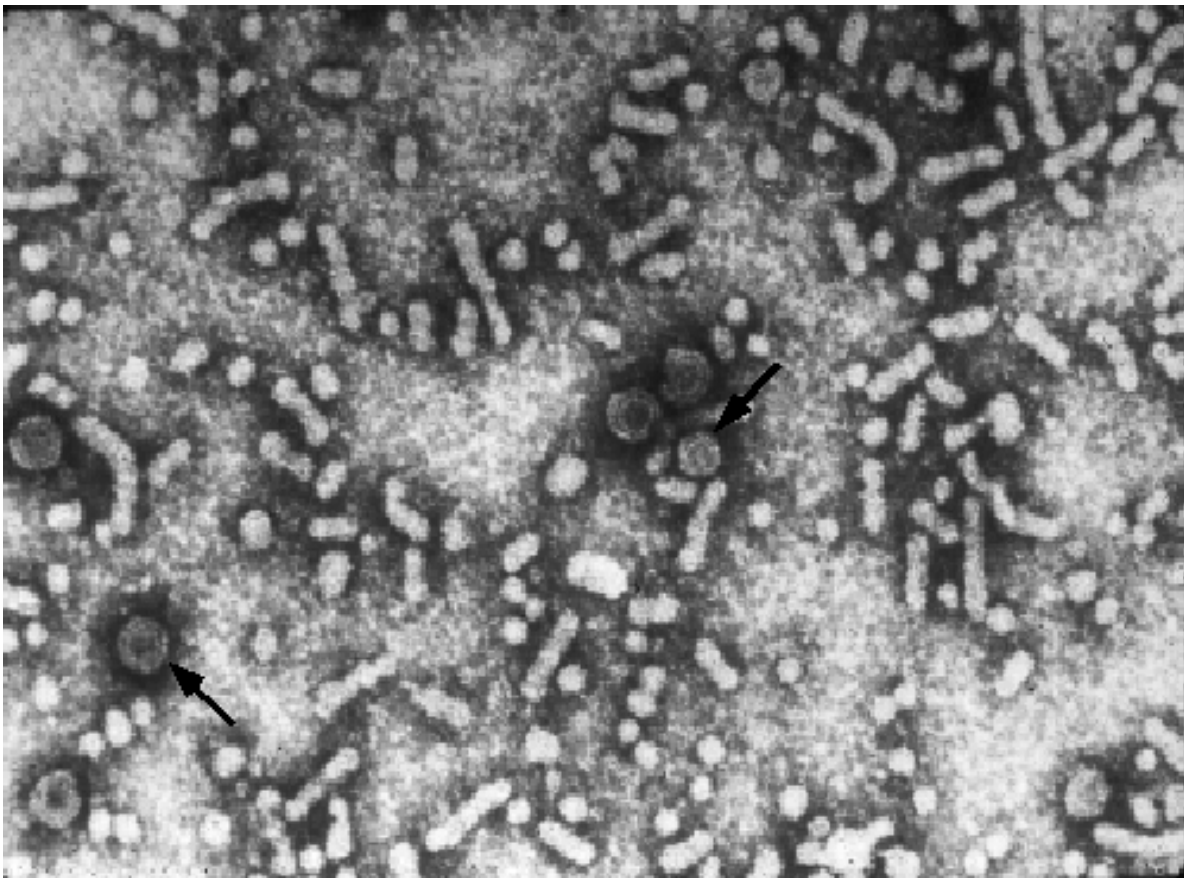

Figure 1-3. Virions and surface antigen particles.

Source: CDC, 2001

Note: The rodlike and spherical particles characterize the surface antigen particles of all the orthohepadnaviruses. The large, 42-nm diameter particles (arrows) are the enveloped virus

1.2.2 Genome organization and gene products

Hepadnaviruses have a characteristic partially double-stranded 3.2 kb DNA genome, which is held in a circular conformation by a short, cohesive overlap between the 5' ends of the two strands (Figure 1-4). All viral mRNAs have the same polarity and are copied from the viral minus-strand DNA, which is itself the product of reverse transcription of one of the largest viral mRNAs, the pregenome. None of the viral RNAs important in HBV replication are known to be spliced. Rather, viral mRNAs for the various gene products are produced from four promoters proximal to the start of the relevant coding sequence. The different RNAs share a common polyadenylation site, located within the viral core gene (Seeger and Mason, 2000, Ganem and Schneider, 2001, Robinson, 1994).

Six functionally distinct mRNAs give rise to seven viral proteins, precore; core; polymerase (pol); L-, M-, and S- envelope proteins; and X. Of these proteins, four (core, L, M, and S) are structural. A fifth, the pol gene product, synthesizes viral DNA. The

remaining two, precore and X, are of uncertain function (Caselmann, 1996, Ganem and Schneider, 2001, Seeger and Mason, 2000).

Two promoters, core and S, are characterized by alternative start sites, which allow them to direct the transcription of mRNAs coding for more than one protein. Core promoter transcripts beginning just upstream or just downstream of the first AUG sequence in the precore ORF (Figure 1-4) direct synthesis of precore and core mRNAs, respectively (Weimer *et al.*, 1987). The core mRNA, also known as the pregenome, is the mRNA for the viral polymerase. Core protein synthesis initiates from the second AUG, located 87 nucleotides (nt) downstream and inframe with the first AUG of the ORF. Pol initiates from an out-of-frame AUG located within the core ORF. PreS2/S transcripts also initiate upstream and downstream of an AUG, this time located within the ORF for the viral envelope proteins and defining the boundary between PreS1 and PreS2. The upstream mRNA encodes the viral M protein, the middle-sized envelope glycoprotein of the HBV envelope containing the PreS2 and S domains. Transcripts initiating downstream of the M protein initiation codon are translated from an inframe AUG 165 nt from the first AUG and direct synthesis of the viral S, which is the most abundant of the three envelope glycoproteins (Ganem and Schneider, 2001, Seeger and Mason, 2000).

A number of researchers have suggested that X may affect multiple processes within cells, most of which may have significant impacts on liver gene expression, cell survival, and viral replication (Becker *et al.*, 1998, Benn and Schneider, 1994, Bouchard *et al.*, 2001a, Caselmann, 1995, Choi *et al.*, 2001, Diao *et al.*, 2001, Elmore *et al.*, 1997, Feitelson *et al.*, 1993, Fischer *et al.*, 1995, Forgues *et al.*, 2001, Hu *et al.*, 1999, Klein and Schneider, 1997, Lee and Yun, 1998, Su and Schneider, 1997, Terradillos *et al.*, 1998, Twu *et al.*, 1993). HBx expression appears to be required for efficient virus replication in cell culture (Bouchard *et al.*, 2001b, Seeger, 1997, Yaginuma *et al.*, 1987), although not all studies have supported this observation (Blum *et al.*, 1992). Experiments with woodchuck hepatitis virus (WHV), a close relative of HBV, have revealed that X is needed for efficient replication in the liver (Chen *et al.*, 1993, Zhang *et al.*, 2001, Zoulim *et al.*, 1994). Recently, it was demonstrated that HBx enhances tumor cell invasion in the chick embryo both *in vivo* and *in vitro* through the induction of cyclooxygenase-2 (COX-2) expression and subsequent activation of membrane-type 1 matrix metalloproteinase (MT1-MMP) (Lara-Pezzi *et al.*, 2002). HBx upregulates the metastatic potential of tumor cells by increasing their ability to degrade the extracellular matrix, traverse the endothelial barrier, and reach the blood stream.

The function of the precore protein is uncertain. It has been known for many years that precore is processed by posttranslational cleavage and that it is secreted from the infected cell as a soluble protein, referred to as HBeAg (Magnius and Espmark, 1972, Ohori *et al.*, 1980, Ou *et al.*, 1986), which can be readily detected by serological assays. The presence of HBeAg is a characteristic marker of efficient virus replication in an HBV carrier. On the other hand, HBeAg is not essential for virus replication, because mutations in the region between the precore and core AUG that block precore translation and HBeAg synthesis are not uncommon in chronic carriers. As a result, although carriers cease to express HBeAg, they continue to produce virus.

In contrast to the nonstructural protein precore, a great deal is understood about the functional activities of the viral polymerase, which is present in limited numbers, one or two molecules per HBV virion (Bartenschlager and Schaller, 1992). Four domains are now recognized (from amino to carboxy terminal end): the terminal protein, spacer, polymerase, and RNase H (Figure 1-4). The terminal protein domain contains the tyr residue that serves as the primer for reverse transcription. The spacer acts as a flexible link to the downstream domains. The polymerase domain contains the active site for both DNA- and RNA-dependent DNA synthesis, and the carboxy terminal RNase H domain is responsible for degradation of the RNA template as it is copied into DNA.

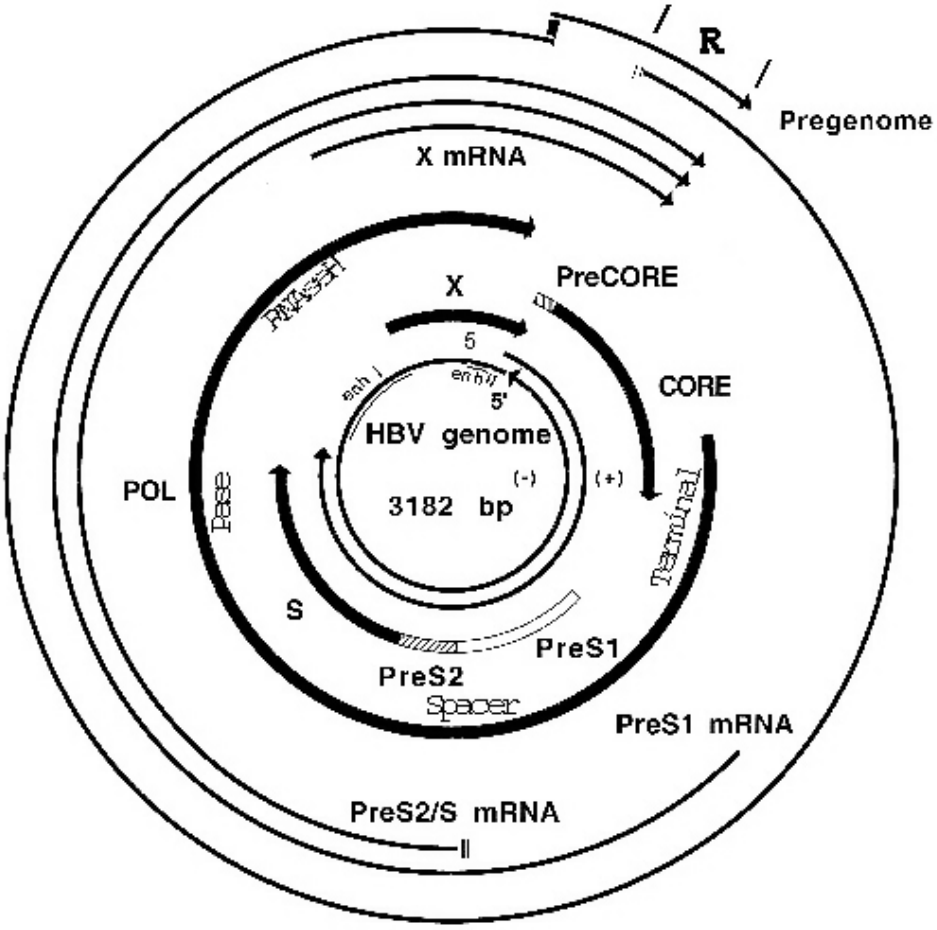

Figure 1-4. Virion structure and organization of HBV

Source: Dr. William Mason (Personal communication)

Note: The 3.3-kb, partially double-stranded DNA genome of HBV is shown as the inner circles. Also shown are the major ORFs on the viral genome and the respective mRNAs for each of the gene products resulting from these ORFs. With the exception of the core and pol gene products, both of which which are translated from pregenomic RNA, each viral protein is translated from a unique mRNA. The pregenome and the slightly larger mRNA for precore are terminally redundant (R). Four viral polymerase domains: the terminal protein, spacer, polymerase, and RNase H domains are indicated.

1.2.3 Regulation of gene expression

Control of viral gene expression has been studied extensively with HBV and, to a lesser extent, with DHBV and WHV (Tennant and Gerin, 2001, Mason *et al.*, 1980). With few exceptions, these studies have employed cell lines of hepatic and extrahepatic origin. Thus, some of the findings may not accurately reveal how virus gene expression is controlled in a natural infection. Nonetheless, one clear finding is that both HBV and DHBV replicate well in liver cell lines, including HepG2 (Glebe *et al.*, 2001) and LMH (Guo *et al.*, 2003), respectively, which continue to transcribe at least some gene products considered specific to differentiated hepatocytes. In contrast, neither replicates well in the cell lines of nonhepatic origin that have been studied. This liver specificity has been explained by the presence of viral promoter and enhancer elements that are dependent upon liver enriched transcription factors. Although liver specificity is not a feature of transcription of every viral mRNA, it appears especially important for transcription of pregenome mRNA (Tang and McLachlan, 2002), the only viral RNA needed for genome replication (see Section 1.2.4). For HBV, the conclusions from cell culture studies are reinforced by the fact that virus replication has been consistently documented only in hepatocytes, although other cell types may become infected (but fail to replicate viral DNA at a detectable level). In addition, there is no clear evidence that pathogenesis of other tissues results from their infection by HBV. DHBV appears to have a somewhat broader tissue specificity than HBV; it infects and replicates genomes in a subset of exocrine and endocrine cells in the pancreas; in the tubular epithelium in the kidney (Halpern *et al.*, 1984, 1985, 1983); and in the yolk sac, liver, and pancreas of the developing embryo (Halpern *et al.*, 1986, Tagawa *et al.*, 1987, Urban *et al.*, 1985). It is unclear whether this finding reflects a difference in transcriptional regulation or a difference in cell surface receptor distribution for the two viruses.

1.2.4 Replication

To a large extent, replication mechanisms have been characterized for DHBV, which is relatively easy to study during a natural infection and in cell culture. To the degree that information is available, these mechanisms appear to apply to HBV. The general replication scheme is illustrated in Figure 1-5. Recent evidence indicated a difference in replication efficiency between certain HBV mutants and their parental counterparts in several hepatoma cell lines (Suk *et al.*, 2002). This development points to a host-factor-independent replication advantage for some mutant strains of HBV in chronic carriers. The general replication mechanisms are the same: when virus infects a hepatocyte, the partially double-stranded DNA is converted to a fully double-stranded and covalently closed circular DNA (cccDNA), from which viral RNAs are then transcribed. This conversion probably requires the viral reverse transcriptase, because cccDNA formation is at least partially inhibited by nucleoside analogs that also inhibit subsequent viral DNA synthesis, as shown with WHV (Moraleda *et al.*, 1997). This interpretation remains somewhat controversial, however; researchers have been unable to demonstrate a similar effect with DHBV (Mason *et al.*, 1987, Kock and Schlicht, 1993), possibly because of the shorter single-stranded region on DHBV genomes (Lien *et al.*, 1987).

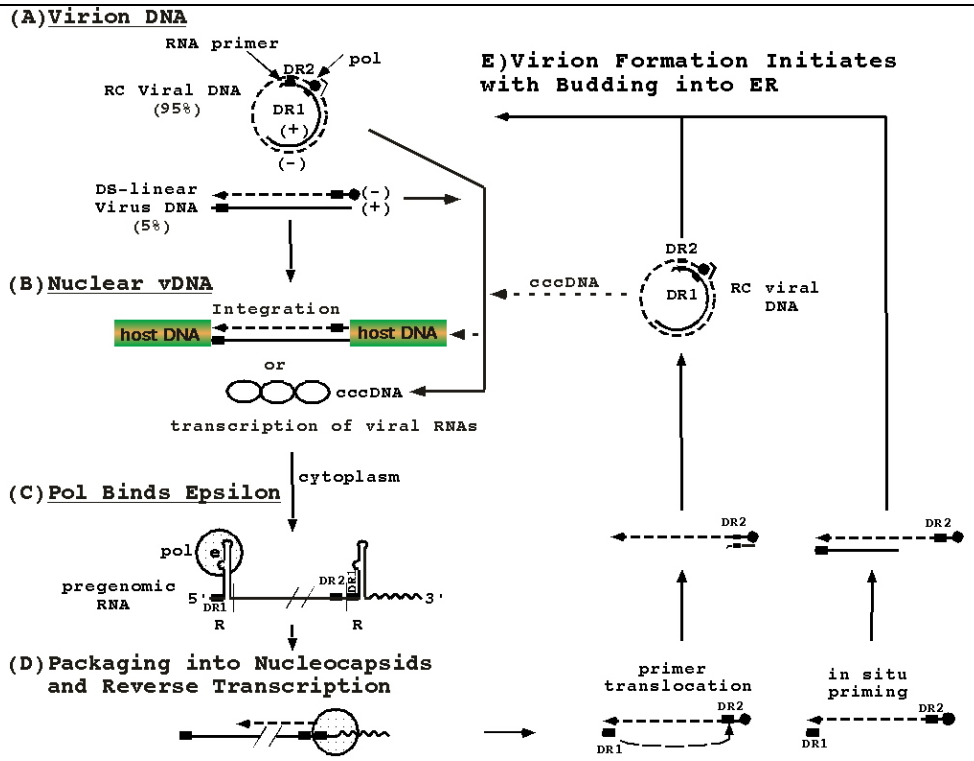

Figure 1-5. Mechanism of hepadnavirus DNA replication based on studies of DHBV and WHV

Source: Unpublished figure (Personal communication, Dr. William Mason)

Note: The two predominant forms of the viral genome, partially double-stranded, relaxed circular DNA and linear, double-stranded DNA, are illustrated at the top left (A). During the initiation of a round of infection, the viral DNA is transported to the nucleus and converted to a cccDNA (B). For linear DNAs, this conversion occurs by illegitimate recombination (Yang *et al.*, 1996). The cccDNA directs the synthesis of multiple viral mRNAs (Figure 1-2), including pregenomic RNA, which is translated both into viral core protein, the nucleocapsid subunit, and into the pol gene product, the viral reverse transcriptase. Reverse transcriptase binds to a stem-loop sequence epsilon in the 5' copy of the terminal redundancy, R (C, and Figure 1-2), and the complex is packaged into viral nucleocapsids. Reverse transcription initiates with copying of four nucleotides from a bulge in the side of epsilon. The polymerase then translocates the four nucleotides to a complementary sequence located in an 11 base region, DR1, six nucleotides downstream from the 5' end of DR1 (D). Reverse transcription then continues to the 5' end of the RNA template, with concomitant degradation of the viral RNA by an RNase H activity of the reverse transcriptase. The last 17 nucleotides of the pregenome, including the CAP and the complete 5' copy of DR1, escape degradation and instead serve as an RNA primer for plus strand synthesis. Prior to initiation of second-strand synthesis, the RNA normally translocates to a sequence DR2, identical to DR1 and located upstream of the terminal redundancy. DNA synthesis continues to the 5' end of the minus strand and then translocates and hybridizes to the 3' end. Translocation is facilitated by a short terminal redundancy on the minus strand created during reverse transcription (the terminal redundancy is removed during cccDNA formation). Occasionally, plus- strand synthesis initiates without primer translocation, giving rise to linear viral genomes. With completion of 50% or more of the plus strand, nucleocapsids are packaged into envelopes by budding into the endoplasmic reticulum (ER). Nucleocapsids also may migrate to the nucleus to facilitate production of additional cccDNA, a process which, in DHBV, is negatively regulated by the viral envelope proteins, presumably through facilitation of budding into the ER. Viral DNA also may integrate into chromosomal DNA. Linear genomes appear to be the predominant substrate for integration.

Following cccDNA formation, viral mRNAs are transported to the cytoplasm and translated into the respective viral proteins (Figure 1-4). Among these proteins, the pregenome serves as mRNA for both the core and pol gene products. It is generally believed that translation of the upstream core ORF is much more efficient (however, see Yao *et al.*, 2000) and that the downstream pol ORF is only occasionally translated by a leaky scanning mechanism. The newly translated reverse transcriptase may bind to a stem-loop structure, epsilon, near the 5' end of the pregenome; this apparently stops further translation of the mRNA and leads to its packaging, along with host chaperones, into immature viral nucleocapsids, produced by assembly of viral core protein dimers (Bartenschlager and Schaller, 1992, Fallows and Goff, 1995, Hu and Seeger, 1996a, 1996b, Junker-Niepmann *et al.*, 1990, Pollack and Ganem, 1993, Wang *et al.*, 1994, Wang and Seeger, 1993, 1992, Zhou and Standring, 1992). Almost all reverse transcription of viral RNA occurs within the nucleocapsids.

The primer for reverse transcription of viral RNA is a tyr residue located in the primer domain of the viral reverse transcriptase (Weber *et al.*, 1994, Zoulim and Seeger, 1994). The protein remains covalently attached to the DNA throughout virus maturation (Bartenschlager and Schaller, 1992, Gerlich and Robinson, 1980). After reverse transcription initiates with the copying of four nucleotides from epsilon (Wang *et al.*, 1994), a translocation takes place to a sequence DR1 in the 3' terminal repeat of the pregenome (Wang and Seeger, 1993). Reverse transcription then continues to the 5' end of the pregenome, with concomitant degradation of the RNA template by the RNAse-H activity of the viral reverse transcriptase. The 5' 17 nucleotides of the pregenome, including the CAP and extending through the 5' copy of DR1, serve as a primer for second (plus) strand synthesis (Lien *et al.*, 1986, Seeger *et al.*, 1986, Will *et al.*, 1987). Normally synthesis initiates after translocation and hybridization of the primer to a site, DR2, which is identical to DR1 (Figure 1-5). DNA synthesis then continues to the 5' end of the template, jumps to the 3' end (Loeb *et al.*, 1997), and continues until the plus strand is partially complete, leaving a single-stranded gap that may constitute up to 50% of genome length (Lutwick and Robinson, 1977, Summers *et al.*, 1975). Occasionally, plus-strand synthesis initiates in the absence of primer translocation and continues toward the 5' end of the minus strand template, producing a double-stranded, but linear, viral DNA (Loeb and Tian, 2001, Staprans *et al.*, 1991) (Figure 1-5).

During viral DNA synthesis, the virus nucleocapsids apparently undergo a maturation process that allows them to interact with the viral envelope protein and during budding the enveloped virus forms into the endoplasmic reticulum; this interaction is probably followed by further processing before release from the infected hepatocyte (Block *et al.*, 1994). The nature of the nucleocapsid maturation process is unclear, but its existence can be deduced from the selective envelopment of nucleocapsids containing double-stranded DNA (Summers and Mason, 1982, Wei *et al.*, 1996).

Studies with DHBV reveal that nucleocapsids also may migrate directly to the nucleus, or at least to nuclear pores, to deliver newly synthesized viral DNA to the nuclear compartment and increase the amount of cccDNA (Tuttleman *et al.*, 1986). cccDNA itself does not replicate. It is estimated that infected cells have, on average, between 5 and 50 copies of cccDNA. With DHBV, new cccDNA formation in infected cells is negatively

regulated by viral envelope proteins (Summers *et al.*, 1990). HBV cccDNA formation may be similarly regulated, although such regulation has yet to be demonstrated (Summers *et al.*, 1990, Ling and Harrison, 1997). New cccDNA synthesis in an infected cell also may depend on the regulated expression of cell proteins, because not all cells types that replicate HBV DNA (i.e., from a transgene) permit for cccDNA formation (Raney *et al.*, 2001).

The precursor of integrated DNA was initially assumed to be the relaxed circular DNA that is the predominant form of the viral genome; however, more recent data suggest that this may not be correct. Animal and cell culture studies suggest that the major precursor to integrated DNA is the minority population of linear viral DNAs. These are able to form generally defective cccDNA molecules by illegitimate recombination and also, apparently, to integrate in end-to-end fashion by the same process (Gong *et al.*, 1999, 1996, Yang *et al.*, 1996, Yang and Summers, 1995, 1998, 1999). Viral DNA integrants in tumors generally show a more complex, highly rearranged structure, which may evolve following integration. Core protein is not usually expressed from integrated DNA, which likely results from integration placing the core promoter distal to the core ORF. Surface and X-protein expression have been observed. In brief, integration of viral DNA into host DNA is a by-product of virus replication.

1.3 Host range and target cells

HBV has a limited host range, primarily infecting humans and the great apes. Within the host, the range of cell types that are infected is not easily deduced because of the difficulty of identifying latent infections. Polymerase chain reaction (PCR) amplification of viral DNAs has the sensitivity to detect a single copy of cccDNA, which is the molecule that defines a cell as being infected. The problem is distinguishing one copy of cccDNA from a huge excess of relaxed circular virion DNA that may be associated with cell surfaces or in surrounding body fluids. Thus, most early studies looked for infection in one of two ways: they assayed either for expression of one or more viral proteins or nucleic acids, or for the forms of viral DNA characteristic of virus DNA replication, as detected by Southern blot analysis. Analyses of tissue sections suggest that HBV infects hepatocytes and cells within the bile duct (Blum *et al.*, 1983), and these and other approaches indicate that hepatocytes, which constitute 60% to 70% of liver-cell mass, are a major site of virus reproduction.

HBV probably also replicates to at least low levels outside the liver {Lanford *et al.*, 1995). Studies of other tissues and organs have reported varied results, complicated in some cases by difficulties in distinguishing infection from passive association. It is generally believed, on the basis of PCR assays and of less sensitive Southern and Northern blot assays, that HBV infects at least one compartment of the leukocyte population in the peripheral circulation (Baginski *et al.*, 1991, Hadchouel *et al.*, 1988, Hoar *et al.*, 1985, Lie-Injo *et al.*, 1983, Pasquinelli *et al.*, 1986, Pontisso *et al.*, 1987, Shen *et al.*, 1986, Stoll-Becker *et al.*, 1997, Yoffe *et al.*, 1986). This idea is supported by the isolation of a cell line of bone marrow-derived lymphoblastoid cells from an HBV carrier that replicated HBV (Elfassi *et al.*, 1984, Romet-Lemonne *et al.*, 1983). The theory also is thought to explain in part the persistent detection of HBV DNA by PCR years after recovery from a “transient” infection (Michalak *et al.*, 1994). The best evidence for leukocyte infection comes, however, from studies with WHV-infected woodchucks. Those studies showed that mitogen stimulation of peripheral blood mononuclear cell (PBMC) cultures from infected animals led to WHV

replication (Korba *et al.*, 1988, Korba *et al.*, 1989). Some studies with nonhuman hosts have suggested that the spleen also may be a site of hepadnavirus replication, as shown by HBV in the chimpanzee (Lieberman *et al.*, 1987), WHV in the woodchuck (Korba *et al.*, 1987), and DHBV in the duck (Jilbert *et al.*, 1987). However, the same effect has not been demonstrated in HBV-infected humans.

By analogy to the studies with DHBV, one might expect that HBV would infect and replicate in kidney and pancreas cells. Some evidence suggests that HBV infects cells of the exocrine pancreas and endocrine islets (Cavallari *et al.*, 1995, Shimoda *et al.*, 1981, Tsukagoshi *et al.*, 1982, Yoshimura *et al.*, 1981), but further research is needed to determine the frequency and significance of this event.

There is no evidence that infection and replication of HBV outside the liver contributes in either a positive or negative way to the pathogenesis associated with transient or chronic HBV infections. Pathogenic effects outside the liver are most likely due either to reduced liver function or to effects of the cellular and humoral immune responses to virus antigens (e.g., immune-complex deposition).

1.4 Summary

Hepatitis B virus is an enveloped DNA virus that is a member of the Hepadnaviridae family, which comprises the genera Orthohepadnavirus (infecting mammals) and Avihepadnavirus (infecting birds). The major site of viral reproduction and pathogenesis is the hepatocyte. Although some HBV infections are transient, chronic infection and the associated liver pathology may progress over time.

The taxonomy of orthohepadaviruses includes four species recognized by the ICTV: HBV, WHV, GSHV, and Woolly monkey HBV. Two species of avihepadnaviruses, DHBV and heron HBV, are currently accepted. There is almost no sequence relationship between the orthohepadnaviruses and the avihepadnaviruses.

The hepadnaviruses have a characteristic, partially double-stranded DNA genome, which is held in a circular conformation by a short, cohesive overlap between the 5' ends of the two strands. The HBV genome, characterized by four overlapping ORFs, codes for the viral polymerase, the core (HBcAg) and precore proteins, the X proteins, and three viral envelope proteins, L, M, and S. The envelope proteins may be assembled into noninfectious structures as ~22-nm rods or spheres. Virions, which consitute a minor population, have an icosahedral nucleocapsid, containing the viral genome and surrounded by an outer membrane 42 nm in diameter that contains the envelope proteins L, M, and S in decreasing order of abundance. The nucleocapsid contains only a single structural protein, C (core), which forms the nucleocapsid together with viral nucleic acids and virus-encoded reverse transcriptase, as well as host protein chaperones, including heat shock protein 90. The precore and X proteins are of uncertain function. However, researchers have suggested that the X protein may affect multiple processes within cells, and that these processes may in turn have significant impact on liver gene expression, cell survival, and viral replication. The precore protein is processed by posttranslational cleavage and is secreted from infected cells as a soluble protein, referred to as HBeAg, which may be detected by serological assays (see Section 2.2.1).

Regulation of viral gene expression has been extensively studied for HBV and DHBV, primarily with cell lines of hepatic and extrahepatic origin. Viral replication occurs best in liver cell lines that continue to transcribe some gene products, considered to be specific to differentiated hepatocytes; this is in contrast to nonhepatic cell lines in which the virus does not replicate well. Replication mechanisms have been characterized for DHBV but appear to apply to HBV as well. When the virus infects a hepatocyte, the partially double-stranded DNA is converted to a circular, fully double-stranded, covalently closed circular DNA (cccDNA), from which viral RNAs are transcribed. Viral mRNAs are transported to the cytoplasm to be translated into the respective viral proteins. The virus nucleocapsid interacts with the viral envelope protein, and during budding the enveloped protein is formed into the endoplasmic reticulum; this is followed by further processing before release from the infected hepatocyte.

HBV has a limited host range, infecting only humans and the great apes. While the difficulty of identifying latent infections complicates the understanding of the range of cell types infected, analyses of tissue sections suggest that HBV infects hepatocytes and bile duct cells in the liver. Although evidence exists for infection and replication of HBV in cells of the kidney and pancreas, no evidence exists for either a positive or negative contribution to the pathogenesis associated with transient or chronic HBV infections. Pathogenic effects outside the liver are most likely due either to reduced liver function or effects of the cellular and humoral immune responses to virus antigens.

2 Human Exposure

2.1 Introduction

The number of Americans who have contracted chronic hepatitis B is estimated to be 1 million to 1.25 million (Hollinger and Liang, 2001). Since HBV is spread principally by parenteral transmission, detection of this virus in donors of blood and blood products is essential to the maintenance of a safe blood supply. The methods currently in use in the United States and approved by the U.S. Food and Drug Administration (FDA) to diagnose acute and chronic infection with HBV are described in this section along with some methods that are not FDA approved but have been used to assess HBV infection in some of the epidemiological studies discussed in Section 3.

The descriptive epidemiology of HBV also is discussed in this section, along with data for the United States and worldwide prevalence and incidence of infection. Although HBV is spread primarily by parenteral transmission, researchers also believe the virus can be spread by contact with infected individuals or their possessions (Hollinger and Liang, 2001). Preventive measures, including “universal precautions” by health-care workers, the widespread use of the HBV vaccine, and the use of therapeutic agents to treat hepatitis B infection, also are discussed in this section as effective measures in limiting the spread of this virus.

2.2 Methods of detection

HBV infection is detected by the presence of HBV-specific proteins, host antibodies against HBV viral proteins, or HBV DNA. An algorithm for the diagnosis and treatment of HBV infection in patients positive for HBsAg is illustrated in Figure 2-1. HBsAg may be detected in serum of patients who have an acute hepatitis B infection within one to three weeks after exposure (Zanetti *et al.*, 1980). The surface antigen, however, usually persists for less than two months from the onset of clinical symptoms. The surface antigen may persist longer in some individuals; however, this “carrier state” does not always lead to progressive liver damage. Antibodies against HBsAg frequently appear weeks to months after recovery from acute hepatitis B infection; the presence of anti-HBsAg indicates an acquired immunity against HBV. The time course of appearance of HBV antigens and the antibodies against these antigens is illustrated in Figure 2-2.

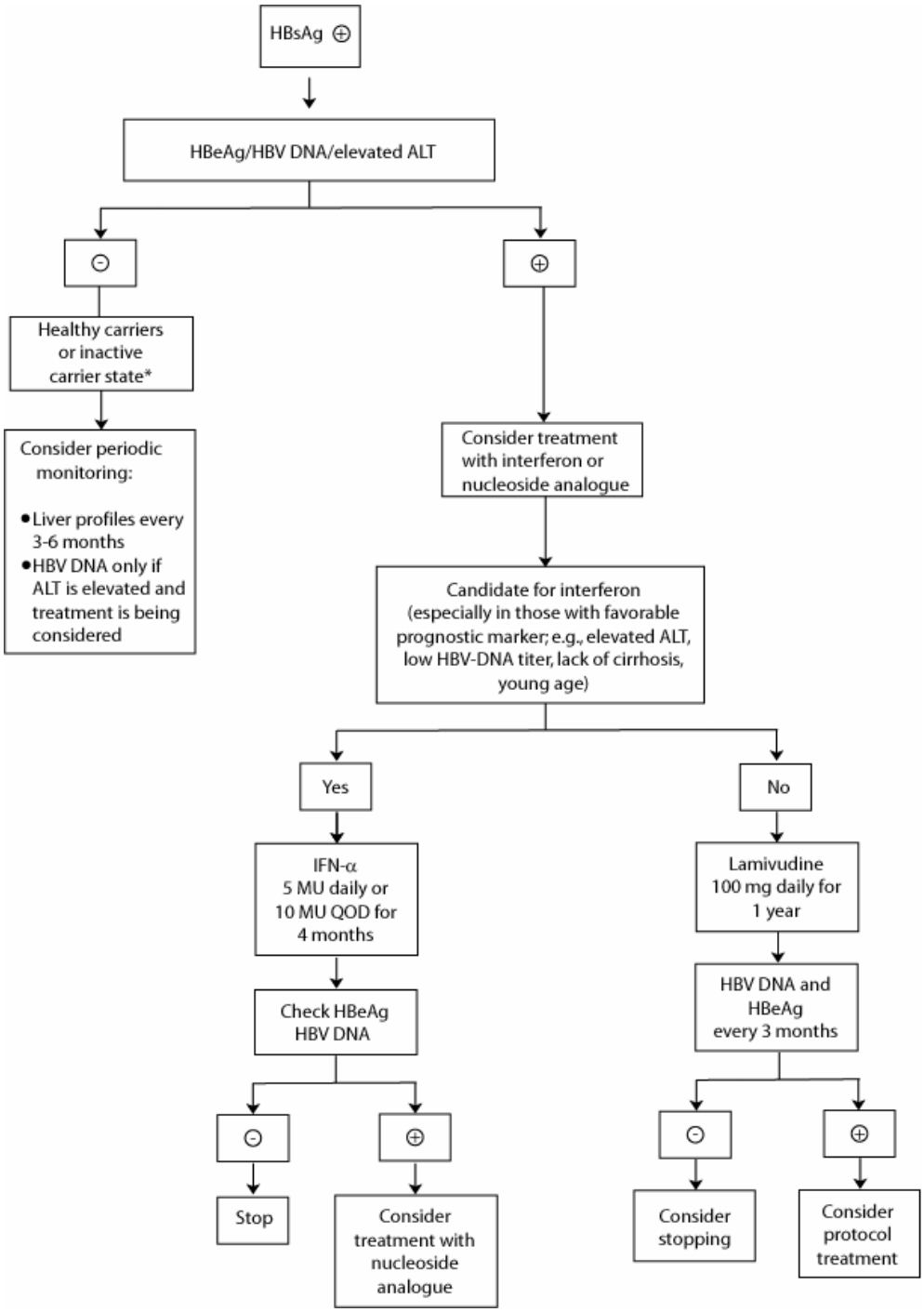

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Figure 2-1. Algorithm for diagnostic testing in patients with positive HBsAg

Source: Younossi, 2000

*If suspicion exists (i.e., elevated alanine aminotransferase) HBV DNA may be positive in those patients with precore mutants ($HBsAg^+$, $HBeAg^-$, HBV DNA^+).

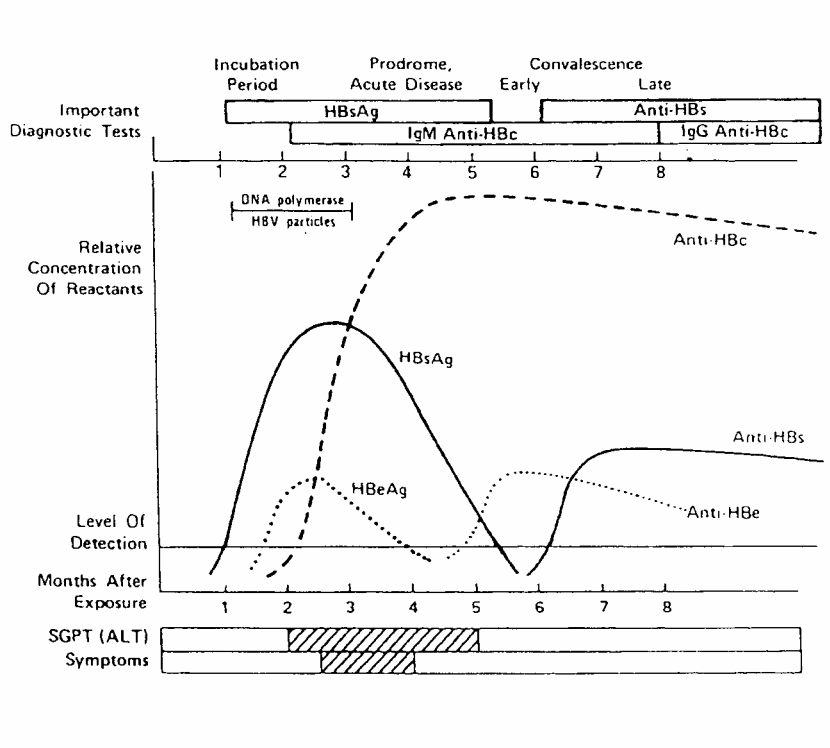

Figure 2-2. Serologic and clinical patterns observed during acute HBV infection

Source: Hollinger and Dienstag, 1995.

The method for detection of HBsAg or the antibody against it in serum of hepatitis patients has evolved toward increasing assay sensitivity from first-generation techniques similar to the immunodiffusion method used in the initial identification of the Australia antigen (Zanetti *et al.*, 1980). As sensitivity increased, the new methods were referred to as second-generation assays. The currently available third-generation methods for detecting HBsAg or the antibody against this antigen (see Tables 2-3, 2-4) include radioimmunoassays (RIAs), enzyme immunoassays (EIAs), reverse passive hemagglutination, and reverse passive agglutination.

2.2.1 Biomarkers (HBsAg, HBeAg, anti-HBc, etc.)

The epidemiological studies reviewed in Section 3 include results obtained with EIA and RIA assays for serum HBsAg, anti-HBs, and anti-HBc that are based on the same methodology as the FDA-approved assays mentioned below. Both EIAs and RIAs are based on binding of either surface or core antigenpresent in the serum with antibodies directed against these peptides *in vitro*. This binding is quantified either by colorimetric absorbance of a colored product formed by enzymatic action (EIA) or by detection of radioactive decay of radionuclides (RIA). As noted above, the assays described in Section 3 and designated as third-generation assays have greater sensitivity than their first- or second-generation predecessors.

Additional assays noted in Section 3 include reverse passive hemagglutination for serum HBsAg, passive hemagglutination for anti-HBs, RIA for HBeAg, RIA for anti-HBe, and

PCR for HBV DNA. Detection of HBV DNA in serum is used as a sensitive test for detection of virus at an early stage of infection; however, molecular assays also may indicate persistence of HBV infection, and they are being used in screening blood donors for HBV infection (e.g., nucleic acid testing) (Wolk *et al.*, 2001). Several molecular methods are available for detection of HBV DNA, including hybrid capture assay, branched DNA (bDNA), liquid hybridization, nucleic acid cross-linking assay, and PCR. The FDA has not yet approved any molecular assay for HBV DNA; thus, laboratory-to-laboratory variation is likely to occur with these assays.

The major HBV-related parameters assayed in clinical laboratory panels and their clinical interpretations are summarized in Table 2-1.

Table 2-1. HBV-related parameters and their interpretation

Parameter	Interpretation
HBsAg	Hepatitis B surface antigen is a marker of infectivity whose presence indicates either acute or chronic HBV infection (see Table 2-2). FDA-approved assays for HBsAg are listed in Table 2-3.
anti-HBs	Antibody to hepatitis B surface antigen is a marker of immunity whose presence indicates an immune response to HBV infection, an immune response to vaccination, or the presence of passively acquired antibody (see Table 2-2). FDA-approved assays for anti-HBs are listed in Table 2-4.
anti-HBc	Antibody to hepatitis B core antigen is a marker of acute, chronic, or resolved HBV infection. It is not a marker of vaccine-induced immunity, but it may be used to determine previous exposure to HBV infection (see Table 2-2). FDA-approved assays for anti-HBc are listed in Table 2-5.
IgM anti-HBc	IgM antibody subclass of anti-HBc indicates recent infection with HBV ($\leq$ 6 months). Its presence indicates acute infection (see Table 2-2).
IgG anti-HBc	IgG antibody subclass of anti-HBc is a marker of past or current infection with HBV. If both IgG anti-HBc and HBsAg are positive (in the absence of IgM anti-HBc), this result indicates chronic HBV infection.
HBeAg	Hepatitis B “e” antigen is a marker of a high degree of HBV infectivity that correlates with a high level of HBV replication. It is primarily used to help determine the clinical management of patients with chronic HBV infection.
Anti-HBe	Antibody to hepatitis B “e” antigen may be present in an infected or immune person. In persons with chronic HBV infection, its presence suggests a low viral titer and a low degree of infectivity.
HBV-DNA	Hepatitis B virus deoxyribonucleic acid is a marker of viral replication that correlates well with infectivity. It is used to assess and monitor the treatment of patients with chronic hepatitis B infection.

Source: Vaccination News 2002.

In its “Recommendations for Preventing Transmission of Infections Among Chronic Hemodialysis Patients” the Centers for Disease Control (CDC) (MMWR 2001a) summarized the interpretation of serologic test results for HBV infection (Table 2-2).

Table 2-2. Interpretation of serologic test results for HBV infection

Serologic markers				
HBsAg*	Anti-HBc† (Total)	Anti-HBc (IgM§)	Anti-HBs¶	Intepretation
–	–	–	–	Susceptible, never infected
+	–	–	–	Acute infection, early incubation**
+	+	+	–	Acute infection
–	+	+	–	Acute, resolving infection
-	+	–	+	Past infection, recovered and immune
+	+	–	–	Chronic infection
–	+	–	–	False positive (i.e., susceptible), past infection, or "low-level" chronic infection
–	–	–	+	Immune if titer is ≥ 10 mIU/mL

Source: MMWR 2001a.

*Hepatitis B surface antigen.

†Antibody to hepatitis B core antigen; total immunoglobulins.

§Immunoglobulin M.

¶Antibody to hepatitis B surface antigen.

**Transient HBsAg positivity (lasting < 18 days) might be detected in some patients during vaccination.

2.2.2 FDA-approved methods

The assays approved by the FDA are based on RIA or EIA methods. FDA-approved assay methods for HBV include those that measure the concentration of HBsAg (Table 2-3) and the antibody against the HBsAg (Table 2-4). Assays for the antibody against the hepatitis B core antigen (HBcAg) also are FDA approved (Table 2-5). The trade name, format, sample type, use, manufacturer, and approval date for assays of each type are given in Tables 2-3, 2-4, and 2-5.

Table 2-3. FDA approved/licensed hepatitis B surface antigen (HBsAg Assay) tests

Trade name	Format	Sample	Use	Manufacturer	Approval date
Auszyme Monoclonal	EIA	Serum/plasma	Donor Screen & Conf. Kit	Abbott Laboratories Abbott Park, IL U.S. License 0043	4/1/1985
Genetic Systems HBsAg EIA 2.0	EIA	Serum/plasma/ cadaveric serum	Donor Screen & Conf. Kit	Bio-Rad Laboratories Blood Virus Division Redmond, WA U.S. License 1109	12/28/1999
Ortho Antibody to HBsAg ELISA Test System 2	EIA	Serum/plasma	Donor Screen & Conf. Kit	Ortho-Clinical Diagnostics, Inc. Raritan, NH U.S. License 1236	7/23/1971
AUK-3	RIA	Serum/plasma	Donor Screen & Conf. Kit	DiaSorin s.r.l. Saluggia, Italy U.S. License 1257	5/9/1986
ETI-MAK-2	EIA	Serum/plasma	Donor Screen & Conf. Kit	DiaSorin s.r.l.	4/18/1995

Source: FDA 2002a.

Table 2-4. FDA approved/licensed antibody against hepatitis B surface antigen (anti-HBs Assay) tests

Trade name	Format	Sample	Use	Manufacturer	Approval date
Ausab	RIA	Serum/plasma	Anti-HBs	Abbott Laboratories Abbott Park, IL U.S. License 0043	2/5/1975
Ausab EIA	EIA	Serum/plasma	Anti-HBs	Abbott Laboratories	11/18/1982
AB-AUK-3	RIA	Serum/plasma	Anti-HBs	DiaSorin s.r.l. Saluggia, Italy U.S. License 1257	6/27/1989
ETI-AB-AUK	EIA	Serum/plasma	Anti-HBs	DiaSorin s.r.l.	4/18/1995

Source: FDA 2002a.

Table 2-5. FDA approved/licensed antibody against hepatitis B core antigen (anti-HBc Assay) tests

Trade name	Format	Sample	Use	Manufacturer	Approval date
Corzyme	EIA	Serum/plasma	Donor screen	Abbott Laboratories Abbott Park, IL U.S. License 0043	3/19/1991
Ortho HBc ELISA Test System	EIA	Serum/plasma	Donor screen	Ortho-Clinical Diagnostics, Inc. Raritan, NH U.S. License 1236	4/18/1991
AB-COREK	RIA	Serum/plasma	Donor screen	DiaSorin s.r.l. Saluggia, Italy U.S. License 1257	3/19/1991
ETI-AB-COREK	EIA	Serum/plasma	Donor screen	DiaSorin s.r.l.	3/19/1991

Source: FDA 2002a.

2.2.3 Liver function and liver enzyme assays

Liver enzyme tests and other liver-related clinical assays are used to monitor liver function during acute or chronic hepatitis (NACB 2000). The National Academy of Clinical Biochemistry (NACB) has recommended guidelines for serum tests to evaluate patients with either known or suspected liver disease, including HBV. The test panel includes the following: serum aspartate aminotransferase (AST), serum amino alanine transferases (ALT), alkaline phosphatase, serum gamma glutamyltransferase (GGT), total bilirubin, direct bilirubin, serum albumin, and prothrombin time. These assays, particularly ALT, provide simple biochemical tests to assess liver disease activity and to establish the severity of hepatitis in the individual (Lok and McMahon 2001).

2.3 Descriptive epidemiology of HBV infection

2.3.1 Prevalence of HBV infection

2.3.1.1 Worldwide

A Fact Sheet published by the World Health Organization reports that an estimated two billion people have been infected with HBV. More than 350 million of these people have chronic infection. WHO has categorized chronic HBV infection as high (prevalence ≥ 8% of the general population of a country or region), intermediate (prevalence 2% to 7%), or low (prevalence < 2%). Areas of high endemicity account for 45% of the global population and include Africa, Asia (east of the Indian subcontinent but excluding Japan), the Pacific Basin, the Amazon Basin, the Arctic Rim, the Asian Republics previously part of the Soviet Republics, and parts of the Middle East, Asia Minor, and the Caribbean. The carrier prevalence of HBV is between 5% and 10% of the general population in the Eastern European countries of Bulgaria, Romania, Albania, and Moldavia. Intermediate endemicity of chronic HBV accounts for 43% of the global population and includes parts of Southern and Eastern Europe, the Middle East, Japan, Western Asia through the Indian subcontinent,

and parts of Central and South America. Low endemicity is reported for most of Western Europe, Australia, and North America. Although average rates are given by countries and regions, the carrier rate is likely to vary within a country or area, particularly among different ethnic groups (Hollinger and Liang 2001). The U. S. Department of Health and Human Services Centers for Disease Control (CDC) has published similar prevalence data, including a map showing the geographic distribution of hepatitis B surface antigen worldwide (Figure 2-3).

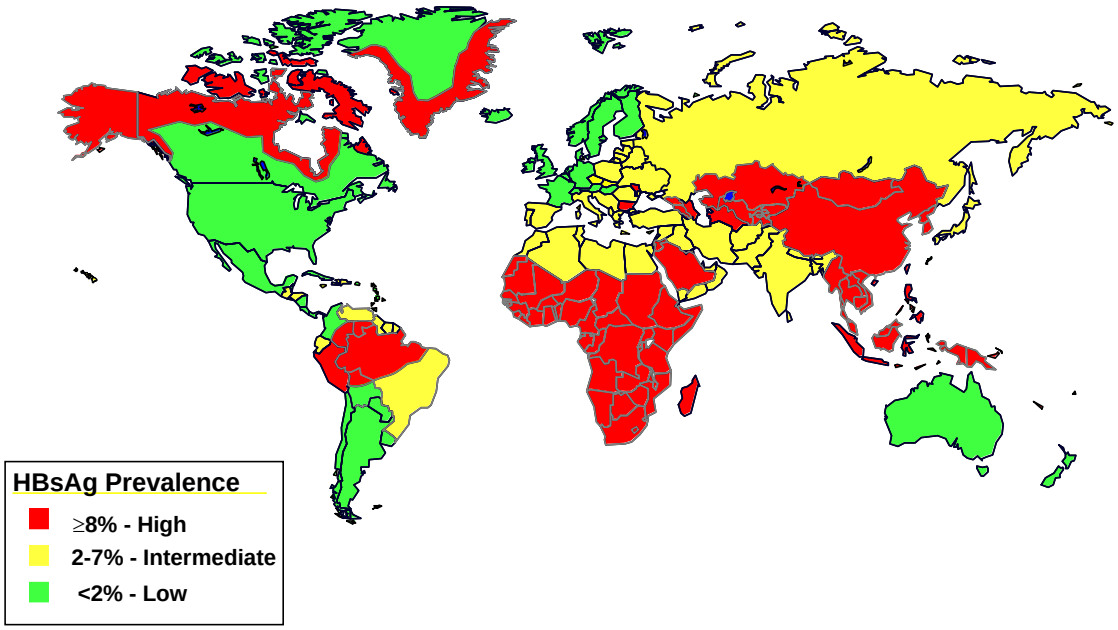

Figure 2-3. Geographic distribution of chronic hepatitis B prevalence for the year 2000

Source: CDC 2002a

2.3.1.2 United States

The prevalence of HBV infection in the United States has been determined from samples collected as part of the National Health and Nutrition Examination Surveys (NHANES) II and III (McQuillan *et al.* 1999). NHANES II covered the period from 1976 to 1980, and NHANES III covered the period from 1988 to 1994. The prevalence data included both resolved and chronic HBV infections. Chronic HBV infection was defined as the presence of both HBsAg and antibody to HBsAg. Past or present HBV infection was defined somewhat differently for NHANES II and NHANES III. For NHANES II, the presence of any two serologic markers (anti-HBsAg [AUSAB, Abbott Laboratories], HBcAg

[CORZYME, Abbott Laboratories], or HBsAg [AYSZYME, Abbott Laboratories]) was considered diagnostic; for NHANES III, the presence of any HBV infection (anti-HBcAg [CORAB, Abbott Laboratories] with confirmation by HBsAg [AUSRIA II, Abbott Laboratories] and anti-HBsAg [AUSAB, Abbott Laboratories]) was based on a positive or borderline result for an antibody to HBV core antigen assay.

The age-specific (ages 6 to 74) figures for prevalence of HBV infection for ethnic groups included in NHANES II and III are illustrated in Figure 2-4. Prevalence increased only slightly with age in most ethnic groups except non-Hispanic blacks, in whom dramatic increases were observed.

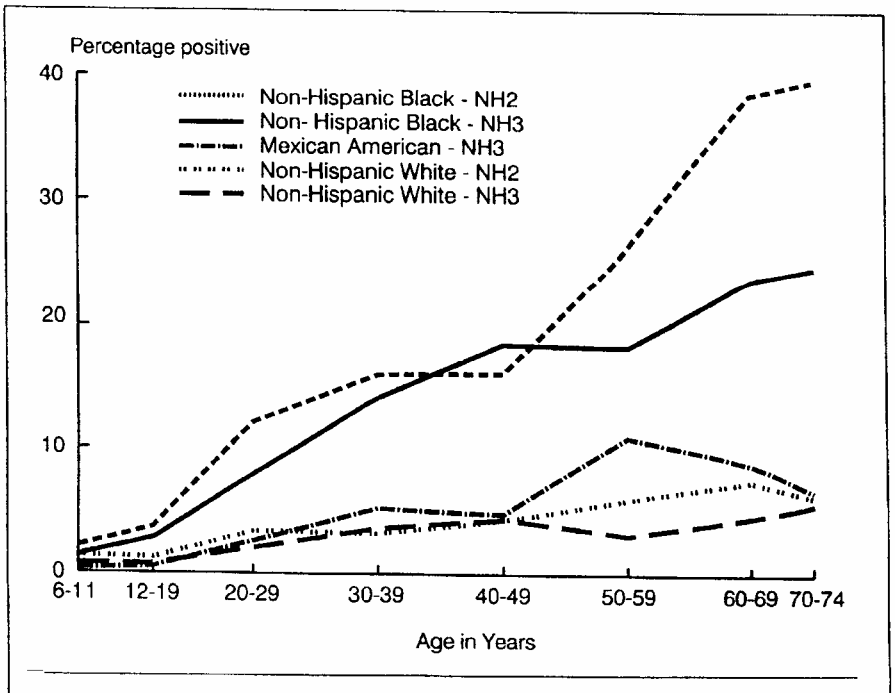

Figure 2-4. Age-specific prevalence of HBV infection, by ethnicity: NHANES II (NH2) and NHANES III (NH3) participants aged 6 to 74 years

Source: McQuillan *et al.* 1999

The overall age-adjusted prevalence of resolved and chronic HBV infection decreased from 5.5% (95% CI = 4.8 to 6.2) in NHANES II to 4.9% (95% CI = 4.3 to 5.6) in NHANES III (not significantly different). Significant decreases were observed between surveys among persons more than 50 years old for some ethnic groups; prevalence among non-Hispanic Whites decreased from 3.6% to 2.6% and among non-Hispanic Blacks from 15.9% to 11.9% ($P < 0.05$, Sattherthwaite-adjusted F statistic). Non-Hispanic Blacks had a significantly elevated ($P < 0.05$) prevalence of HBV infection compared to non-Hispanic Whites and Mexican Americans in both surveys. McQuillan *et al.* (1999) interpreted the decrease in HBV prevalence in these populations as reflecting the loss of infected persons

in an older cohort. The incidence of hepatocellular carcinoma in these populations was not addressed in this study.

2.3.2 Incidence of HBV infection

2.3.2.1 Worldwide

Estimates of the annual incidence of HBV range from 3% to 5% among children in areas of Asia and Africa where the virus is endemic (Hollinger and Liang 2001). The epidemiological pattern in these areas is different from that in North America and Western Europe. Most infections in the high endemic areas occur in infants and children who probably contract the disease through maternal-neonatal transmission or early childhood contact. Percutaneous transmission of HBV through contaminated needles or other unsafe injection methods may account for 8 million to 16 million HBV infections in adults and children (Kane *et al.* 1999). Maddrey (2000) reviewed HBV as a public health issue worldwide. Although HBV vaccines are now widely available, new infections are still common. Approximately one million people become infected each year in Europe. Although corresponding data are not available for regions of Asia and Africa where HBV is endemic, Maddrey (2000) suggested that infection rates are likely to be much higher in those areas, consistent with the high prevalence of HBV.

2.3.2.2 United States

A total of 8,036 cases of acute symptomatic hepatitis B was reported to the CDC in 2000; the number of new cases represents a greater than 60% decrease compared to 10 years earlier, when 21,102 cases were reported (MMWR 2002a;see Figure 2-5). The number of cases of HBV reported annually in the United States has decreased steadily since 1985, largely due to a decline in incidence among homosexual men and intravenous drug users. The highest rate of reported cases in 2000 was among individuals between 25 and 39 years of age (incidence rate = 5.76/100,000). A higher number of cases was reported for males (4,981 cases [incidence rate = 3.74/100,000]) than for females (2,997 cases [incidence rate = 2.15/100,000]). Similar incidence rates and trends of acute viral hepatitis were reported in a study conducted by the CDC of four counties in the United States, which were considered typical of disease incidence and demographic makeup of the United States as a whole. The incidence rate of acute HBV infection in 1998 was 3.3 per 100,000, which had decreased from 13.8 per 100,000 in 1987 (Goldstein *et al.* 2002).

The CDC believes that number of symptomatic cases reported is much smaller than the actual number of new infections. The most recent CDC estimate of new infections that occurred between 1995 and 1999 was 105,000 per year. Earlier CDC estimates put the number of new infections in the United States for the two decades prior to 1999 at 200,000 to 300,000 annually (McQuillan *et al.* 1999). The CDC also noted that infections among infants and young children are likely to be underestimated because most infections in this group are asymptomatic.

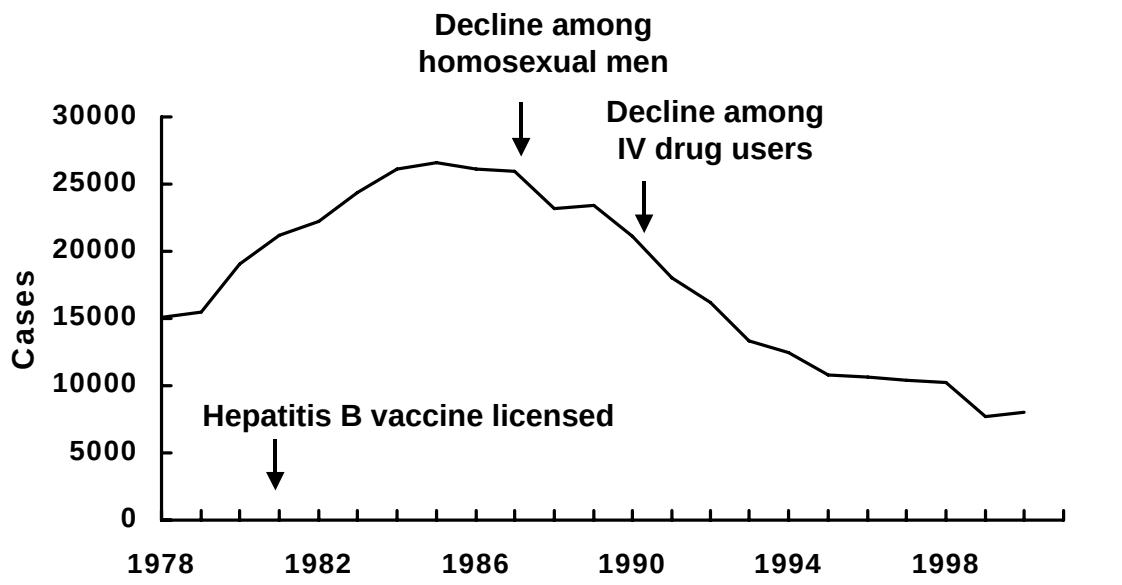

Figure 2-5. Reportable symptomatic HBV in the United States (1978 to 2000)

Source: CDC 2002b

Note: The incidence of symptomatic cases is smaller than the actual number of new infections.

2.3.3 HBV incidence and prevalence in United States blood donors

The risk of transfusion-transmitted HBV has been estimated to be 1 in 63,000 (Glynn *et al.* 2000). Factors that have contributed to the current low risk include donor education, donor screening, and improved laboratory test procedures. To determine changes over time in infection rates of HBV in the United States donor population, Glynn *et al.* (2000) used cross-sectional survey data from the Retrovirus Epidemiology Donor Study sponsored by the National Heart, Lung, and Blood Institute, which collected data from a total of 1.9 million volunteer blood donors between January 1991 and December 1996. No statistically significant trend was found in the prevalence of HBsAg among first-time donors. The proportion of HBsAg-positive, first-time donors was approximately 0.2%, and the overall estimated incidence rate for HBV was 2.66 (95% CI = 2.04 to 3.41) per 100,000 person-years. The authors also reported that the prevalence of HBV-positive, first-time donors seemed to have remained constant since the late 1970s when a prevalence of 0.26% was reported.

2.3.4 Risk factors for HBV infection

According to CDC sentinel surveillance study (Figure 2-6), the risk factors for acquisition of HBV infection in order of importance are (1) heterosexual transmission, either through heterosexual contact with a person with acute or chronic HBV infection or having more

than one heterosexual partner, (2) injected drug use, (3) homosexual transmission (i.e., men who have sex with men), (4) household contact with a person with acute or chronic HBV infection, and (5) health-care workers (i.e., occupational exposure to blood). However, about one-third of HBV infections was not associated with any known risk factor. Other risk factors that contribute to only a small percentage of HBV infections are chronic hemodialysis, institutionalization in a home for the developmentally disabled, and blood transfusion from an infected donor (Goldstein *et al.* 2002, Alter *et al.* 1990).

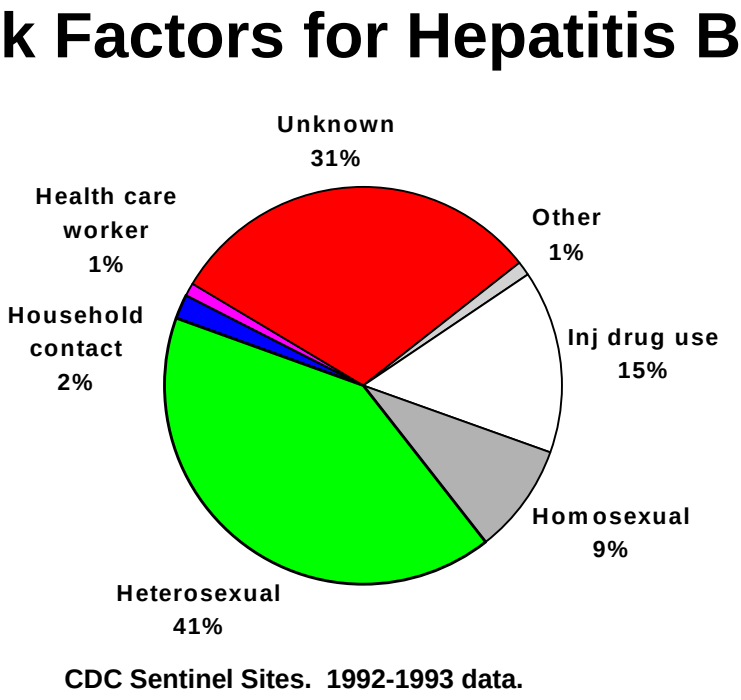

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Figure 2-6. Risk factors for acute HBV infection in the United States

Source: CDC 2002b

Overall, The CDC sentinel surveillance study of four counties in the United States (see Section 2.3.2.2) identified a recognized risk factor for 66.0% of the 3,296 people with HBV infection who were interviewed between 1982 and 1998. The predominant risk factors for infection were heterosexual exposure to an infected partner or multiple partners, intravenous drug use, and men who have sex with men. Collectively, these three risk factors accounted for about 88% of the cases for which a risk factor could be identified. Nevertheless, significant declines in the cases associated with these risk factors have occurred over the past two decades. For example, cases associated with heterosexual exposure declined by 50.7% between 1992 and 1994 among whites, cases among intravenous drug users declined by 90.6% between 1988 and 1998. Cases among homosexual men declined by 63.5% between 1982 and 1986 but has not changed much

since then (Goldstein *et al.* 2002). The risk for many groups has decreased with improvements in blood screening and availability of a vaccine for HBV (Hollinger and Liang 2001). The CDC has suggested that continued implementation of a comprehensive immunization strategy (see Section 2.5.1.2) has the potential to eliminate transmission of HBV within the United States.

While comparable data on risk factors are not available for acquisition of HBV infection worldwide, perinatal transmission of the virus from an infected mother to her offspring either at birth or during childhood has been suggested to be the most important mechanism explaining the maintenance of a population of HBV carriers (Hollinger and Liang 2001). Perinatal transmission is proposed as the explanation for carrier status of at least 23% of carriers in Asia and 8% in Africa.

2.3.5 Transmission

The major modes of transmission of HBV are parenteral, contact-associated, maternal-neonatal, and nosocomial (Hollinger and Liang 2001). The WHO (2000) listed the major routes of infection as perinatal (from mother to baby at birth), child-to-child transmission, unsafe injections and transfusions, and sexual contact. Occupational transmission is also a major concern, and the CDC has published recommendations for reducing those risks for health-care personnel. Although HBV shares similar modes of transmission with human immunodeficiency virus (HIV) (i.e., contact with blood or body fluids of an infected person), the infectivity of HBV is 50 to 100 times greater than that of HIV. HBV transmission cannot occur through casual contact in the workplace or through contaminated food or water; however, as noted below, transmission is possible within households. The IARC Working Group (1994) reported three life stages at which HBV infection occurs by different modes of transmission (i.e., at birth, in early childhood, and in adult life). The most common modes of transmission worldwide are from infected mother to child (neonatal), from infected child to child by contact within the household (early childhood), and from the reuse of unsterilized needles or syringes and sexual transmission (adult). In many countries of the developing world, almost all children become infected with HBV. The pattern of transmission differs in many of the industrialized countries of Western Europe and North America. The majority of infections in these countries results from sexual activity and intravenous drug use during young adulthood.

Modes of transmission of HBV infection in the United States and other developed countries fall into two major categories, parenteral and sexual exposure. As noted above (Section 2.3.4), the predominant risk factors for infection were heterosexual exposure to an infected partner or multiple partners, intravenous drug use, and men who have sex with men.

2.3.5.1 Parenteral transmission

Although parenteral or percutaneous transmission of HBV is still a principal method by which the virus is spread in the United States, the risk of transmission by blood transfusion or administration of blood products has decreased dramatically in recent years (Hollinger and Liang 2001).

The risk of transmission of HBV by parenteral exposure remains high among users of illicit drugs who share contaminated needles or syringes. By 1988, 27% of patients with HBV infection in the four CDC sentinel counties reported that they used parenteral drugs, making intravenous drug use the major risk factor for acquiring HBV at that time (Alter *et al.* 1990). HBV infection also is common in some hemodialysis units, where outbreaks are caused by cross-contamination among patients (MMWR 2001a). Preventive measures, such as segregating HBV-infected patients and their equipment from other patients, resulted in 70% to 80% reduction in the incidence of HBV infection among dialysis patients.

2.3.5.2 Contact-associated transmission

In the United States, the transmission of HBV by sexual contact, either heterosexual or homosexual, with an infected person is currently the most common route of transmission (Hollinger and Liang 2001). As illustrated in Figure 2-6, 50% of HBV cases are attributable to sexual contact; 41% of those are due to heterosexual contact, 9% to homosexual contact (see also Section 2.3.4). HBV-infection rates have historically been higher among homosexual men. However, within that population, two major factors—modifications to high-risk behavior, aimed at preventing HIV infection, and the availability of the HBV vaccine—have dramatically decreased the frequency of the disease in this group in recent years.

The risk of contact-associated transmission also includes nonsexual routes of transmission within households (e.g., through shared razors, towels, or toothbrushes) (Hollinger and Liang 2001). Household surfaces also may be contaminated from skin lesions of HBV-infected persons.

2.3.5.3 Maternal-neonatal transmission

Maternal-to-neonatal transmission has been proposed as the most common route of transmission of HBV in parts of the world where HBV is endemic in a large portion of the population (e.g., Asia and Africa) (Hollinger and Liang 2001). Transplacental transmission does not appear to be an important means of transferring HBV infection, because neonatal infection is less common when an acute HBV infection occurs during the first or second trimester of pregnancy. HBsAg may be present in maternal blood, amniotic fluid, cord blood, breast milk, and vaginal secretions. Although the means by which perinatal infection occurs have not been defined, proposed routes of transmission of HBV include amniotic fluid swallowed by the fetus *in utero*, maternal blood swallowed during delivery, and transmission through minor abrasions or across mucous membranes. Although HBV could theoretically be transmitted postnatally through breast-feeding, current data do not support an increased risk to breast-fed infants of HBV-positive mothers. In a populaton of 369 infants born to women with chronic HBV, of whom 268 bottle fed and 101 were breast fed, only nine infants, all of whom were bottle fed (3% of 268), were positive for HBsAg (Hill *et al.* 2002). The authors were not able to separate prenatal and postnatal transmission in this study; however, they concluded that breast feeding of infants by mothers with chronic HBV does not pose any additional risk of transmission of the virus when appropriate immunoprophylaxis with hepatitis B immune globulin and hepatitis B vaccine is used.

2.3.5.4 Nosocomial transmission

Transmission of HBV from health-care workers to patients in health-care settings is rare (Hollinger and Liang 2001); however, such transmission can occur. Reports include cases attributed to HBV-positive oral surgeons and dentists, infections associated with obstetric-gynecologic surgeons, cardiac surgeons, and other health-care workers, such as inhalation therapists. Most of the health-care worker-to-patient transmission can be prevented with the use of protective equipment, primarily gloves.

The risk of HBV infection to health-care workers depends on the degree of contact with blood in the workplace and with the HBeAg status of the person who is the source of that blood (MMWR 2001b). Percutaneous injuries can transmit HBV infection efficiently; the risk of developing serologic evidence of HBV infection has been reported to be 37% to 62% if the blood source was both HBsAg and HBeAg positive. The risk of developing clinical hepatitis under the same circumstances was 22% to 31%. The risk associated with body fluids other than blood is much lower. Although HBsAg has been found in other body fluids (including breast milk, bile, cerebrospinal fluid, feces, nasopharyngeal washings, saliva, semen, sweat, and synovial fluid), the concentration of HBsAg in these fluids can be 100- to 1,000-fold higher than the concentration of infectious HBV particles. Thus, body fluids other than blood are not likely to be efficient vehicles for transmission of HBV because they contain small amounts of infectious HBV.

2.4 Clinical disease other than cancer

Hepatitis refers to inflammation of the liver. Acute hepatitis resulting from HBV infection or other causes has a common set of symptoms during a period of several weeks. These symptoms include yellowing of the skin and eyes (jaundice), dark urine, extreme fatigue, nausea, vomiting, and abdominal pain. The patient may recover from these symptoms after several months to a year; however, when the patient cannot clear the virus, HBV also can cause a chronic infection with risk of liver cirrhosis or liver cancer.

2.4.1 Natural history

Yuen and Lai (2000) contrasted the natural history of chronic HBV infection acquired during birth or early childhood, which is most common in the Asian population, with infection acquired during adulthood, which is most common in the Caucasian population. The natural history of HBV infection is characterized by three phases: (1) the immune tolerance phase, (2) the immune clearance phase, and (3) the phase of residual integrated HBV. The disease process in hepatitis is illustrated in Figure 2-2. The risk that a person will be chronically infected and become a carrier is strongly related to age at infection (Figure 2-7). The risk of acquiring chronic infection also is greater in males than in females, with a ratio of approximately 1.6:1 (London 1979). The sex ratio for carrier status increases with age because males tend to have a longer duration of chronic infection (Coursaget *et al.* 1987). As noted in Section 3, hepatocellular carcinoma is a disease with a strong male dominance, which is observed across all populations.

The pathogenesis of HBV infections is described in Section 6.1; however, some outcomes of the host immune response are listed in Table 2-6. Most of the damage to the host

organism is self-inflicted, i.e., it is caused by adverse effects of the host immune responses to HBV-infected hepatocytes (Hilleman 2001).

Table 2-6. Pathogenesis of hepatitis B

Hepatitis B virus is not cytopathogenic. Damage results from host immune response
Outcomes of host immune response:
Vigorous- usually results in spontaneous resolution
Mild- may result in persistent infection
Slow resolution
Persistent infection
Chronic hepatitis
Fulminant
Chronic-active hepatitis: remission and exacerbation
Mortality– 25% (cirrhosis, cancer)
Random integration of viral DNA segments into host cell genome

Source: Hilleman 2001, by permission

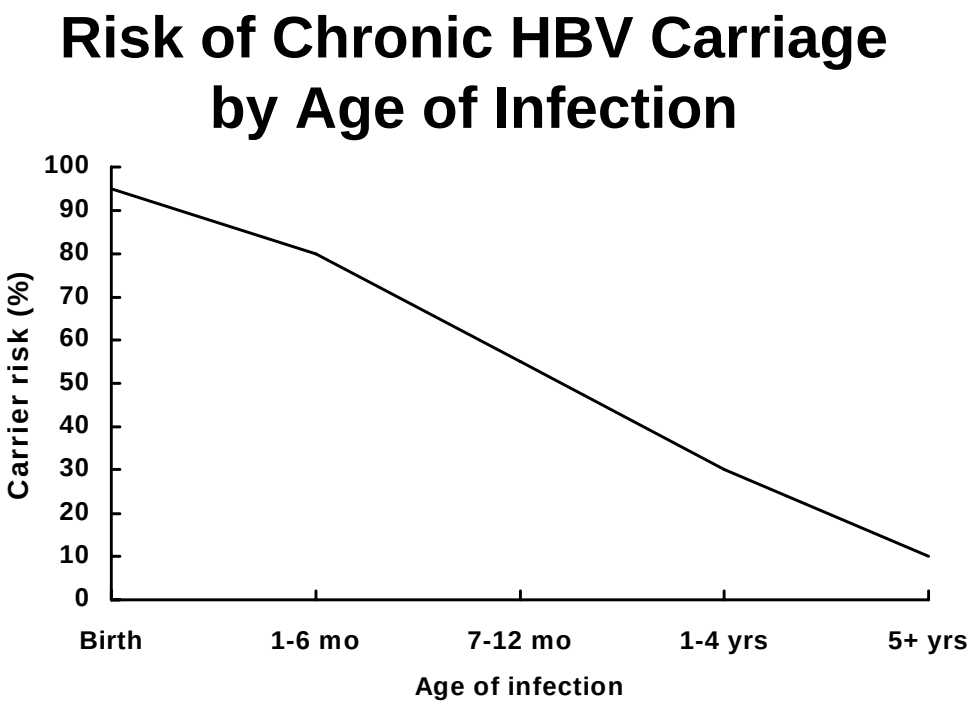

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Figure 2-7. Risk of chronic HBV carriage by age of infection

Source: CDC 2002b

2.4.1.1 Chronic HBV infection acquired during childhood

When infection is acquired during early childhood, an immunotolerance phase lasting two or more decades is common. Following this phase is an immuneclearance phase of variable duration, the endpoint of which is HBeAg seroconversion to anti-HBe. The major factors cited by Yuen and Lai (2000) to explain the predisposition of young children to chronic HBV infection are the relative immaturity of the immune system and induction of T-cell tolerance to nucleocapsid-derived HBV peptides as a result of soluble HBeAg in the mother crossing the placenta.

The liver damage associated with chronic HBV infection is believed to occur primarily during the immune-clearance phase described above; however, these findings are based on patients who present at clinics and may not represent the subclinical course of the disease. The course of the disease may include multiple episodes of acute exacerbation that may be accompanied by increased serum ALT. About five to eight weeks prior to an episode of acute exacerbation, elevations have been observed in serum HBV DNA, HBeAg concentration, and HBeAg/anti-HBe immune complex formation. The peak in serum ALT levels, which may represent maximal liver damage, is followed one to four weeks later by a peak in anti-HBe antibodies. Researchers have suggested that pattern may reflect an increase in viral replication and accumulation of viral proteins in the serum and within cells that reach a threshold level sufficient to elicit specific immune responses. These immune

responses are postulated to initiate the liver injury associated with the episodes of acute exacerbation. Chronic HBV infection acquired during adulthood

Chronic HBV infection acquired during adulthood may result in either chronic hepatitis or a state referred to as a “healthy carrier” (Yuen and Lai 2000). “Healthy carriers” usually have normal serum aminotransferase levels, undetectable or very low HBV DNA, normal or minimally abnormal liver biopsies, and integrated or undetectable HBV DNA in the liver. The term “healthy carrier” may, however, be misleading since chronic HBV infection is associated with increased risk of liver and other cancers (see Section 3).

2.4.2 Viral clearance

Sherlock (1987) reported that approximately 10% of patients diagnosed with acute HBV infection were still positive for HBsAg in the serum after six months. These individuals are considered to be “carriers,” a condition that can persist. A distinct sex difference exists for development of carrier status, males being six times more likely to become carriers of HBV than females. HBV is not cytopathic for hepatocytes; therefore, viral clearance is considered to be a result of the immune response (Guidotti 2002). Patients who successfully clear the virus after acute infection with HBV mount a multispecific polyclonal cytotoxic T lymphocyte (CTL) cell response to a number of HBV-encoded antigens. The absence of such a response, or a weak response, is associated with development of chronic infection (see Section 6.1.2). Thus, the ability of the infected individual to mount an immune response is believed to determine whether HBV infection persists or is cleared. While the key factors that affect the outcome of an infection with HBV are not defined, it is reasonable that the clinical outcome would depend on viral, host, and environmental factors (Han *et al.* 2002).

The efficiency level for clearance of HBV infection may differ among HBV carriers from different geographic regions in which there are also varying degrees of risk of hepatocellular carcinoma. In a cross-sectional study of serological markers of HBV infection, Evans *et al.* (1998) compared adult male carriers from China (Haimen City), Senegal, and West Africa, and Asian-Americans living in or near Philadelphia, Pennsylvania for prevalence of HBV DNA positivity. Among Senegalese men in their 20s, 14.5% were HBV DNA positive by Southern blot assay; however, the prevalence fell to 0% for men in their 50s. Among Chinese men the prevalence was 29.4% for those in their 20s; 20.6% of men in their 50s also were positive. Among Asian-Americans, the prevalence was 36.8% for men in their 20s and 4.6% for those in their 50s. The authors concluded that the differences in clearance of HBV among different populations were not sufficient to explain differences in the risk of hepatocellular carcinoma in the same populations.

2.4.3 Co-infection with HBV and HCV

Coinfection with both HBV and HCV is associated with more severe liver disease and with resistance to interferon therapy (Villa *et al.* 2001, see Section 2.5). Cirrhosis develops more often in patients with coinfection than in those with HCV alone. Epidemiological studies (see Section 3.4) also support a synergistic effect of HBV and HCV coinfection on risk of hepatocellular carcinoma.

2.5 Preventive measures, including vaccines, and therapy

The therapeutic measures for HBV are of limited efficacy; therefore, the emphasis has been on prevention of HBV infections through reduction of contact with potentially contaminated body fluids in health-care settings ("universal precautions") or by vaccination in the general population.

2.5.1 Preventive measures

2.5.1.1 Universal precautions

The Occupational Safety & Health Administration (OSHA) on January 1, 1992 (OSHA 1992) established a "bloodborne pathogens final standard," which covers potential exposure to infectious material, including semen, vaginal secretions, cerebrospinal fluid, synovial fluid, pleural fluid, pericardial fluid, peritoneal fluid, amniotic fluid, saliva in dental procedures, any body fluid visibly contaminated with blood, and all body fluids in situations where it is difficult or impossible to differentiate between them. This standard also includes unfixed human tissues or organs and any cell, tissue, or organ cultures containing HIV or HBV, including those from experimental animals infected with either virus (see Section 2.6 and Table 2-8).

The central feature of the OSHA standard is the concept of "universal precautions," which are emphasized in medical and laboratory settings throughout the United States. The principal of "universal precautions" calls for all body fluids/materials to be treated as infectious and establishes a number of specific practices to be followed. These practices include provisions for hand-washing and procedures to minimize needlesticks and splashing and spraying of blood, procedures to ensure appropriate packaging of specimens and regulated wastes, and instructions to ensure proper labeling or decontamination of contaminated equipment. In addition, employees must have access to and must use personal protective equipment such as gloves, gowns, masks, mouthpieces, and resuscitation bags to reduce the potential for contact with body fluids. The standard specifies methods for safely disposing of contaminated sharps and sets forth standards for containers for these items and other regulated waste. Microbiological laboratories must follow standard microbiological practices and additional practices intended to minimize exposure of employees working with concentrated viruses and to reduce the risk of accidental exposure for other employees at the facility. Employers also are required to provide the following: (1) a written schedule for decontamination following contact with potentially infectious materials, (2) hazard communication through orange or orange-red biohazard symbols on warning labels of containers with potentially infectious materials, (3) information and training of employees, (4) HBV virus vaccination within 10 working days of assignment, (5) post-exposure evaluation and follow-up including a confidential medical evaluation, and (6) maintenance of medical records for each employee with occupational exposure.

2.5.1.2 HBV vaccines

Prior to the late 1980s, plasma-derived vaccines were developed in the United States, but today their manufacture is limited to a few countries in Asia and Southeast Asia (Hollinger and Liang 2001). The FDA currently approves three hepatitis B vaccines. All three are subunit viral vaccines derived from HBsAg produced in yeast cells. Recombivax HB is

marketed by Merck & Co., Inc., Engerix-B by GlaxoSmithKline, and Twinrix (a combined hepatitis A and hepatitis B vaccine) by GlaxoSmithKline (FDA 2002b). Hollinger and Liang (2001) reported that clinical trials involving 40,140 vaccine doses found no clinically adverse effects; however, adverse reactions including anaphylaxis, immediate hypersensitivity reactions, and delayed hypersensitivity reactions have been reported, although rarely.

The first official recommendation for the use of hepatitis B vaccine was published in 1982 and covered those groups known to be at high risk for HBV infection. The CDC (MMWR 1991), through its Advisory Committee on Immunization Practices (ACIP), has recommended universal HBV vaccination of infants, regardless of the HBsAg status of the mothers, and adolescents at high risk of infection because of intravenous drug use or multiple sex partners. In addition, vaccination of persons at high risk included: (1) persons with occupational risk, (2) clients and staff of institutions for the developmentally disabled, (3) hemodialysis patients, (4) recipients of certain blood products, (5) household contacts and sex partners of HBV carriers, (6) adoptees from countries where HBV infection is endemic, (7) international travelers, (8) injecting drug users, (9) sexually active homosexual and bisexual men, (10) sexually active heterosexual men and women, and (11) inmates of long-term correctional facilities. Current recommendations (CDC 2002c) also include a 12th category, all unvaccinated adolescents.

The most recent recommended childhood immunization schedule provided by CDC (MMWR 2002b) and approved by the ACIP, the American Academy of Pediatrics, and the American Academy of Family Physicians includes the recommendation that all infants should receive the first dose of hepatitis B vaccine soon after birth and before hospital discharge. As of September 2002, school or daycare requirements for vaccination against hepatitis B had been implemented by 44 states and the District of Columbia (IAC 2002). The recommendations for adolescent/adult immunization for hepatitis B (CDC 2002c) call for three intramuscular doses, with the second dose one to two months after the first and the third dose four to six months after the first. The incidence of acute hepatitis B among children and among health-care workers has decreased as a result of the vaccination program (MMWR 2002a).

Internationally, WHO (2000) is encouraging national immunization programs to include the HBV vaccine. Although 116 countries, including most countries in Eastern and Southeast Asia, the Pacific Islands, Australia, North and South America, Western Europe, and the Middle East included HBV vaccine in national programs as of March 2000, many low-income countries do not yet use the vaccine. Cost is an obstacle to the adoption of the vaccine in sub-Saharan Africa, the Indian subcontinent, and Newly Independent States of the former Soviet Union.

Approximately 14% of the population may not respond to vaccination for HBV, as indicated by the production of low levels of anti-HBsAg after vaccination (Alper *et al.* 1989). Risk factors for low response may include human leukocyte antigens (HLA), age, body mass index, smoking status, and gender (Alper *et al.* 1989, Wood *et al.* 1993).

2.5.2 Therapy

Treatment of chronic hepatitis B infection has the aim of limiting long-term cirrhosis-related complications, because complete eradication of the virus from an infected individual is not considered a reasonable therapeutic endpoint (Yuen and Lai 2001). Therapeutic agents for HBV include immunomodulators, antiviral agents, and combination therapy.

Immunomodulators include nonspecific agents, such as interferon alpha (recombinant, pegylated), thymosin α_1, levamisole, interleukin-2, interleukin-12, and others (Hollinger and Liang 2001). Interferon alpha, is a glycoprotein cytokine produced by leukocytes in response to various stimuli, including viral infection; it is one of three licensed therapies for chronic hepatitis and is sometimes used in combination with the antiviral, lamivudine. Viral-specific agents include therapeutic vaccines, and cell-based therapy involves adoptive immune transfer.

Antiviral agents (nucleoside/nucleotide analogues) include lamivudine, adefovir dipivoxil, entacavir, emtricatabine, β-L-2'-deoxythymidine, famciclovir, and other nucleoside analogues. Lamivudine and adefovir dipivoxil are the other approved therapies for chronic hepatitis. Lamivudine is a pyrimidine derivative in the “unnatural” L-configuration (Torresi and Locarnini 2000). Lamivudine is the negative enantiomer of a dideoxy analogue of cytidine and acts as a reverse transcriptase inhibitor through its 5'-triphosphate metabolite. (AIDSinfo 2003b). Adefovir dipivoxil is a diester prodrug of the acyclic nucleotide analog of adenosine monophosphate, adefovir. Adefovir diphosphate acts as an inhibitor of HBV polymerase by competing with the natural substrate deoxyadenosine triphosphate; this inhibition causes DNA chain termination after incorporation of adefovir diphosphate into viral DNA (AIDSinfo 2003a). Other antiviral therapies include glycosylation inhibitors and gene therapy by antisense RNA, ribozyme, or targeted drug delivery.

Combinations of drugs appear to be the most effective strategy for HBV therapy (Schalm *et al.* 2002). The combination of interferon and lamivudine may induce a sustained response, while lamivudine plus adefovir is considered the best choice for long-term antiviral therapy. Other combination therapies include lamivudine plus therapeutic vaccines, steroid priming followed by lamivudine or interferon alpha, and multiple nucleoside analogues such as lamivudine plus famciclovir.

Long-term beneficial responses (i.e., inhibition of viral replication) to interferon alpha were reported in only about 35% of patients with HBeAg-positive chronic HBV infection, while beneficial responses were reported for 16% to 20% of patients treated with lamivudine. Lewin *et al.* (2002) also reported that pilot studies of interferon alpha in combination therapy with lamivudine support an increased rate of response; however, even the combined therapy was effective in only about one-third of patients.

2.6 Regulations

The FDA has set regulations for human blood and blood products that require testing for HBsAg, regulations that require that human tissue donors be assessed for their risk for hepatitis, and regulations that require that specimens from the donors be tested for hepatitis

B. OSHA has set rules for the recording of occupational injuries that could lead to hepatitis B infection and also requires that employers make available the hepatitis B vaccine to all employees who have had occupational exposure to HBV. TheU.S. Public Health Service has set regulations for transporting hepatitis B-associated materials. See Tables 2-7, 2-8, and 2-9 for a summary of these regulations.

Table 2-7. FDA regulations regarding human blood and blood products

Regulatory citation	Regulatory action
21 CFR 610 - PART 610 - GENERAL BIOLOGICAL STANDARDS. Promulgated 38 FR 32056, 11/20/73. U.S. Codes: 21 U.S.C. 321, 351, 352, 353, 355, 360, 371; 42 U.S.C. 216, 262, 263, 263a, 264.	Each donation of blood, plasma, or serum to be used in preparing a biological product shall be tested for the presence of hepatitis B surface antigen.
21 CFR 640 - PART 640 - ADDITIONAL STANDARDS FOR HUMAN BLOOD AND BLOOD PRODUCTS. Promulgated: 38 FR 32089, 11/20/73. U.S. Codes: 21 U.S.C. 321, 351, 352, 353, 355, 360, 371; 42 U.S.C. 216, 262, 263, 263a, 264.	No individual shall be used as a source of whole blood if he has a history of viral hepatitis, a history of close contact within 12 months of donation with an individual having viral hepatitis, a history of having received within 12 months of donation human blood or any derivative which the FDA has advised the blood establishment is a possible source of viral hepatitis. Each unit of source plasma shall be nonreactive to a test for hepatitis B surface antigen.
21 CFR 660 - PART 660, SUBPART A - ANTIBODY TO HEPATITIS B SURFACE ANTIGEN. Promulgated: 40 FR 29711, 7/15/75. U.S. Codes: 21 U.S.C. 321, 351, 352, 353, 355, 360, 371; 42 U.S.C. 216, 262, 263, 263a, 264.	Hepatitis B surface antigen: the source shall be plasma or blood, obtained aseptically from animals immunized with hepatitis B surface antigen which have met the outlined requirements on processing and materials.
21 CFR 1270 - PART 1270 - HUMAN TISSUE INTENDED FOR TRANSPLANTATION. Promulgated: 62 FR 40444, 7/29/97. U.S. Codes: 42 U.S.C. 216, 243, 264, 271.	Donor must be questioned to elicit whether he or she is at increased risk for hepatitis; the physical assessment of donor must be performed to check for any signs of hepatitis infection; and the donor's medical records must be checked for hepatitis. Donor specimens must be tested for hepatitis B.

Table 2-8. OSHA regulations for recording and treating occupational injuries that can lead to hepatitis B

Regulatory citation	Regulatory action
29 CFR 1904 and 1952 - PARTS 1904 AND 1952 - OCCUPATIONAL INJURY AND ILLNESS RECORDING AND REPORTING REQUIREMENTS. Promulgated: 66 FR 5916, 1/19/01. U.S. Codes: 29 U.S.C. 657, 673.	Rules for the recording of occupational injuries involving punctures, cuts, and lacerations caused by needles or other sharp objects contaminated with potentially infectious materials that could lead to hepatitis B.

Regulatory citation	Regulatory action
29 CFR 1910 - PART 1910, SUBPART Z - TOXIC AND HAZARDOUS SUBSTANCES. Promulgated: 39 FR 35896, 10/4/74, as amended numerous times. U.S. Codes: 29 U.S.C. 653, 655, 657.	The employer shall make available the hepatitis B vaccine to all employees who have had occupational exposure to pathogenic microorganisms, including hepatitis B. Universal precautions should be observed to prevent contact with blood or other potentially infectious materials. Hepatitis B is mentioned under the section on exposure to vinyl chloride; a program of medical surveillance is required for each employee exposed to vinyl chloride in excess of the action level. A medical history shall be taken, including the employee's past history of hepatitis, and on repeated abnormal serum tests - tests shall be taken for hepatitis B antigen.

Table 2-9. U.S. Public Health Service regulations materials associated with hepatitis B

Regulatory citation	Regulatory action
42 CFR 72 - PART 72 - INTERSTATE SHIPMENT OF ETIOLOGIC AGENTS. Promulgated: 45 FR 48627, 7/21/80. U.S. Codes: 42 U.S.C. 246, 271; 31 U.S.C. 9701: 18 U.S.C. 3559, 3571; 42 U.S.C. 262 note.	Rules for transporting of diagnostic specimens, biological products, and packing requirements for hepatitis B-associated materials.

The regulations in Tables 2-7, 2-8, and 2-9 have been updated through the 2001 Code of Federal Regulations, December 31, 2001.

2.7 Summary

HBV is one of the major causes of viral hepatitis in the United States. An estimated 1 to 1.25 million Americans have contracted chronic hepatitis B. Detection is based on assays for one of several proteins coded for in the HBV genome or antibodies that the host produces against these proteins. The FDA currently approves enzyme immunoassays or radioimmunoassays for the hepatitis B surface antigen, antibody against the hepatitis B surface antigen, and antibody against the hepatitis B core antigen.

The prevalence of HBV infection, including both current and resolved infections, in the United States population remained relatively constant from the late 1970s (5.5% prevalence in NHANES II) through the early 1990s (4.9% prevalence in NHANES III). Worldwide, the prevalence of chronic HBV infection has been reported to range from < 2% (low) in Western Europe, Australia, and North America; to 2% to 7% (intermediate) in parts of Southern and Eastern Europe, the Middle East, Japan, Western Asia through the Indian subcontinent, and parts of Central and South America; to ≥ 8% (high) in Africa, Asia (east of the Indian subcontinent but excluding Japan), the Pacific Basin, the Amazon Basin, the Arctic Rim, the Asian republics previously part of the USSR, and parts of the Middle East.

The incidence rate for HBV infection in the United States has been estimated at approximately 105,000 new cases each year, although only a small fraction, roughly 8,000,

of this number is actually reported to the Centers for Disease Control. In parts of Asia and Africa where HBV is endemic, estimates of the annual incidence of infection among children range from 3% to 5%.

The major modes of transmission of HBV are parenteral, contact-associated, maternal-neonatal, and nosocomial. The natural history of HBV infection differs for those acquired at birth or during early childhood and those acquired during adulthood, which is the most common pattern for the United States population.

Therapeutic measures for HBV are of limited efficacy; thus, the emphasis has been on disease prevention through “universal precautions” in health-care settings and vaccination of infants and at-risk adults in the population at large. Therapeutic agents currently in use include immunomodulators, which are not specific for HBV, antiviral agents, and combination therapies from both classes.

3 Human Cancer Studies

3.1 Introduction

The cancer that is singularly associated with hepatitis B infection is hepatocellular carcinoma, the primary histological subgroup of primary liver cancer in humans. In the United States, where primary liver cancer is relatively rare, hepatocellular carcinoma constitutes 70% to 75% of primary liver cancer cases. The incidence of liver cancer in the United States between 1996 and 2000 was 5 per 100,000 (NCI 2003). In East/Southeast Asia and sub-Saharan Africa, where primary liver cancer is one of the most commonly occurring cancers, hepatocellular carcinoma constitutes well over 90% of all primary liver cancers (Parkin *et al.* 1997).

Hepatocellular carcinoma is a disease with a strong male predominance, which is observed across all populations. Rates in men are at least two to three times higher than the corresponding rates in women. This sex ratio is especially pronounced in high-risk regions (Yeh *et al.* 1989, Parkin *et al.* 1997). Similarly, as noted in Section 2.4.1, the risk of acquiring chronic HBV infection also is greater in men than in women, with a ratio of approximately 1.6:1 (London 1979).

In the United States, the incidence of hepatocellular carcinoma has increased steadily over the past two decades, such that the overall age-adjusted rate during the period from 1991 to 1995 is approximately 70% higher than that during the period from 1976 to 1980. Rates of increase have been comparable between black and white men; further, these increases are more pronounced than those observed in either black or white women (El-Serag and Mason 1999).

Strictly speaking, chronic HBV infection should be defined by two positive readings of serum HBsAg tests taken six months apart. In epidemiological studies, however, two blood draws instead of one is highly impractical. Given that adult noncarriers of HBV are highly unlikely to show positivity for HBsAg, a single testing of HBsAg is considered a valid measure for chronic carrier state. The assays of choice (i.e., recognized to be both highly sensitive and specific) for measuring serum HBsAg are RIAand EIA. The reverse passive hemagglutination test is considered less sensitive than RIA or EIA. As noted in Section 2, assay sensitivity of the RIAs and EIAs for HBV or antibodies against HBV has increased from first to second to third generation assays.

Other serologic markers of HBV infection that are commonly measured in epidemiological studies are antibodies to the hepatitis B core antigen (anti-HBc, a marker of past or current infection) and antibodies to HBsAg (anti-HBs, a marker of immunity). Although abundant evidence exists that serologic markers other than HBsAg do not predict hepatocellular carcinoma risk in populations with endemic HBV infection and high hepatocellular carcinoma incidence, recent data from populations with low HBV infection rates suggest that anti-HBc positivity in the absence of HBsAg and anti-HBs is another predictor of hepatocellular carcinoma in these lower risk populations. Anti-HBc positivity in the absence of other HBV serologic markers can suggest low-level chronic infection (see Table 2.2).

Another oncogenic viral agent responsible for large numbers of hepatocellular carcinoma cases in humans is the hepatitis C virus (HCV). HCV was identified and characterized in 1989 (Choo *et al.* 1989). An EIA developed to detect antibodies to a specific HCV antigen (C100-3) was subsequently termed the "first-generation" anti-HCV assay. Later, EIAs comprising additional antigens besides C100-3 were developed. These second- or third-generation anti-HCV assays are more specific and sensitive than the first-generation assay. Confirmatory recombinant immunoblot assays (RIBA) using different viral antigens also were developed and have been used frequently to confirm anti-HCV EIA results.

Established nonviral risk factors for hepatocellular carcinoma include dietary aflatoxin intake, excessive alcohol consumption, and exogenous use of estrogen (oral contraceptives) and androgen. Cigarette smoking has been shown in multiple analytic epidemiological studies to be a risk factor for hepatocellular carcinoma, independent of alcohol intake. However, given the strong positive correlation between use of tobacco and alcohol in most populations, especially in the West, where both exposures are relatively prevalent, many researchers consider the relationship between cigarette smoking and hepatocellular carcinoma to be inconclusive (Yu *et al.* 2000).

3.2 IARC evaluation

IARC evaluated the carcinogenic risk to humans of chronic HBV infection (IARC 1994) and concluded that "*there is sufficient evidence in humans for the carcinogenicity of chronic infection with hepatitis B virus*." As a result, HBV was given a Group 1 classification ("*the agent is carcinogenic to humans*").

IARC reviewed 15 cohort studies conducted in diverse populations, ranging from various Asian populations with high to intermediate incidence of hepatocellular carcinoma to low-risk U.S. whites and Europeans. In all studies, chronic HBV carrier state was determined by the presence of HBsAg in serum. All studies showed a greater risk of hepatocellular carcinoma in HBsAg-positive than in HBsAg-negative subjects; estimates of relative risk were between 5 and 148.

Case-control data are more numerous and have a wider range of study populations than the cohort investigations. There were studies in Africa and Asia (high-risk regions), as well as North America and Europe (low-risk regions). Overall results are consistent and show a strong association between HBsAg positivity and hepatocellular carcinoma risk regardless of race-ethnicity and geography. Estimates of relative risk range between 5 and 30.

Some studies evaluated other potential risk factors for hepatocellular carcinoma in conjunction with HBV infection, including HCV infection, dietary aflatoxin, cigarette smoking, alcohol drinking, and hormone use in women. In all instances, the strong association between HBsAg positivity and hepatocellular carcinoma risk remained after adjustment for these viral and/or chemical exposures.

There was no evidence from any of the published studies (cohort or case-control) that chronic HBV infection increases the risk of any other cancer besides hepatocellular.

3.3 Studies published after the IARC review

The following section summarizes human cancer studies published after the 1994 IARC review. The studies are separated by study design, case-control versus prospective (cohort and nested case-control) and, whenever applicable, are grouped according to study geographical location.

3.3.1 Case-control studies

Case-control studies published after the IARC review are described in Table 3-1.

3.3.1.1 Asia

Fukuda *et al.* (1993) studied 368 incident cases of hepatocellular carcinoma, in people aged 40 to 69 years (287 men, 81 women), who were residents of Fukuoka or Saga prefecture in Northern Kyushu, Japan, and diagnosed at the Kurume University Hospital between April 1986 and May 1992. Controls were inpatients of two university-affiliated general hospitals in Kurume, judged to be free of chronic hepatitis or hepatic cirrhosis, and matched to the index cases by age (5-yr age groups), sex, residence (prefecture), and time of hospitalization (within two months of the case interview). The case:control ratio was 1:1 for male cases and 1:4 for female cases. Serum HBsAg status was abstracted from hospital records, with the method of assessment being either reverse passive hemagglutination or EIA. Serum anti-HCV status was tested using a first-generation EIA assay (anti-C100-3). Study subjects were interviewed in person, using a structured questionnaire, for information on medical history, family history of liver disease, use of tobacco and alcohol, and usual dietary habits. In men, the age-matched odds ratio (OR) for hepatocellular carcinoma in HBsAg-positive versus HBsAg-negative subjects was 9.7; the corresponding 95% confidence interval (CI) was 2.9 to 31.7. The comparable OR in women was 7.6 (95% CI = 2.0 to 29.4).

Cordier *et al.* (1993) conducted a hospital-based, case-control study in Hanoi, Vietnam between 1989 and 1992, involving 152 male patients with hepatocellular carcinoma and 241 male hospital controls of similar ages, who were admitted to the same hospitals for reasons other than cancer or liver disease. Second-generation EIAs were used to assess serum HBsAg and anti-HCV status. Study subjects were interviewed in person, using a structured questionnaire, for information on use of tobacco and alcohol, lifetime occupational history, and exposure to pesticides, including Agent Orange. The age-adjusted OR for HBsAg positivity was 61.7 (95% CI = 30 to 128).

Pyong *et al.* (1994) conducted a hospital-based, case-control study involving 90 Korean patients (68 men, 22 women) newly diagnosed with hepatocellular carcinoma at the Kyowa Hospital in Osaka, Japan, between January 1989 and December 1992. Controls were 249 Korean patients admitted to the same hospital, during the same time period as the cases, who were 40 to 89 years of age, and without a history of liver disease or any smoking- or alcohol-related conditions, including ischemic heart disease, lung cancer, peptic ulcer, or pancreatitis. Serum HBsAg status was assessed by a reverse passive hemagglutination method. A first-generation EIA was used to assess anti-HCV status. All subjects were interviewed in person to obtain information on use of tobacco and alcohol and history of blood transfusion. Among anti-HCV negative subjects, the OR for HBsAg positivity was

58.2 (95% CI = 15.3 to 221.0) after adjustment for age, sex, tobacco smoking, alcohol drinking, and history of blood transfusion.

Okuno *et al.* (1994) studied 186 newly diagnosed hepatocellular carcinoma patients (168 men, 18 women) at the Guangxi Medical University Affiliated Hospital in Nanning City, Guangxi Province, Southern China, between January 1991 and September 1992. Controls were 48 apparently healthy workers (30 men, 18 women) given a routine physical examination at the same hospital in August 1992. HBsAg was measured by a reverse passive hemagglutination method, and anti-HCV was tested by a second-generation EIA. A total of 70.4% of cases tested positive for HBsAg versus 10.4% of the controls. Based on tabular data, the crude OR for HBsAg positivity was calculated to be 20.5 (95% CI = 7.4 to 56.3).

Park *et al.* (1995) studied 540 patients with hepatocellular carcinoma (433 men, 107 women) admitted to the Kosin University Hospital in Pusan, Korea between July 1992 and February 1994. Controls were 808 apparently healthy residents of Pusan City (431 men, 377 women), who received their annual, routine physical examinations at the same hospital between September 1992 and October 1993. All control subjects were free of biochemical or clinical features of liver disease at enrollment. Presence of serum HBsAg was tested by RIA. A second-generation EIA was used to determine anti-HCV. HCV RNA was tested by nested reverse transcriptase (RT)-polymerase chain reaction (PCR). The OR for HBsAg positivity with adjustment for anti-HCV was 37.9 (95% CI = 31.4 to 45.7). [Note: It is not clear if the reported OR was also adjusted for age and sex.]

Sun *et al.* (1996) conducted a population-based, case-control study in seven townships in Taiwan involving 58 incident cases (51 men, 7 women) of hepatocellular carcinoma and 225 controls matched to the index cases by age (within five years), gender, and township of residence. Second-generation EIAs were used to assess HBsAg and anti-HCV status. HCV RNA was assessed by RT-PCR. The matched OR for HBsAg positivity was 27.6 (95% CI = 10.7 to 70.9) with adjustment for anti-HCV.

Tsai *et al.* (1996) studied 361 newly diagnosed patients with hepatocellular carcinoma (303 men, 58 women), who were consecutively admitted to the Kaohsiung Medical College Hospital in Taiwan between January 1991 and December 1993. Controls were apparently healthy subjects who entered the same hospital for a physical check-up during the study period; they were individually matched (one control per case) to the index cases by age (within five years) and gender. All subjects were tested for HBsAg, HBeAg, and antibodies to HBeAg (anti-HBe) by RIA, and anti-HCV by second-generation EIA. The matched OR for HBsAg positivity was 68.4 (95% CI = 20.5 to 227.8) after adjustment for anti-HCV and HBeAg.

Shin *et al.* (1996) conducted a hospital-based, case-control study in Pusan, Korea, between August 1990 and August 1993. The 203 cases (159 men, 44 women) were newly diagnosed hepatocellular carcinoma patients admitted consecutively to the Inje University Pusan Paik Hospital. Two groups of control subjects were individually matched to the index cases by age (within four years) and sex; control populations consisted of 203 apparently healthy subjects who entered the same hospital for a routine checkup, and 203 hospital inpatients

free of cancer or liver disease. Subjects were tested for HBsAg, anti-HBc, and anti-HBs by RIA, and anti-HCV by a second-generation EIA. In-person interviews using a structured questionnaire were administered to all subjects to obtain information on medical history, family history of cancer and liver disease, smoking and drinking habits, and occupational history. The two control groups yielded similar results and were combined in the reporting of data in the study report. The matched OR for HBsAg positivity was 87.4 (95% CI = 22.2 to 344.3) after adjustment for anti-HCV, *Clonorchis sinensis* in stool, history of blood transfusion, history of acute hepatitis, history of liver fluke, alcohol drinking, and tobacco smoking.

Tanaka *et al.* (1996) studied 91 newly diagnosed patients with hepatocellular carcinoma (73 men, 18 women), 40 to 69 years of age, residents of Fukuoka or Saga Prefecture, Northern Kyushus, Japan, who were admitted to the Kyushu University Hospital between December 1985 and June 1989. Controls were 410 residents of Fukuoka City (291 men, 119 women), 40 to 69 years of age, who had a physical examination at a public health center near the Kyushu University Hospital between January 1986 and July 1989. Controls were frequency matched to the cases by age and had no known history of liver disease. Serum HBsAg status was determined by a reverse passive hemagglutination method. A second-generation RIA with confirmation by a second-generation recombinant immunoblot assay (RIBA) was used to ascertain anti-HCV status. HCV RNA was assessed by nested RT-PCR. The sex- and age-adjusted OR for HBsAg positivity was 16.0 (95% CI = 6.4 to 39.7). Among anti-HCV negative subjects, the comparable OR was 293.7 (95% CI = 68.7 to 1,255.6).

Yu *et al.* (1997a) conducted a case-control study in four areas of southeastern China with relatively high incidence of hepatocellular carcinoma. A total of 359 cases and an equal number of individually matched controls (matched by age within five years, sex, and location) were enrolled (number of cases in each location ranged from 71 to 100). No information was reported detailing how the cases and controls were identified and selected. Serum HBsAg and anti-HCV status were tested using Chinese-manufactured EIA kits; it was unclear whether the anti-HCV assay was first or second generation. The matched OR for HBsAg positivity was 6.6 (95% CI = 4.7 to 9.0). Among anti-HCV negative subjects, the comparable OR was 6.1 (95% CI = 4.2 to 8.8).

Okada *et al.* (1998) conducted a hospital-based, case-control study of hepatocellular carcinoma among patients with chronic liver disease at the National Cancer Center Hospital in Tokyo, Japan, between January 1992 and December 1993. Cases were 141 consecutive patients (110 men, 31 women) with hepatocellular carcinoma and underlying chronic liver disease, between 25 and 81 years of age. Controls were 151 consecutive patients (96 men, 55 women) with chronic liver disease but no evidence of hepatocellular carcinoma (aged 25 to 79 years), admitted to the same hospital during the study period. All subjects were tested for HBsAg by reverse passive hemagglutination, anti-HBs by passive hemagglutination, anti-HBc by EIA, and anti-HCV by a second-generation EIA. The OR for HBsAg positivity after adjustment for age, sex, anti-HCV, and anti-HBc was 4.7 (95% CI = 1.7 to 12.7). Anti-HBc positivity without anti-HBs or HBsAg also was predictive of hepatocellular carcinoma risk (OR = 2.6, 95% CI = 1.5 to 4.7).

Zhang *et al.* (1998a, 1998b) studied 152 patients (136 men, 16 women) with hepatocellular carcinoma admitted to four hospitals in Henan Province, China between January 1994 and October 1995. Controls were 115 patients (99 men, 16 women) admitted to the same hospitals during the study period, with ages similar to those the cases, and judged to be free of liver disease. Study subjects were tested for HBsAg and anti-HBs by RIA, anti-HBc and anti-HCV by EIA (unknown generation), HBV DNA by PCR, and HCV RNA by RT-PCR. Personal interviews using a structured questionnaire were conducted to solicit information on medical history, history of cigarette smoking and alcohol drinking, usual diet, history of blood transfusion, and family history of cancer and liver disease. HBsAg positivity rates in cases and controls were 63.2% and 5.2%, respectively (test of difference in rates, $P < 0.001$). Among anti-HCV negative subjects, the age- and sex-adjusted OR for HBsAg positivity was 28.8 (95% CI = 11.2 to 78.8). After adjustment for personal and family history of liver disease, alcohol drinking, cigarette smoking, dietary aflatoxin intake, and history of blood transfusion, the comparable OR increased to 44.6 (95% CI = 12.5 to 158.5).

3.3.1.2 Africa

Bile *et al.* (1993) conducted a hospital-based, case-control study in Mogadishu, Somalia in 1989, involving 62 chronic liver disease patients (49 hepatocellular carcinoma, 13 other liver disease) admitted to two main referral centers. For each case, a hospital control patient, matched for age (within five years) and gender was selected [no further details on control selection were given]. Serum HBsAg status was measured by RIA, while anti-HCV was tested using a second-generation EIA. All reported data referred to the 62 chronic liver disease patients and their matched controls; therefore, risk for hepatocellular carcinoma alone cannot be estimated from the published paper. Among anti-HCV negative subjects, the matched OR for HBsAg positivity was 3.3 (95% CI = 1.1 to 9.9).

Cenac *et al.* (1995) studied 26 (Sahelian African patients 19 men, 7 women) with hepatocellular carcinoma, who were admitted to the Hospital National in Niamey, Niger between June 1983 and June 1985. Forty-seven Sahelian African patients (24 men, 23 women) who had no history of liver disease and were admitted to the Department of Internal Medicine in the same hospital during the study period served as the comparison group. Serum HBsAg was measured by RIA. Anti-HCV status was assessed using a second-generation EIA. HBsAg positivity rates in cases and controls were 73.1% and 29.8%, respectively (test of difference in rates, $P < 0.0001$). Based on tabular presentation, the crude OR for HBsAg was calculated to be 6.4 (95% CI = 2.1 to 18.9).

Kew *et al.* (1997) studied 231 South African Black patients with hepatocellular carcinoma and hospital controls individually matched (by age within two years, sex, race, rural/urban/rural-urban background, hospital, medical versus surgical ward) admitted to four Johannesburg hospitals. Serum HBsAg was measured by RIA. A second- or third-generation EIA was used to test for anti-HCV. HCV RNA was measured by nested RT-PCR. The matched OR for HBsAg positivity was 21.8 (95% CI = 8.9 to 53.4). Among anti-HCV negative subjects, the comparable OR was 23.3 (95% CI = 9.2 to 59.4).

3.3.1.3 Americas

Yu *et al.* (1997b) conducted a population-based, case-control study among non-Asians of Los Angeles County, California, between 18 and 74 years of age. A total of 111 incident cases of hepatocellular carcinoma and 128 community controls were assessed for HBsAg, anti-HBc, and anti-HBs by RIA, and anti-HCV by a second-generation EIA with confirmation by a second-generation RIBA. Ten cases, but no controls, were positive for HBsAg (lower 95% CI = 5.6). Anti-HBc positivity, with and without concurrent anti-HBs positivity, was significantly related to risk of hepatocellular carcinoma, with higher risk associated with anti-HBc without anti-HBs. The overall OR for anti-HBc positivity was 7.4 (95% CI = 3.6 to 15.4).

Yuan *et al.* (1999) reported on the latest results of the ongoing population-based Los Angeles Study, which was included in the meta-analysis of Donato *et al.* (1998; see Section 3.4.1). The latest findings were based on 144 non-Asian patients with hepatocellular carcinoma and 252 community controls of similar age, gender, and race. HBsAg and anti-HBc were independent serum predictors of hepatocellular carcinoma risk in this low-risk study population. The risk associated with HBsAg positivity was considerably stronger than that associated with anti-HBc positivity. Eleven cases and no controls were positive for HBsAg (lower 95% CI = 7.7). The age- and sex-adjusted OR for HBsAg(-)/anti-HBc(+) was 4.2 (95% CI = 2.3 to 7.8).

A hospital-based case-control study consisting of 115 cases with hepatocellular carcinoma and 230 non-liver cancer controls matched for sex, age, and year of diagnosis was studied to evaluate risk factors for hepatocellular cancer in the United States as well as possible synergisms between the risk factors (Hassan *et al.* 2002). All subjects were tested for HBsAg, anti-HBc, and anti-HCV using second-generation EIAs. Exposure to other risk factors, including alcohol and diabetes mellitus, was obtained by interviews and medical records. Multivariate analysis adjusting for anti-HCV, alcohol consumption, cigarette smoking, and diabetes mellitus was performed and an OR of 12.6 (95% CI = 2.5 to 63.1) was calculated for HBsAg seropositivity. A higher risk for hepatocellular carcinoma was observed in individuals positive for both HBsAg and anti-HBc (OR = 23.8; 95% CI = 3.9 to 141.6; reference group, HBsAg negative and anti-HBc individuals. The population attributable risk percentage for HBsAg was 16%. The combined effect for chronic hepatitis infection and heavy alcohol consumption (see Section 3.6.2) also was evaluated.

3.3.1.4 Europe

Arico *et al.* (1994) conducted a hospital-based, case-control study of hepatocellular carcinoma among patients with liver cirrhosis. Cases were 62 consecutive patients (50 men, 12 women) admitted to a district hospital in Turin, Italy between 1986 and 1992, in whom a diagnosis of hepatocellular carcinoma in the presence of liver cirrhosis was made for the first time. Two hospital control groups were selected. The first group consisted of 310 individually matched (by age within five years, sex, and hospital) inpatients free of a history of liver disease; 97 asymptomatic inpatients with a first diagnosis of liver cirrhosis constituted the second group. All subjects were tested for HBsAg and anti-HBs by EIA. HBsAg was slightly higher in cirrhotic controls (8 patients, 8.2% tested positive) than in non-cirrhotic controls (7 patients, 2.3% tested positive). Personal interviews were

conducted by trained personnel to obtain information on alcohol consumption. After adjustment for lifetime alcohol intake, the matched OR for HBsAg positivity relative to noncirrhotic controls was 10.7 (95% CI = 4.9 to 20.5). The comparable OR using cirrhotic controls was 6.8 (95% CI = 1.4 to 32.3).

Peters *et al.* (1994) studied 86 patients with hepatocellular carcinoma and underlying liver cirrhosis, who were seen at the University Hospital of Mainz in Germany between 1986 and 1993. (The cases consisted of 74 men, 14 women, as reported in the paper, although one table reports the number as 71 men.) Controls were patients individually matched (by age, within five years, and sex to the index case) with liver cirrhosis but no evidence of hepatocellular carcinoma, seen at the same hospital during the study period. All subjects were tested for HBsAg, anti-HBs, anti-HBc, HBeAg, anti-HBe, anti-delta antibody by RIA. Subjects admitted to the hospital prior to January 1991 were tested for anti-HCV using a first-generation EIA. All subsequent study subjects were tested for anti-HCV by a second-generation EIA. Information on cigarette smoking and alcohol drinking was abstracted from patient charts. The matched OR for HBsAg positivity was 1.1 (95% CI = 0.5 to 2.5). The comparable OR for anti-HBc positivity was 1.5 (95% CI = 0.8 to 2.9).

Goritsas *et al.* (1995) studied 51 consecutively admitted patients (48 men, 3 women) with hepatocellular carcinoma at the Patras University Hospital in Patras, Greece between October 1989 and October 1992. Controls were patients individually matched (by sex and age within five years) and seen at the same hospital during the study period; they did not have a history of liver disease or cancer. Study subjects were tested for HBsAg, anti-HBs, and anti-HBc by EIA; and for anti-HCV by a second-generation EIA with confirmation by a second-generation RIBA. Information on alcohol and tobacco intake was obtained through personal interviews. The matched OR for HBsAg positivity after adjustment for anti-HCV, heavy alcohol intake, and tobacco smoking was 2.2 (95% CI = 1.5 to 3.2).

Hadziyannis *et al.* (1995) conducted a hospital-based, case-control study in Athens, Greece, involving 65 incident cases of hepatocellular carcinoma and two groups of hospital controls individually matched to the index cases by age (within five years) and gender (65 metastatic liver cancer patients and 65 patients hospitalized for eye, ear, nose, or throat conditions). Study subjects were tested for HBsAg, anti-HBc, and anti-HBs by EIA, and for anti-HCV by a second-generation EIA with confirmation by a second-generation RIBA. All subjects were interviewed in person using a structured questionnaire to obtain information on medical history, diet, and other lifestyle factors. Combining the two groups of control subjects, the matched OR for HBsAg positivity after adjustment for anti-HCV was 18.8 (95% CI = 8.2 to 43.2). Further adjustment for potential confounders, including history of blood transfusion, cigarette smoking, alcohol drinking, coffee drinking, and history of diabetes did not materially change the HBsAg-hepatocellular carcinoma association.

A hospital-based, case-control study was conducted in two hospitals in Göteborg, Sweden during 1984 to 1991 (Kaczynski *et al.* 1996). Seventy-three patients (55 men, 18 women) with hepatocellular carcinoma were compared to 32 patients with other cancers (n = 21) or benign liver disease (n = 11), who served as control subjects. Serum HBsAg testing was

performed by RIA. A third-generation EIA with confirmation by a third-generation RIBA was used to determine anti-HCV status. None of the cases were positive for HBsAg.

Donato *et al.* (1997) studied 172 newly diagnosed hepatocellular carcinoma patients in the two major hospitals in the province of Brescia in northern Italy. Other hospital patients without a history of liver disease or cancer served as the control group. They were frequency matched to the cases by age (5-year age groups), sex, and date/hospital of admission. Subjects were tested for HBsAg by EIA, anti-HCV by a third-generation EIA with confirmation by RIBA, and HCV RNA by nested RT-PCR. Information on alcohol intake was obtained through personal interviews. The OR for HBsAg positivity, after adjustment for age, sex, residence, anti-HCV, HCV RNA, and alcohol intake was 11.4 (95% CI = 5.7 to 22.8). Among heavy drinkers (> 80 g/day ethanol), the comparable OR was 64.7 (95% CI = 20 to 210).

Tagger *et al.* (1999) reported on the latest results of the ongoing hospital-based Brescia Study, which was included in the meta-analysis of Donato *et al.* (see Section 3.4.1). The latest findings were based on 305 cases of hepatocellular carcinoma and 610 hospital controls. HBsAg and anti-HBc alone were independent serum predictors of hepatocellular carcinoma risk in this intermediate-risk study population. The risk associated with HBsAg was considerably stronger than that associated with anti-HBc alone. Relative to subjects with no HBV serological markers, the OR for anti-HBc alone after adjustment for age, sex, alcohol intake, and HCV infection was 1.9 (95% CI = 1.1 to 3.2). The comparable OR for HBsAg positivity was 18.8 (95% CI = 10.0 to 35.2).

Chiesa *et al.* (2000) studied 142 patients (116 men, 26 women) with hepatocellular carcinoma in the presence of liver cirrhosis and 21 patients (19 men, 2 women) with hepatocellular carcinoma but without cirrhosis, who were admitted to two main hospitals in Brescia, Italy between 1995 and 1997. Controls were 610 patients admitted to the same hospitals during the study period who were free of liver disease. Serum HBsAg and HBV DNA status were measured by EIA and PCR, respectively. A third-generation EIA and RT-PCR were used to test for anti-HCV and HCV RNA, respectively. The age- and sex-adjusted OR for HBsAg and/or HBV DNA positivity in the presence of cirrhosis was 17.6 (95% CI = 9.0 to 34.4). The comparable OR in the absence of cirrhosis was 20.3 (95% CI = 5.7 to 72.6).

Kuper *et al.* (2000) studied 333 incident cases of hepatocellular carcinoma treated at three teaching hospitals in Athens, Greece during January 1995 to December 1998. Two groups of hospital controls admitted to the same hospitals during the study period, and similar in age and sex distributions to the cases were accrued. The first group consisted of 272 patients with metastatic liver cancer, while the second group consisted of 360 patients hospitalized for eye, ear, nose, or throat conditions. All subjects were tested for HBsAg and anti-HCV by third-generation EIAs. Combining the two control groups, the OR for HBsAg positivity after adjustment for age, sex, schooling, and anti-HCV was 48.8 (95% CI = 30.5 to 78.3). Among anti-HCV negative subjects, the comparable OR was 53.4 (95% CI = 33.0 to 86.2).

3.3.2 Prospective studies (nested case-control and cohort)

Prospective studies (nested case-control and cohort studies) on HBV published after the IARC review are summarized below and described in Table 3-2. Kato *et al.* (1994) assembled a cohort of 401 patients (273 men, 128 women) with cirrhosis in Nagasaki, Japan, from April 1977 to March 1992. The patients showed no evidence of and did not exhibit a history of hepatocellular carcinoma at enrollment. Baseline blood samples collected from study subjects were tested for HBsAg by RIA and anti-HCV by second-generation EIA or RIA. By March 1993 (mean follow-up time period, 4.4 yrs), 127 incident cases of hepatocellular carcinoma had developed within the cohort. The 5-year cumulative risk of hepatocellular carcinoma among subjects negative for both HBsAg and anti-HCV was 12.4%. The comparable figure in subjects positive for HBsAg alone was 21.2%, and the difference between the two rates was statistically significant ($P < 0.05$).

A population-based cohort of 9,775 men aged 30 to 85 years from six townships in Taiwan was accrued between September 1984 and February 1986. At baseline, a structured personal interview and a blood specimen were obtained from each study subject. The interview solicited information on history of tobacco and alcohol use, usual dietary habits, and family and personal history of liver disease. The cohort was actively followed on an annual basis until March 1992. Chang *et al.* (1994) conducted a nested case-control analysis on 38 newly diagnosed cases of primary liver cancer and 152 controls within the cohort. Control subjects were matched to the index cases by age (within one year), township of residence, and date of recruitment. A reverse passive hemagglutination method was used to determine serum HBsAg status at baseline. Negative samples were rechecked using RIA. Anti-HCV status was determined by a second-generation EIA. The matched OR for HBsAg positivity was 81.8 (95% CI = 9.1 to 740.7) after adjustment for anti-HCV, vegetable intake, and personal history of chronic liver disease.

A population-based cohort of 18,244 Chinese men, 45 to 64 years of age, in Shanghai was recruited between January 1986 and September 1989. A personal interview was conducted and blood and urine specimens were collected from each participant at baseline.Yuan *et al.* (1995) studied 76 incident cases of hepatocellular carcinoma within the cohort and 410 control cohort subjects individually matched to the cases by age (within one year), time of blood sample collection (within one month), and neighborhood of residence. Serum HBsAg was measured by RIA, while anti-HCV was assessed by a second-generation EIA. The matched OR for HBsAg positivity was 15.0 (95% CI = 4.4 to 51.6).

Nomura *et al.* (1996) conducted a nested case-control analysis on 24 incident cases of hepatocellular carcinoma and 72 age-matched cohort controls from a cohort of 5,924 Japanese-American men in Hawaii accrued between 1967 and 1970 and followed for cancer occurrence until 1992 (The Japan-Hawaii Cancer Study). Serum HBsAg status was determined by RIA. All subjects were first tested by a first-generation EIA for anti-HCV; positive samples were retested by RIBA. The matched OR for HBsAg positivity was 43.0 (95% CI = 5.7 to 325.5).

Tsai *et al.* (1997) followed a cohort of 400 patients (290 men, 110 women) with nonalcoholic cirrhosis at the Kaohsiung Medical College Hospital in Taiwan from January 1989 to December 1994. Eighty new cases of hepatocellular carcinoma occurred in this

cohort after 1,185 person-years of follow-up. Baseline samples collected from study subjects were tested for HBsAg by RIA and for anti-HCV by second-generation EIA. The incidence of hepatocellular carcinoma among subjects negative for HBsAg and anti-HCV at baseline was 2.0%. The comparable figure for subjects who were HBsAg(+) and anti-HCV(–) at baseline was 6.6%. The relative risk of hepatocellular carcinoma for HBsAg positivity alone was 4.1 (95% CI = 1.2 to 13.3) after adjustment for age, sex, and other potential confounders.

Ikeda *et al.* (1998) followed a cohort of 2,215 patients (1,544 men, 671 women) with chronic viral hepatitis diagnosed at the Toranomon Hospital in Tokyo, Japan, between January 1980 and August 1995. Eighty-nine cases of hepatocellular carcinoma developed within the cohort after a median follow-up period of 4.1 years (range, 0.1 to 16.3 years). Serum HBsAg and anti-HCV status at baseline were established using RIA and second-generation EIA, respectively. HBsAg positivity was a significant risk factor for hepatocellular carcinoma development among cohort members ($P = 0.02$).

Mori *et al.* (2000) recruited a population-based cohort of 3,059 residents (981 men, 2,078 women) of Saga Prefecture, Japan, aged 30 years or older, during June 1992. At baseline, a blood specimen and a written questionnaire requesting medical and family histories and use of tobacco and alcohol were collected from each participant. Baseline serum samples were tested for HBsAg by reverse passive hemagglutination and anti-HCV by second-generation EIA. By March 1997 (median follow-up time period, 4.8 years), 22 incident cases of hepatocellular carcinoma (14 in men, 8 in women) had developed among cohort members. The age- and sex-adjusted relative risk of hepatocellular carcinoma for HBsAg positivity at baseline was 7.3 (95% CI = 1.6 to 32.6).

Yang *et al.* (2002) followed 11,893 men for the development of hepatocellular carcinoma from 1991 to 2000 in Taiwan. At baseline, serum samples were tested for HBsAg and HBeAg by RIA, anti-HCV by second-generation EIA, anti-HBeAg by ELISA, and HBV DNA by a branched chain DNA assay. The men were questioned about sociodemographic characteristics, diet, cigarette smoking, consumption of alcohol, betel-nut chewing, their medical and surgical history, and any family history of hepatocellular carcinoma or liver cirrhosis. A total of 111 men developed cancer, as ascertained from the National Cancer Registry. The incidence rate of hepatocellular carcinoma was 1,169 cases per 100,000 person-years among men who were positive for both HBsAg and HBeAg, 324 per 100,000 person-years for those who were positive for HBsAg only, and 39 per 100,000 person-years for those who were negative for both. After adjustment for age, sex, the presence or absence of antibodies against hepatitis C virus, cigarette smoking, and use or nonuse of alcohol, the relative risk was 9.6 (95% CI = 6.0 to 15.2) for men who were positive for HBsAg alone and was 6-fold higher (60.2; 95% CI = 35.5 to 102.1) for men who were positive for both HBsAg and HBeAg, as compared to men who were negative for both. Nested case-control analyses were done on 130 individuals positive for HBsAg and negative for HBeAg (44 cases and 86 matched controls). Of those, 92 percent were positive for anti-HBe, but the proportion did not differ between cases and controls. In contrast, cases with detectable serum HBV DNA in the case-control study had an increased risk for developing hepatocellular carcinoma than individuals without detectable serum HBV DNA

(OR = 3.9, 95% CI = 1.6 to 9.2); however, these results are based on small numbers (17 cases and 12 controls). The ORs increased with increasing levels of HBV DNA.

Two cohorts of adults (male and female) between 25 and 64 years of age from Haimen City, China were followed for eight years for the development and mortality of hepatocellular carcinoma (Evans 2002). The male cohort consisted of 58,454, of whom 15% were HBV carriers; and the female cohort consisted of 25,340 women, of whom 10.7% were HBV carriers. Serum HBsAg status was determined by RIA from blood taken at enrollment; 15% of the men and 10.7% of the women were HBV carriers. Individuals provided samples of blood and answered a questionnaire regarding risk factors and demographic information at enrollment. Hepatocellular cancer and incidence and/or mortality rates were obtained from hospital records, township doctors, and death certificates. A total of 900 men and 77 women died from hepatocellular carcinoma during follow-up. The cumulative risk of death from hepatocellular carcinoma during the 8-year follow-up was 8% for HBsAg-positive males and 2% for HBsAg-positive females. Multivariate analyses, adjusting for age and all candidate risk factors, showed a relative risk of 18.8 (95% CI = 16.0 to 22.1) for men and 33.2 (95% CI = 17.0 to 65.0) for women. No significant interaction (multiplicative) was found between HBsAg status and acute hepatitis history, family history, occupation, smoking, or alcohol use.

3.4 Effects of HBV

3.4.1 Meta-analysis of Donato et al. 1998

Donato *et al.* (1998) undertook a meta-analysis of 32 epidemiological studies investigating the relationship between HBV/HCV coinfection and hepatocellular carcinoma risk. The studies in the meta-analysis were selected by doing a Medline search for studies published between 1993 and 1997 and using the IARC monograph literature search for studies published prior to 1993.

Case-control studies (including nested case-control studies) were included in the analysis if the cases included hepatocellular carcinoma patients, and controls were subjects without chronic liver diseases. Only studies that used the following serological markers of chronic infection were included in the analysis: HBsAg for HBV infection and anti-HCV (tested with ELISA) or HCV RNA (anti-HCV/HCV RNA; detected by RT-PCR) for HCV infection. When data were available from anti-HCV and HCV RNA tests, the anti-HCV data only were used to classify subjects as positive or negative for HCV infection. Studies with fewer than 10 hepatocellular carcinoma cases were excluded, because they could not detect any case positive for both HBsAg and anti-HCV/HCV RNA. Only studies that reported, or allowed computation, of estimates of the ORs or RRs for each HBsAg and anti-HCV/HCV RNA combination were included.

Of the 32 studies reviewed by Donato et al. 11 were part of the IARC evaluation (IARC 1994) and 21 were published afterwards (see Table 3-4 for a list of all 32 studies). One study was reported only as an abstract (Yang et al. 1996) with no subsequent full-length publication. The remaining 20 studies are described in the section above (Section 3.3) and include 17 case-control studies:

- Fukuda *et al*. 1993,
- Cordier *et al*. 1993,
- Pyong *et al*. 1994,
- Okuno *et al*. 1994,
- Park *et al*. 1995,
- Sun *et al*. 1996,
- Tsai *et al*. 1996,
- Shin *et al*. 1996,
- Tanaka *et al*. 1996,
- Yu *et al*. 1997a,
- Bile *et al*. 1993,
- Cenac *et al*. 1995,
- Kew *et al*. 1995,
- Yu *et al*. 1997b,
- Hadziyannis *et al*. 1995,
- Kaczynski *et al*. 1996,
- Donato *et al*. 1997;

and three nested case-control studies:

- Chang *et al*. 1994,
- Yuan *et al*. 1995,
- Nomura *et al*. 1996.

Findings from these studies on the combined effect of HBV and HCV coinfection on hepatocellular carcinoma risk are reported in Section 3.5.

Table 3-3 shows the results of the meta-analysis. HBsAg positivity was strongly associated with hepatocellular carcinoma (OR = 13.7, 95% CI = 12.2 to 15.4). The summary OR from studies employing hospital controls was similar to that derived from studies employing community controls (15.2 versus 15.7). A stronger association between HBV infection and hepatocellular carcinoma risk was noted in areas where both infection and cancer incidence are high (East and Southeast Asia, and sub-Saharan Africa) than in areas such as Japan and Southern Europe where both HBV infection and hepatocellular carcinoma incidence are at intermediate levels (summary OR of 16.7 in the former versus 8.5 in the latter).

3.4.2 Recent studies not included in the Donato meta-analysis.

Section 3.3 also reviews 10 case control studies (Okada *et al*. 1998, Zhang *et al*. 1998a, 1998b, Yuan *et al.* 1999, Hassan *et al.* 2002, Arico *et al*. 1994, Peters *et al*. 1994, Goritsas *et al*. 1995, Tagger *et al.* 1999, Chiesa *et al.* 2000, Kuper *et al*. 2000) and six cohort studies (Kato *et al*. 1994, Tsai *et al*. 1997, Ikeda *et al*. 1998, Mori *et al*. 2000, Yang *et al*. 2002, and Evans *et al*. 2002) that were not included in the 1998 meta-analysis by Donato *et al.* Some of those studies used patients without liver disease as controls similar to the controls in the studies included in the meta-anlaysis; others used patients with liver disease as controls. All of the studies that used patients without liver disease found positive associations between hepatocellular carcinoma risk and HBsAg seropositivity, with risk estimates ranging from ~ 2 to ~50. Most studies adjusted for anti-HCV and/or reported risk in anti-HCV-negative individuals.

Five studies evaluated the role of chronic hepatitis and the development of hepatocellular carcinoma with respect to cirrhosis or liver disease. Three case-control studies (Arico *et al*. 1994, Peters *et al.* 1994, and Okada *et al*. 1998) used cirrhotic controls, one case-control study evaluated hepatocellular carcinoma both with and without cirrhosis, and a cohort study followed a cohort of cirrhosis patients for the development of hepatocellular carcinoma. All but one study (Peter *et al.* 1994) reported that HBsAg was a risk for hepatocellular development in the presence of cirrhosis.

3.5 Combined effects of HBV and HCV

This section describes the results of studies investigating the combined effect of HBV and HCV including a meta-analysis performed by Donato *et al*. in 1998 (See section 3.4.1) and four studies evaluating both HBV and HCV that are not part of that analyses. All individual studies have been described in Sections 3.1 and 3.2.

3.5.1 Meta-analysis of Donato et al. 1998.

Table 3-4 shows the interactive effect of HBV and HCV infections on hepatocellular carcinoma risk observed in the individual studies included in the Donato *et al*. (1998) meta-analysis, and Table 3-5 shows the overall meta-analysis results derived from a total of 4,596 cases of hepatocellular carcinoma and 7,002 control subjects. The summary OR of hepatocellular carcinoma risk in individuals positive for HBsAg alone was similar to the OR for those positive for anti-HCV/HCV RNA alone; both were approximately 20. A synergistic (greater than the sum of each infection alone) effect of HBV/HCV coinfection was noted; the summary OR for the combined presence of HBsAg and anti-HCV/HCV RNA was 135. Studies employing community controls tend to yield larger estimates of the interactive effect than studies with hospital controls, which showed only additive effects. Larger estimates were also observed in studies from high-risk areas than from intermediate- or low-risk areas. One should be cautious in interpreting the magnitude of OR differences in HBV/HCV coinfection across subgroups of studies (Table 3-5, last column). The number of coinfected subjects in each study was very small (see Table 3-4); thus the subgroup risk estimates were highly unstable.

3.5.2 Additional studies

Table 3-6 shows the results of four studies on the interactive effects of HBV and HCV published after the IARC review that were not part of the Donato *et al.* (1998) meta-analysis. The studies by Yuan *et al*. (1999) and Tagger *et al*. (1999) exhibited a synergistic effect of HBV/HCV coinfection on risk that was consistent with the meta-analysis results of Donato *et al.* (1998). All four studies suffer from relatively small numbers of cases and controls in the HBV/HCV coinfection category.

3.5.3 Geographical considerations

HBV infection is prevalent in sub-Saharan Africa and East and Southeast Asia, where the carrier rate can be as high as 20% to 25% in adult men. In these regions of endemic disease, primary HBV infection usually occurs in early childhood, and the virus is believed to contribute to the development of 80% or more of hepatocellular carcinoma cases in the population (See Section 3.2, Yu *et al.* 2000, Alter, 2003). Donato's meta-analysis reported a higher risk for HBsAg positivity in high-risk areas than in low-risk areas (See Sections

3.4.1 and 3.5.1 and Tables 3-3 and 3-5). On the other hand, the role of HCV infection is relatively minor in the occurrence of hepatocellular carcinoma in these high-risk areas. In Asia and Africa, where the role of HCV in development of hepatocellular carcinoma is relatively minor, the summary OR from Donato's meta-analysis associated with HCV infection alone (11.5) is less than half the summary OR (31.2) derived from studies in Japan and the Mediterranean countries, where growing evidence exists that HCV infection is largely responsible for the increasing incidence of hepatocellular carcinoma in these low- to intermediate-risk regions (El-Serag and Mason 2000, Yu *et al.* 2000).

In the United States, the HBV carrier rate in the general population is below 1%, although rates in at-risk subgroups, such as intravenous drug users, homosexual men, patients on hemodialysis, hemophiliacs, etc., can be quite high (See Section 2.3.1). It is estimated that approximately one in four cases of hepatocellular carcinoma in the non-Asian segment of the U.S. population is HBV related. Recent evidence points to a decreasing role of HBV infection in the development of hepatocellular carcinoma in the United States, while HCV infection is a likely culprit behind the increasing incidence of hepatocellular carcinoma nationwide (Yu *et al*, 2000).

3.6 Modifying effects of nonviral cofactors on HBV-hepatocellular carcinoma

3.6.1 Aflatoxins

Aflatoxins are among the most potent hepatocarcinogens in animals (Wogan 1992). Humans are exposed to these mycotoxins through ingestion of moldy foods, a consequence of poor storage of susceptible grains. Highly exposed populations are primarily those residing in sub-Saharan Africa and East and Southeast Asia. Ross *et al.* (1992) and Qian *et al.* (1994) provided the first set of analytical epidemiological data to definitively link dietary aflatoxin exposure to hepatocellular carcinoma development in humans. Their data also suggest a synergistic (greater than the multiplicative product of each exposure alone) effect of HBV infection and dietary aflatoxin intake on hepatocellular carcinoma development. Other human studies in China and Taiwan have supported their finding of a potential viral-chemical interaction. Synergy between aflatoxin and hepadnaviruses also has been observed in animals (see sections 4.1.2.2, 4.2.2.4, and 6.5.3). Although the mechanism of this interaction is unknown, levels of aflatoxin albumin adducts have been reported to be higher in HBsAg-positive adolescents than HBsAg-negative adolescents (Chen *et al*. 2001; see Section 6.5.3 for a discussion of possible mechanisms).

The following paragraphs briefly describe several studies that investigated the interactive effect of these two hepatocarcinogenic agents in populations in which both exposures are prevalent. Results of these studies are summarized in Table 3-7.

The Shanghai Cohort Study (Yuan *et al.* 1995) was described in Section 3.3.2. Between January 1986 and September 1989, 18,244 male residents of Shanghai, China, aged 45 to 64 years, were enrolled in a prospective study. At baseline, personal interview and a blood specimen were collected from each participant. By February 1992, 55 incident cases of hepatocellular carcinoma had occurred among cohort members. A nested case-control analysis was conducted, in which multiple controls were matched to each case by age (within one year), time of urine sample collection (within one month), and neighborhood of

residence (Qian *et al.* 1994). A total of 267 controls were selected. For these cases and controls, baseline urine specimens were tested for six aflatoxin metabolites, including the major aflatoxin DNA adduct, aflatoxin-N7-guanine. The matched OR for the presence of urinary aflatoxin-N7-guanine was 9.1 (95% CI = 2.9 to 29.2) after adjustment for HBsAg positivity and cigarette smoking. The matched OR for the combined presence of serum HBsAg and urinary aflatoxin metabolites relative to the absence of both was 59.4 (95% CI = 16.6 to 212.0).

McGlynn *et al.* (1995) studied 52 patients (43 men, 9 women) with hepatocellular carcinoma treated at the Zhong Shan Hospital in Shanghai, China, between 1991 and 1992. Controls were 116 healthy residents (81 men, 35 women) of Haimen City, which is within the catchment area of Zhong Shan Hospital. Cases and controls were tested for HBsAg using a third-generation EIA and genotyped for two polymorphic genes, glutathione S-transferase M1 (GSTM1) and epoxide hydrolase (EPHX), by PCR. Both GSTM1 and EPHX are involved in aflatoxin detoxification in hepatocytes. The GSTM1-null genotype was associated with an increased risk for hepatocellular carcinoma (OR = 1.9, 95% CI = 0.94 to 3.63). Likewise, the presence of one or two copies of the low-activity allele “2” of the EPHX gene was associated with elevated hepatocellular carcinoma risk (OR = 3.3, 95% CI = 0.39 to 28.6). A reduced capacity to detoxify aflatoxin would lead to increased hepatic exposure to mutagenic aflatoxin metabolites; thus, the EPHX-2 genotype can serve as a surrogate marker for increased aflatoxin exposure to the target cells, especially in a population, such as the residents of Shanghai, who are universally exposed to dietary aflatoxin through widespread contamination of staple grains. Relative to subjects possessing the EPHX-1/1 genotype and negative for HBsAg, the OR for the combined presence of HBsAg and EPHX-2 genotype was 77.3 (95% CI = 8.9 to 665.8).

From July 1990 to June 1992, a community-based cancer screening program was conducted in seven townships of Taiwan. All residents born between 1927 and 1961 were invited to participate, and 25,618 subjects (12,024 men, 13,594 women) were enrolled. At baseline, a personal interview, a fasting blood specimen, and a spot urine specimen were collected from each participant. By June 1995, 56 incident cases of hepatocellular carcinoma had occurred among enrollees. A nested case-control analysis was conducted, in which four controls were matched to each case by age (within five years), sex, date of recruitment (within three months), and township of residence. Serum samples from 56 cases and 220 controls were tested for HBsAg by EIA, and anti-HCV by a second generation EIA. Their urine samples were tested for aflatoxin metabolites by competitive ELISA, using monoclonal antibody AF8E11, which can detect AFB_1 and selected derivatives (AFB_2, AFM_1, AFG_1 and AFP_1) but not AFB_1-guanine. The matched OR for the combined effects of high (above median level) versus low (below median level) urinary aflatoxin metabolites was 3.8 (95% CI = 1.1 to 12.8) after adjustment for HBsAg positivity. The matched OR for HBsAg positivity and high (above median level) urinary aflatoxin metabolites versus HBsAg negativity and low (below median level) urinary aflatoxin metabolites was 111.9 (95% CI = 13.8 to 905.0) (Wang *et al.* 1996).

Lunn *et al.* (1997) investigated the role of chronic HBV infection and aflatoxin in hepatocellular carcinoma development in a case-control study of 105 cases and 37 controls from Taiwan. Serum HBsAg was detected by RIA and aflatoxin was assessed by

measuring aflatoxin DNA-adducts in liver tumor and nontumor tissue of the cases and nontumor liver tissue from the controls. The risk of developing hepatocellular carcinoma was approximately four times higher (OR = 67.6, 95% CI = 12.2 to 373.2) in individuals with markers for both virus and chemical than in HBsAg-negative individuals with detectable aflatoxin DNAadducts (OR = 17.0; 95% CI2.8 to 103.9) or HBsAg-positive individuals without detectable aflatoxin DNA-adducts (17.4; 95 % CI, 3.4 to 90.3). Test for interaction was not performed.

3.6.2 Alcohol

Three studies have provided some evidence that heavy alcohol intake may enhance the carcinogenic effect of HBV infection (see Table 3-8). In the Italian case-control study (Donato *et al.* 199; see Section 3.3.1), the OR for HBsAg positivity and light drinking (0 to 80 g/day ethanol) was 9.1 (95% CI = 3.7 to 22.5), while the comparable ORs for HBsAg(-)/ heavy drinking (> 80 g/day ethanol) and HBsAg(+)/heavy drinking were 4.2 (95% CI = 2.4 to 7.4) and 64.7 (95% CI = 20 to 210), respectively. In the Japanese cohort study of patients with viral hepatitis (Ikeda *et al.* 1998; see Section 3.3.2), the relative risk for heavy alcohol intake (lifetime intake of 500+ kg ethanol) was 2.0 (95% CI = 1.1 to 3.6) in HBsAg-negative patients. The comparable OR in HBsAg-positive patients was 8.4 (95% CI = 2.7 to 25.9). The third study, a case-control study in the United States (Hassan *et al.* 2002; see Section 3.3.1) reported an OR of 53.9 (95% CI = 7.0 to 415.7) for subjects who had chronic hepatitis infection (either HBV or HCV) and were heavy drinkers (≥ 80 mL of ethanol per day) compared to an ORs of 19.1 (95% CI = 4.1 to 89.1) for subjects without chronic hepatitis infection who did not drink heavily and an OR of 2.4 (95% CI = 1.3 to 4.4) for heavy drinkers without chronic hepatitis. Test for interaction on the additive scales was significant; synergy index (S) = 2.7 (95% CI = 1.1 to 5.2). The synergy index compares ORs of the joint effects versus the ORs of each risk factor in the absence of the other. In contrast, a cohort study from China did not report any interaction between HBV and alcohol consumption (Evans *et al*. 2002; see Section 3.3.2). In contrast to the other studies, alcohol consumption in the Chinese study was not an independent risk factor for liver cancer. One possible reason for the differences observed between the Chinese study and the other studies may be that the Chinese used a lower intake value to assess alcohol consumption (≥ 4 drinks per week) than the other studies (most of which assessed drinking as ≥ 80 g of ethanol per day).

3.7 HBV genotypes

Various methods involving antigenic typing and DNA sequencing have been used to divide HBV isolates into at least six subgroups. The new classification system is based on the complete sequencing of the viral genomes and the establishment of genomic differences between individual subgroups (Clarke and Bloor 2002). There is no clear indication at present that these subgroups differ in their association with hepatocellular carcinoma, although some investigators have suggested the possibility (Ding *et al.* 2001, Kao *et al.* 2000, Widell *et al.* 2000).

3.8 Biomarkers of HBV infection

It has been repeatedly shown that HBsAg is the only serologic marker that predicts hepatocellular carcinoma risk in populations with endemic HBV infection and

hepatocellular carcinoma incidence (sub-Saharan Africa, East and Southeast Asia). However, recent data from populations with relatively low HBV infection rates have suggested that anti-HBc positivity alone is also a significant risk factor for hepatocellular carcinoma in low- to intermediate-risk regions (United States, Mediterranean countries, Japan). The magnitude of the observed risk associated with anti-HBc alone is considerably lower than that associated with HBsAg. It is recognized that anti-HBc positivity in the absence of other HBV serologic markers can suggest low-level chronic infection (see Table 2.2). As mentioned in Table 2-1 and depicted in Figure 2-2, the HBeAg correlates with a high level of HBV replication, and disappearance of this antigen and appearance of anti-HBeAg indicates clinical remission. A recent cohort study in Taiwan suggests that the HBeAg in the presence of HBsAg may be associated with an even higher risk of hepatocellular carcinoma than HBsAg alone (Yang *et al*. 2002; see Section 3.3.1). This study also found that anti-HBeAg did not appear to be risk factor in a nested case-control analysis of men who were HBsAg positive but HBeAg negative.

3.9 Summary

In 1994, IARC published its monograph on the carcinogenic risk of chronic HBV infection to humans, and the Group reached the conclusion that "*the agent is carcinogenic to humans*." Cohort and case-control studies conducted in diverse populations by race/ethnicity and geography published since the IARC review further strengthen the recognized association between chronic HBV infection and the development of hepatocellular carcinoma. The overall OR for hepatocellular carcinoma in the Donato *et al*. (1998) meta-analysis for HBsAg positivity was 13.7 (95% CI = 12.2 to 15.4). The recent studies generally assessed chronic HBV infection using relatively sensitive and specific serological markers of infection; many also included information on use of alcohol and tobacco, medical history, and dietary aflatoxin (for studies in high-intake areas). These studies support a strong association between HBV and hepatocellular carcinoma that remains after adjustment for these potential confounders. Taken together, the large body of analytic epidemiological data on HBV infection and hepatocellular carcinoma risk provides some of the strongest supportive evidence linking environmental exposure to the development of a human cancer.

Consistent and abundant data support a synergistic effect of HBV and HCV coinfection on risk of hepatocellular carcinoma. Data also support dietary aflatoxin and heavy alcohol intake acting as cofactors that enhance the risk of hepatocellular carcinoma in a chronic HBV carrier.

No evidence exists from any of the published studies (cohort or case-control) that chronic HBV infection increases the risk of any other cancer besides hepatocellular carcinoma.

Table 3-1. Case control studies of chronic hepatitis published after the 1994 IARC review: Odds ratios for hepatocellular carcinoma in subjects testing positive for HBsAg versus subjects testing negative

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Asia				
Fukuda *et al.* 1993 Japan	Hospital-based, case-control study Cases: 368 incident cases of HCC (287 men, 81 women) aged 40 to 69 yrs, residents of Fukuoka or Saga prefecture, diagnosed at the Kurume University Hospital between April 1986 and May 1992 Controls: inpatients of two university-affiliated general hospitals in Kurume (287 men, 198 women), judged to be free of chronic hepatitis or hepatic cirrhosis, matched to index cases by age (5-yr age groups), sex, residence (prefecture), and time of hospitalization (within two months of the case interview)	HBsAg: reverse passive hemagglutination or EIA (data from hospital records) (10.7%/1.3%) Anti-HCV: 1st-generation EIA	Men: 9.7 (2.9–31.7) Women: 7.6 (2.0–29.4)	Age-matched OR in men only Age-matched OR in women only Analysis (Wilcoxon matched-pairs signed ranks tests, McNemar's test or conditional logistic regression model) was performed using the following case-control sets: 287 male 1:1 sets, 41 female 1:1sets, 3 female 1:3 sets and 37 female 1:4 sets

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Cordier *et al.* 1993 Vietnam	Hospital-based, case-control study (men only) in Hanoi, Vietnam 1989–1992 Cases: 152 male patients with HCC Controls: 241 male hospital controls of similar ages, admitted to the same hospitals for reasons other than cancer or liver disease	HBsAg: 2nd-generation EIA (92.6%/18.3%) Anti-HCV: 2nd-generation EIA	61.7 (30.0–128.0)	OR age-adjusted
Pyong *et al.* 1994 Japan	Hospital-based, case-control study Cases: 90 Korean patients (68 men, 22 women) newly diagnosed with HCC at the Kyowa Hospital in Osaka, Japan between January 1989 and December 1992 Controls: 249 Korean patients admitted to the same hospital during the same period as the cases; ages 40–89 years, with no history of liver disease or smoking- or alcohol-related conditions, including ischemic heart disease, lung cancer, peptic ulcer, or pancreatitis	HBsAg: reverse passive hemagglutination (16.7%/3.6%) Anti-HCV: 1st-generation EIA	Anti-HCV(-): 58.2 (15.3–221.0)	OR for anti-HCV negative subjects only; OR adjusted for age, sex, tobacco smoking, alcohol drinking, and history of blood transfusion

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Okuno *et al.* 1994 China	Hospital-based, case-control study Cases: 186 newly diagnosed HCC patients (168 men, 18 women) at the Guangxi Medical University Affiliated Hospital in Nanning City, Southern Guangxi January 1991–September 1992 Controls: 48 (apparently healthy workers 30 men, 18 women) given a routine physical examination at the same hospital in August 1992	HBsAg: reverse passive hemagglutination (70.4%/10.4%) Anti-HCV: 2nd-generation EIA	20.5 (7.4–56.3)	OR unadjusted for age/sex

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Park *et al.* 1995 Korea	Hospital-based, case-control study Cases: 540 patients (433 men, 107 women) with HCC, admitted to Kosin University Hospital in Pusan, Korea between July 1992 and February 1994 Controls: 808 apparently healthy residents of Pusan City, (431 men, 377 women) who received their annual, routine physical examination at the same hospital between September 1992 and October 1993. All control subjects were free of biochemical or clinical features of liver disease at enrollment	HBsAg-RIA (61.1%/5.2%) Anti-HCV: 2nd-generation EIA HCV RNA: nested RT-PCR	37.9 (31.4–45.7)	OR adjusted for anti-HCV
Sun *et al.* 1996 Taiwan	Population-based, case-control study in seven townships in Taiwan Cases: 58 incident cases of HCC (51 men, 7 women) Controls: 225 controls matched to index cases by age (within five years), sex, and township of residence	HBsAg: 2nd-generation EIA (82.8%/12.9%) Anti-HCV: 2nd-generation EIA HCV-RNA: RT-PCR	27.6 (10.7–70.9)	Age/sex-matched OR further adjusted for anti-HCV

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Tsai *et al.* 1996 Taiwan	Hospital-based, case-control study Cases: 361 newly diagnosed patients with HCC(303 men, 58 women), who were consecutively admitted to the Kaohsiung Medical College Hospital in Taiwan between January 1991 and December 1993 Controls: apparently healthy subjects who entered the same hospital for a physical check-up during the study period; they were individually matched (one control per case) to the index cases by age (within five years) and sex	HBsAg (80.3%/20.7%), HBeAg, Anti-HBe: RIAs Anti-HCV: 2nd-generation EIA	68.4 (20.5–227.8)	Age/sex-matched OR further adjusted for anti-HCV and HBeAg

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Shin *et al.* 1996 Korea	Hospital-based, case-control study in Pusan, Korea, August 1990–August 1993 Cases: 203 newly diagnosed HCC patients (159 men, 44 women) admitted consecutively to the Inje University Pusan Paik Hospital Controls: two groups individually matched to the index cases by age (within four years) and sex: 203 apparently healthy subjects who entered the same hospital for a routine checkup, and 203 hospital inpatients free of cancer or liver disease	HBsAg (65.5%/3.5%), anti-HBc, anti-HBs: RIAs Anti-HCV: 2nd-generation EIA	87.4 (22.2–344.3)	Age/sex-matched OR further adjusted for anti-HCV, *Clonorchis sinensis* in stool, history of blood transfusion, history of acute hepatitis, history of liver fluke, alcohol drinking, and tobacco smoking

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Tanaka *et al.* 1996 Japan	Hospital-based, case-control study Cases: 91 newly diagnosed patients with HCC (73 men, 18 women), 40–69 years old, residents of Fukuoka or Saga Prefecture, who were admitted to the Kyushu University Hospital between December 1985 and June 1989 Controls: 410 residents of Fukuoka City (291 men, 119 women), 40–69 years old, who had a physical examination at a public health center near the Kyushu University Hospital between January 1986 and July 1989. Controls were frequency-matched to the cases by age and had no known history of liver disease	HBsAg: reverse passive hemagglutination (20.9%/2.0%) Anti-HCV: 2nd-generation immunoradio-metric assay (IRMA); confirmation by RIBA HCV-RNA: RT-PCR	Total: 16.0 (6.4–39.7) Anti-HCV(-): 293.7 (68.7–1,255.6)	Age/sex-adjusted OR Comparable OR for anti-HCV-negative subjects only

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Yu *et al.* 1997b China	Multiregion, hospital-based, case-control study in four areas of southeastern China with a relatively high incidence of HCC Cases: 359 cases Controls: an equal number of individually matched (by age within five years, sex, and location [number of cases in each location ranged from 71 to 100]) No information provided on how cases and controls were identified and selected	HBsAg: EIA (Chinese-manufactured kits) (66.5%/22.9%) Anti-HCV: EIA (Chinese-manufactured kits; 1st or 2nd generation not specified)	Total: 6.6 (4.7–9.0) Anti-HCV(-): 6.1 (4.2–8.8)	Age/sex/location-matched OR Comparable OR for anti-HCV negative subjects only

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Okada *et al*. 1998 Japan	Hospital-based, case-control study: HCC among patients with chronic liver disease at the National Cancer Center Hospital in Tokyo, Japan, January 1992-December 1993 Cases: 141 consecutive patients with HCC and underlying chronic liver disease (110 men, 31 women), 25-81 years old Controls: 151 consecutive patients with chronic liver disease but no evidence of HCC (96 men, 55 women), 25-79 years old, who were admitted to the same hospital during the study period	HBsAg: reverse-passive hemagglutination (16.0%/9.0%) Anti-HBs: passive hemagglutination Anti-HBc: EIA Anti-HCV: 2nd-generation EIA	Total: 4.7 (1.7–12.7) HBc(+)/HBs(-)/ HBsAg(-): 2.6(1.5–4.7)	OR adjusted for age, sex, anti-HCV, and anti-HBc OR for anti-HBc positivity without anti-HBs or HBsAg

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Zhang *et al.* 1998a China	Hospital-based, case-control study Cases: 152 patients (136 men, 16 women) with HCC admitted to four hospitals in Henan Province, China, January 1994–October 1995 Controls: 115 patients (99 men, 16 women) admitted to the same hospitals during the study period, with similar ages as the cases and judged to be free of liver disease	HBsAg, anti-HBs: RIAs (63.2%/5.2%) Anti-HBc, anti-HCV: EIAs (unknown generation) HBV DNA: PCR HCV RNA: RT-PCR	Anti-HCV(-): 28.8 (11.2–78.8) Adjusted for other factors: 44.6 (12.5–158.5)	Age/sex-adjusted OR for anti-HCV negative subjects only OR further adjusted for personal and family history of liver disease, alcohol drinking, cigarette smoking, dietary aflatoxin intake, and history of blood transfusion
Africa				
Bile *et al.* 1993 Somalia	Hospital-based, case-control study in Mogadishu, Somalia in 1989 Cases: 62 chronic liver disease patients (49 HCC, 13 other liver disease) admitted to two main referral centers in Mogadishu Controls: for each case, one hospital control patient, matched for age (within five years) and sex, [no further details given]	HBsAg: RIA (41.9%/11.3%) Anti-HCV: 2nd-generation EIA	Anti-HCV(-): 3.3 (1.1–9.9)	Age/sex-matched OR for anti-HCV-negative subjects only

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Cenac *et al.* 1995 Niger	Hospital-based, case-control study Cases: 26 Sahelian African patients (19 men, 7 women) with HCC, who were admitted to the Hospital National in Niamey, Niger beween June 1983 and June 1985 Controls: 47 Sahelian African patients (24 men, 23 women) admitted to the Department of Internal Medicine in the same hospital during the study period, who had no history of liver disease	HBsAg: RIA (73.1%/29.8%) Anti-HCV: 2nd-generation EIA	6.4 (2.1–18.9)	OR unadjusted for age/sex
Kew *et al.* 1997 South Africa	Hospital-based, case-control study Cases: 231 South African black patients with HCC Controls: individually matched (by age within two years, sex, race, rural/urban/rural-urban background, hospital, medical versus surgical ward) hospital controls admitted to four Johannesburg hospitals	HBsAg: RIA (53.2%/8.2%) Anti-HCV: 2nd- or 3rd-generation EIA HCV-RNA: nested RT-PCR	Total: 21.8 (8.9–53.4) Anti-HCV(-): 23.3 (9.2–59.4)	Age/sex/race-matched OR Comparable OR for anti-HCV negative subjects only

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Americas				
Yu *et al.* 1997b USA	Population-based, case-control study among non-Asians of Los Angeles County, California, 18–74 years old Cases: 111 incident cases of HCC Controls: 128 community control	HBsAg (9.0%/0.0%), anti-HBc, anti-HBs: RIA Anti-HCV: 2nd-generation EIA; confirmation by RIBA	Total: – (5.6,-)[a] Anti-HBc (+): 7.4 (3.6–15.4)	Age/sex/race-adjusted OR for anti-HBc positivity
Yuan *et al.* 1999 USA	Population-based, case-control study (Los Angeles Study) Cases: 144 non-Asian patients with HCC Controls: 252 community controls of similar age, gender, and race	HBsAg: RIA (7.6%/0.0%) Anti-HBsAg, anti-HBcAg: EIA Anti-HCV: 1st- or 2nd-generation EIA HCV RNA: nested RT-PCR	Total: – (7.7,-)[a] Anti-HBc(+)/ HBsAg(-): 4.2 (2.3-7.8)	Age/sex/race-adjusted OR for anti-HBc positivity without HBsAg
Hassan *et al.* 2002 USA	Hospital-based, case-control study Cases: 115 (87 men, 28 women) patients with HCC from University of Texas M.D. Anderson Cancer Center Controls: 230 (174 men, 56 women) with malignant neoplasms other than HCCs	HBsAg (14.7%/0.9%) and anti-HBc: ELISA (2nd generation)	HBsAg(+) and antiHBc(+): 23.8(3.9–141.6)	OR adjusted for anti-HBc, alcohol consumption, cigarette smoking, and diabetes

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Europe				
Arico *et al.* 1994 Italy	Hospital-based, case-control study of HCC among patients with liver cirrhosis Cases: 62 consecutive patients (50 men, 12 women) admitted to a district hospital in Turin, Italy, 1986–1992, in whom a diagnosis of HCC in the presence of liver cirrhosis was made for the first time Controls: One group of 310 inpatients, individually matched (by age within five years, sex, and hospital), and with no history of liver disease; one group of while 97 asymptomatic inpatients with a first diagnosis of liver cirrhosis	HBsAg (19.4%/2.3%) and anti-HBs: EIA	Noncirrhotic controls: 10.7 (4.9–20.5) Cirrhotic controls: 6.8 (1.4–32.3)	Age/sex-matched OR further adjusted for alcohol intake using noncirrhotic controls

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Peters *et al.* 1994 Germany	Hospital-based, case-control study Cases: 86 patients (74 men, 14 women, although one table lists no. men as 71) with HCC and underlying liver cirrhosis, seen at the University Hospital of Mainz in Germany between 1986 and 1993 Controls: patients with liver cirrhosis but no evidence of HCC, individually matched (by age within five years and sex to the index case), seen at the same hospital during the study period	HBsAg: RIA (25.0%/22.0%), Anti-HBs, anti-HBc, HBeAg, anti-HBe, anti-delta antibody: RIAs Anti-HCV: 1st- or 2nd-generation EIA	Total: 1.1 (0.5–2.5) Anti-HBc(+): 1.5 (0.8–2.9)	Hospital-based, case-control study age/sex matched OR Comparable OR for anti-HBc positivity
Goritsas *et al.* 1995 Greece	Hospital-based, case-control study Cases: 51 consecutively admitted patients (48 men, 3 women) with HCC at Patras University Hospital in Patras, Greece, October 1989–October 1992 Controls: patients seen at the same hospital during the study period, individually matched (by sex and age within five years), who had no history of liver disease or cancer	HBsAg: EIA (60.8%/7.8%), Anti-HBs, anti-HBc: EIAs Anti-HCV: 2nd-generation EIA; confirmation by RIBA	2.2 (1.5–3.2)	Age/sex-adjusted OR further adjusted for anti-HCV, heavy alcohol intake, and tobacco smoking

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Hadziyannis *et al.* 1995 Greece	Hospital-based, case-control study in Athens, Greece Cases: 65 incident cases of HCC Controls: two groups of hospital controls individually matched to the index cases by age (within five years) and sex (65 metastatic liver cancer patients and 65 patients hospitalized for eye, ear, nose, or throat conditions)	HBsAg: EIA (61.5%/9.2%), Anti-HBc, anti-HBs: EIAs Anti-HCV: 2nd-generation EIA; confirmation by RIBA	18.8 (8.2–43.2)	Age/sex-adjusted OR was further adjusted for anti-HCV
Kaczynski *et al.* 1996 Sweden	Hospital-based, case-control study conducted in two hospitals in Goteborg, Sweden from 1984 to 1991. Cases: 73 patients with HCC (55 men, 18 women) Controls: 32 patients with other cancers (n = 21) or benign liver disease (n = 11)	HBsAg: RIA (0.0%/–) Anti-HCV: 3rd-generation EIA; confirmation by RIBA	–[b]	No cases positive for HBsAg

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Donato *et al.* 1997 Italy	Hospital-based, case-control study Cases: 172 newly diagnosed HCC patients in the two major hospitals in the province of Brescia in northern Italy Controls: other hospital patients with no history of liver disease or cancer, frequency-matched to the cases by age (5-year age groups), sex, and date/hospital of admission	HBsAg: EIA (23.8%/5.4%) Anti-HCV: 3rd-generation EIA; confirmation by RIBA HCV-RNA: nested RT-PCR	11.4 (5.7–22.8)	OR adjusted for age/sex/residence/anti-HCV, HCV RNA/alcohol intake
Tagger *et al.* 1999 Italy	Hospital-based, case-control study (Brescia study) Cases: 305 cases of HCC Controls: 610 hospital controls	HBsAg: EIA (24.4%/4.1%), Anti-HBs, anti-HBc: EIAs anti-HCV: 3rd-generation EIA; confirmation by RIBA HCV genotypes: nested RT-PCR	Total: 18.8 (10.0–35.2) Anti-HBc (+): 1.9 (1.1–3.2)	Age/sex-adjusted OR further adjusted for alcohol intake and HCV infection Comparable OR for anti-HBc

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Chiesa *et al.* 2000 Italy	Hospital-based, case-control study Cases: 142 patients (116 men, 26 women)with HCC with liver cirrhosis and 21) HCC cases (19 men, 2 women) without cirrhosis, who were admitted to two main hospitals in Brescia, Italy 1995–1997. Controls: 610 patients admitted to the same hospitals during the study period, who were free of liver disease	HBsAg: EIA (25.3%/–[c]) HBV DNA: PCR Anti-HCV: 3rd-generation EIA HCV RNA: RT-PCR	HBsAg(+) and/orHBV DNA(+), with cirrhosis: 17.6 (9.0–34.4) HBsAg(+) and/or HBV DNA(+), without cirrhosis: 20.3 (5.7–72.6)	Age/sex-adjusted OR for HBsAg(+) and/or HBV DNA(+) in the presence of cirrhosis Comparable OR in the absence of cirrhosis

Reference and location	Study population	Exposure and HBsAg prevalence (cases/controls)	OR (95% CI)	Comments
Kuper *et al.* 2000 Greece	Hospital-based, case-control study Cases: 333 incident cases of HCC treated at three teaching hospitals in Athens, Greece January 1995–December 1998 Controls: Two groups of hospital controls admitted to the same hospitals during the study period, and similar in age and sex distributions to the cases. The first group consisted of 272 patients with metastatic liver cancer; the 2nd group consisted of 360 patients hospitalized for eye, ear, nose, or throat conditions	HBsAg: 3rd-generation EIA (62.8%/5.0%) Anti-HCV: 3rd-generation EIA	Total: 48.8 (30.5–78.3) Anti-HCV(-): 53.4 (33.0–86.2)	OR adjusted for age/sex/schooling/anti-HCV Comparable OR for anti-HCV negative subjects only

[a]No controls were HBsAg positive; therefore, no finite OR estimate can be calculated.

[b]None of the cases were positive for HBsAg.

[c]Number of controls was not given in this study.

Table 3-2. Prospective cancer studies (nested case-control and cohort studies) of chronic hepatitis published since the 1994 IARC review

Reference and location	Cohort/duration of follow-up	Cases/controls or # of cases that developed cancer	Exposure and HBsAg seroprevalence[a]	Effect	Comments
Kato *et al.* 1994 Japan	Cohort of cirrhosis patients: 401 patients (273 men, 128 women) with cirrhosis April 1977–March 1992 in Nagasaki, Japan. Patients had no history of and showed no evidence of HCC at enrollment Follow-up- mean, 4.4 yrs	127 individuals developed HCC	HBsAg: RIA (38%) Anti-HCV: 2nd-generation EIA or RIA	5-yr cumulative risk, 21.2% in HBsAg(+) alone vs. 12.4% in HBsAg(-)/anti-HCV(-)	$P < 0.05$, test for difference in cumulative risk

Reference and location	Cohort/duration of follow-up	Cases/controls or # of cases that developed cancer	Exposure and HBsAg seroprevalence[a]	Effect	Comments
Chang *et al.* 1994 Taiwan	Population-based cohort: 9,775 men 30-85 years of age from six townships in Taiwan accrued between September 1984 and February 1986. The cohort was actively followed on an annual basis until March 1992. Nested case-control analysis Follow-up- range 0.5 to 6.0 yrs	Cases: 38 newly diagnosed cases of primary liver cancer Controls: 152 controls matched by age (within one year), township of residence, and date of recruitment to the index cases	HBsAg: reverse passive hemagglutination; negative samples rechecked by RIA (63.2%/6.6%) Anti-HCV: 2nd generation EIA	RR[b] = 81.8 (95% CI[c] = 9.1–740.7)	Age-matched OR further adjusted for anti-HCV, vegetable intake, and personal history of chronic liver disease
Yuan *et al.* 1995 China	Population-based cohort: 18,244 Chinese men, ages 45 to 64 years, in Shanghai recruited January 1986–September 1989 nested case-control analysis Follow-up- average, 5.3 yrs	Cases: 76 incident cases of HCC within the cohort Controls: 410 control cohort subjects individually matched to the cases by age (within one year), time of blood sample collection (within one month), and neighborhood of residence	HBsAg: RIA (65.8%/11.9%) Anti-HCV: 2nd-generation EIA	RR = 15.0 (95% CI = 4.4–51.6)	Age-matched OR

Reference and location	Cohort/duration of follow-up	Cases/controls or # of cases that developed cancer	Exposure and HBsAg seroprevalence[a]	Effect	Comments
Nomura *et al.* 1996 USA	Population-based, male cohort: 5,924 Japanese-American men in Hawaii accrued between 1967 and 1970 and followed for cancer occurrence until 1992 (The Japan-Hawaii Cancer Study) Nested case-control analysis Follow-up- 19 yrs	Cases: 24 incident cases of HCC Controls: 72 age-matched cohort controls	HBsAg: RIA (62.5%/2.8%) Anti-HCV: 1st-generation EIA; positive samples retested by RIBA	RR = 43.0 (95% CI = 5.7–325.5)	Age-matched OR
Tsai *et al.* 1997 China	Cohort of non-alcoholic cirrhosis patientst: 400 patients (290 men, 110 women) with nonalcoholic cirrhosis at Kaohsiung Medical College Hospital January 1989–December 1994. Follow-up- 1,185 person-yrs	80 subjects developed HCC	HBsAg: RIA (69%) Anti-HCV: 2nd-generation EIA	Anti-HCV(+): RR = 4.1 (95% CI = 1.2–13.3)	RR for anti-HCV(-) subjects only; adjusted for age, sex, and other potential confounders

Reference and location	Cohort/duration of follow-up	Cases/controls or # of cases that developed cancer	Exposure and HBsAg seroprevalence[a]	Effect	Comments
Ikeda *et al.* 1998 Japan	Cohort of chronic viral hepatitis patients: 2,215 (1,544 men, 671 women) patients with chronic viral hepatitis diagnosed at the Toranomon Hospital in Tokyo, Japan January 1980–August 1995 Follow-up- median, 4.1 yrs range, 0.1–16.3 yrs	89 patients developed HCC	HBsAg: RIA (30%) Anti-HCV: 2nd-generation EIA	*P* = 0.02	RR not given, only *P* value for test of RR = 1
Mori *et al.* 2000 Japan	Population-based cohort: 3,059 (981 men, 2,078 women) residents of Saga Prefecture, Japan, aged 30 years or older, during June 1992 Follow-up- 13,983.9 person-years; median, 4.8 yrs	Cases: 22 (14 men, 8 women) developed HCC	HBsAg: reverse passive hemagglutination (2%) Anti-HCV: 2nd-generation EIA	RR = 7.3 (95% CI = 1.6–32.6)	Age/sex-adjusted RR

Reference and location	Cohort/duration of follow-up	Cases/controls or # of cases that developed cancer	Exposure and HBsAg seroprevalence[a]	Effect	Comments
Yang *et al.* 2002 Taiwan	Cohort of 11,893 men living in Taiwan with no evidence of HCC, during 1991 and 1992 Follow-up- 92,359 person-yrs	111 individuals HCC Nested case-control analysis 130 subjects positive for HBsAg and negative for HBeAg Cases: 44 Controls: 86 (matched)	HBsAg and HBeAg: RIA (23.8%; 30-39 yrs) (21.9%; 40-49 yrs) (18.9%; 50-59 yrs) (12.5%; >60 yrs) Anti-HCV: 2nd-generation EIA Anti-HBeAg- ELISA serum HBV DNA-branched chain DNA assay	Cohort +HBsAg: RR = 9.6 (95% CI = 6.0–15.2) +HBsAg and +HBeAg : RR = 60.2 (95% CI = 35.5–102.1) Nested case-control analysis: HBV DNA OR = 3.9 (95% CI = 1.6-9.2) Anti-HBeAg Cases vs. controls: $P = 0.16$	Adjusted for age/sex/anti-HCV/cigarette smoking/alcohol use Nested case-control analysis, test for trend for increasing amounts of HBV DNA, $P = 0.003$

Reference and location	Cohort/duration of follow-up	Cases/controls or # of cases that developed cancer	Exposure and HBsAg seroprevalence[a]	Effect	Comments
Evans *et al.* 2002 China	Cohort of 83,794 adults (58,454 men and 25,340 women) in Haimen City, China, followed from 1992-1993 to 2000 Follow-up- 434,718 person-yrs (men) 181,362 person-yrs (women)	Cases: 900deaths from HCC (men) occurred 77 deaths from HCC (women) occurred	HBsAg: RIA (15%, men) (10.7%, women) Anti-HCV: EIA	Men: RR = 18.8 (95% CI = 16.0-22.0) Women: RR = 33.2 (95% CI = 17.0–65.0)	HBsAg positivity was the major risk factor for HCC development RRs (multi-variate- Cox proportional hazard model) adjusted for age and all candidate risk factors

[a]Percentage prevalence at baseline.
[b]Relative risk.
[c]95% confidence interval.

Table 3-3. Summary results of the meta-analysis of Donato *et al.* (1998): Odds ratios for hepatocellular carcinoma in subjects testing positive for hepatitis B surface antigen positive versus subjects testing negative

	Number of studies	HBsAg positivity OR (95% CI)[a]
Total studies	32	13.7 (12.2–15.4)
Geographical area[b]		
High-risk area of HCC (sub-saharan Africa, East & Southeast Asia)	14	16.7 (14.5–19.3)
Intermediate to low-risk area of HCC (Japan, Southern Europe, North America)	4	8.5 (5.8–12.6)
Type of controls[b]		
Hospital	9	15.2 (11.9–19.4)
Community	12	15.7 (13.4–18.4)

[a]Odds ratio (95% confidence interval) for anti-HBsAg positivity regardless of HCV status.
[b]Only studies using 2nd- or 3rd-generation HCV assays included.

Table 3-4. Results of individual studies included in the meta-analysis of Donato *et al.* 1998: Odds ratio for hepatocellular carcinoma in subjects stratified jointly by HBV and HCV serology

	HBsAg-negative, anti-HCV-negative	HBsAg-positive, anti-HCV-negative		HBsAg-negative, anti-HCV-positive		HBsAg-positive, anti-HCV-positive	
Study	**Cases/controls**	**Cases/controls**	**OR (95% CI)[a]**	**Cases/controls**	**OR (95% CI)[a]**	**Cases/controls**	**OR (95% CI)[a]**
Bile *et al.* 1993[c]	14/52	23/6	14.2 (4.9–41.7)	22/3	27.2 (7.1–104)	3/1	11.1 (1.1–116)
Bruix *et al.* 1989[b,d]	20/163	4/3	10.9 (2.3–52.1)	67/10	54.6 (24.3–123)	5/0	–
Cenac *et al.* 1995[c]	5/32	15/12	8.0 (2.4–26.0)	2/2	6.4 (0.7–56.3)	4/1	25.6 (2.4–278)
Chang *et al.* 1994[c]	10/138	23/10	31.7 (11.9–84.7)	4/4	13.8 (3.0–63.6)	1/0	–
Chuang *et al.* 1992[b,d]	16/267	87/104	14.0 (7.8–24.9	13/8	27.1 (9.8–74.8)	12/5	40.1 (12.6–128)
Cordier *et al.* 1993[c]	8/194	138/44	76.1 (34.7–167)	3/2	36.4 (5.3–249)	0/0	–
Coursaget *et al.* 1992[b,d]	25/82	22/51	1.4 (0.7–2.8)	2/0	–	0/1	-
Dazza *et al.* 1993[c,d]	52/163	115/27	13.4 (7.9–22.5)	8/4	6.3 (1.8–21.7)	3/0	-
Di Bisceglie *et al.* 1991[b,d]	80/96	6/0	–	12/2	7.2 (1.6–33.1)	1/0	-
Donato *et al.* 1997[c]	66/292	37/17	9.6 (5.1–18.4)	65/22	13.1 (7.5–22.9)	4/1	17.5 (2.2–439)
Fukuda *et al.* 1993[b]	8/166	17/1	353 (41.6–2,993)	150/9	346 (130–919)	2/0	–
Hadziyannis *et al.* 1995[c]	20/116	37/11	19.5 (8.6–44.5)	5/2	14.5 (2.6–79.9)	3/1	17.4 (1.7–176)
Kaczynski *et al.* 1996[c]	57/30	0/0	–	7/1	3.7 (0.4–31.3)	0/0	–
Kaklamani *et al.* 1991[b,d]	71/373	42/29	7.6 (4.5–13.0)	29/29	5.3 (3.0–9.3)	43/1	226 (30.6–1,667)
Kew *et al.* 1997[c]	69/197	103/18	23.3 (9.2–59.4)	39/15	6.6 (2.7–15.7)	20/1	82.5 (8.9–762)
Nomura *et al.*	9/69	15/2	57.5 (11.3–294)	0/1	–	0/0	–

	HBsAg-negative, anti-HCV-negative	HBsAg-positive, anti-HCV-negative		HBsAg-negative, anti-HCV-positive		HBsAg-positive, anti-HCV-positive	
Study	**Cases/controls**	**Cases/controls**	**OR (95% CI)[a]**	**Cases/controls**	**OR (95% CI)[a]**	**Cases/controls**	**OR (95% CI)[a]**
1996[c]							
Okuno *et al.* 1994[c]	54/43	122/5	19.4 (7.3–51.8)	1/0	–	9/0	–
Park *et al.* 1995[c]	149/753	314/42	37.8 (26.2–54.5)	61/13	23.7 (12.7–44.3)	16/0	–
Pyong *et al.* 1994[b]	9/220	14/9	58.2 (15.3–221)	66/20	92.4 (33.8–252)	1/0	–
Saito *et al.* 1990[b,d]	66/133	49/0	-	136/15	18.3 (9.9–33.6)	2/0	–
Shin *et al.* 1996[c]	40/371	111/14	73.5 (38.6–140)	17/9	17.5 (7.3–41.9)	2/0	–
Simonetti *et al.* 1992 [b,d]	46/197	15/4	16.1 (5.1–50.7)	133/11	51.8 (25.9–104)	18/0	–
Stroffolini *et al.* 1992[c,d]	11/80	11/6	13.3 (4.1–43.3)	38/13	21.3 (8.7–51.8)	5/0	–
Sun *et al.* 1996[c]	8/186	42/29	32.9 (14.5–81.9)	2/10	4.6 (0.6–23.3)	6/0	–
Tanaka *et al.* 1996[c]	3/372	17/8	294 (68.7–1,256)	69/30	340 (96.5–1,196)	2/0	–
Tsai *et al.* 1996[c]	22/278	232/73	40.1 (23.5–69.0)	49/8	77.3 (30.5–204)	58/2	366 (83.4–1,601)
Xu *et al.* 1990 [b,d]	11/46	35/4	36.6 (10.7–125)	1/0	–	3/0	–
Yang *et al.* 1996[c,d]	15/68	62/11	25.6 (10.9–59.8)	0/1	–	10/0	–
Yu *et al.* 1991[b,d]	12/104	101/21	41.7 (19.5–89.2)	5/2	21.7 (3.8–124)	9/0	–
Yu *et al.* 1997a[c]	95/249	184/79	6.1 (4.2–8.9)	19/21	2.4 (1.7–4.8)	42/1	110 (14.9–811)
Yu *et al.* 1997b[c]	66/123	8/0	–	35/5	13.0 (4.7–44.2)	2/0	–
Yuan *et al.* 1995[c]	26/358	49/49	13.8 (7.9–24.1)	0/1	–	1/0	–

[a]Reference group = HBsAg(-)/anti-HCV(-).

[b]Used 1st-generation anti-HCV tests.

[c]Used 2nd- or 3rd-generation anti-HCV or HCV RNA tests.

[d]Reviewed by IARC 1994; not reviewed in the previous sections of this document.

Table 3-5. Summary results of the meta-analysis of Donato *et al.* 1998: Odds ratios of hepatocellular carcinoma in subjects stratified jointly by HBV and HCV serology (relative to subjects negative for both HBsAg and anti-HCV antibodies)

	Number of studies (cases/controls)	HBsAg-positive, anti-HCV-negative OR (95% CI) (cases/controls)	HBsAg-negative, anti-HCV-positive OR (95% CI) (cases/controls)	HBsAg-positive, anti-HCV-positive OR (95% CI) (cases/controls)
Total studies	32 (4,596/7,002)	20.4 (18.0–23.2) (2,050/689)	23.6 (20.0–28.1) (1,060/273)	135 (79.7–242) (287/15)
Geographical area[a]				
High-risk area of HCC (sub-Saharan Africa, East & Southeast Asia)	14 (2,538/3,613)	20.8 (17.8–24.3) (1,533/419)	11.5 (8.8–15.0) (227/93)	191 (86.1–494) (175/6)
Intermediate to low risk area of HCC (Japan, Southern Europe, North America)	4 (393/971)	18.8 (11.8–30.3) (102/42)	31.2 (20.9–47.4) (177/67)	75.6 (15.6–614) (14/2)
Type of controls[a]				
Hospital	9 (1,040/1,338)	24.0 (18.4–31.3) (475/128)	12.6 (9.1–17.4) (198/69)	34.6 (14.2–101) (41/5)
Community	12 (2,090/3,236)	19.2 (16.2–22.9) (1,183/335)	16.2 (12.1–21.7) (248/98)	420 (143–1,732) (150/3)

[a]Only studies using 2nd- or 3rd-generation HCV assays included.

Table 3-6. Results of other case-control studies: Odds ratios of hepatocellular carcinoma in subjects stratified jointly by HBV and HCV serology (relative to subjects negative for both anti-HBV and anti-HCV antibodies)

	HBV(-)/HCV(-)	HBV(+)/HCV(-)		HBV(-)/HCV(+)		HBV(+)/HCV(+)	
Study	**Cases/controls**	**Cases/controls**	**OR (95% CI)**	**Cases/controls**	**OR (95% CI)**	**Cases/controls**	**OR (95% CI)**
Zhang *et al.* 1998a	51/105	84/6	28.8 (11.2–78.8)	5/4	2.6 (0.6–12.0)	12/0	– (5.9,–)[a]
Yuan *et al.* 1999	68/228	30/19	5.2 (2.7–10.0)	24/4	20.1 (6.6– 61.2)	22/1	45.5 (5.9–352.2)
Tagger *et al.* 1999	39/221	63/24	21.1 (11.1–40.0)	36/8	35.6 (14.5–87.1)	11/1	132.0 (15.3–890)
Kuper *et al.* 2000	83/574	198/28	53.4 (33.0–86.2)	41/9	32.3 (15.0–69.4)	11/2	46.2 (9.9–216.6)

[a]No controls in this category; therefore, no finite OR estimate can be calculated.

Table 3-7. Odds ratios of hepatocellular carcinoma in subjects stratified jointly by HBsAg serology and aflatoxin exposure

		HBsAg(-)		HBsAg(+)	
Reference and location	**Aflatoxin assessment**	**Cases/ controls**	**OR (95% CI)**	**Cases/ controls**	**OR (95% CI)**
Qian *et al.* 1994[a] Shanghai, China	Urinary aflatoxin metabolites				
	Negative	5/134	1.0	9/24	7.3 (2.2–24.4)
	Positive	13/102	3.4 (1.1–10.0)	23/7	59.4 (16.6–212.0)
McGlynn *et al.* 1995 China	Epoxide hydrolase genotype				
	allele “1” homozygote	1/25	1.0	3/5	15.0 (1.2–184.0)
	One or two copies of allele “2”	10/75	3.3 (0.39–28.6)	34/11	77.3 (8.9–665.8)

Reference and location	Aflatoxin assessment	HBsAg(-) Cases/ controls	HBsAg(-) OR (95% CI)	HBsAg(+) Cases/ controls	HBsAg(+) OR (95% CI)
Wang *et al.* 1996 Taiwan	Aflatoxin:albumin adducts				
	Non-detectable	7/101	1.0	14/17	17.4 (3.7–81.9
	Detectable	1/52	0.3 (0.0–3.6)	30/9	70.0 (11.8–415.4)
	Urinary aflatoxin metabolites				
	Low	2/61	1.0	10/14	22.8 (3.6–143.4)
	High	4/53	1.7 (0.3–10.8)	22/8	111.9 (13.8–905.0)
Lunn *et al.* (1997) Taiwan	Aflatoxin:DNA adducts				
	Nondetectable	3/18	1.0	18/3	17.0 (2.8–103.9)
	Detectable	23/10	17.4 (3.4–90.3)	61/6	67.6 (12.2–373.2)

[a]Study population is the same as that reported by Yuan *et al.* (1995) in Table 3-1.

Table 3-8. Odds ratios of hepatocellular carcinoma in subjects stratified jointly by HBsAg serology and alcohol intake level

		HBsAg(-)		HBsAg(+)	
Reference and location[a]	**Alcohol status**	**Cases/controls**	**OR (95% CI)**	**Cases/controls**	**OR (95% CI)**
Donato *et al.* 1997	Ethanol intake				
Brescia, Italy	0–80 g/day	56/197	1.0	16/14	9.1 (3.7–22.5)
	> 80 g/day	75/117	4.2 (2.4–7.4)	25/4	64.7 (20.0–210.0)
Ikeda *et al.* 1998	Lifetime ethanol intake				
Tokyo, Japan	< 500 kg	–[c]	1.0	–[c]	1.0
	500+ kg	–[c]	2.0 (1.1–3.6)	–[c]	8.4 (2.7–25.9)
Hassan *et al.* 2002	Ethanol intake				
Texas, USA	0–80 g/day	–[c]	1.0[d]	–[c]	19.1 (4.1–89.1)[d]
	> 80 g/day	–[c]	2.4 (1.3–4.4)[d]	–[c]	53.9 (7.0–415.7)[d]

[a]Effects for Evans et al. were not given; it was only stated that no significant interaction was found between alcohol and HBsAg status.
[b]Number of cases was not given in this cohort study report.
[c]Results are for chronic hepatitis virus infection (either HBV or HCV).
[d]Results are for chronic hepatitis virus infection (either HBV or HCV).

4 Studies of Cancer in Experimental Animals

Animal models have been valuable resources in studies of replication, pathogenesis, and carcinogenesis of hepadnaviruses. Indeed, the replication cycle of hepadnaviruses was deduced from studies in ducks. Woodchucks and ground squirrels have been particularly useful in shaping our understanding of the molecular mechanisms of hepadnavirus-induced hepatic carcinogenesis. The utility of animal hepadnaviruses stems from the limited host range of HBV, as well as the difficulty of *in vitro* propagation. A variety of experimental animal models of hepadnavirus infection are available; however, only great apes, gibbons, and tree shrews are susceptible to infection with human HBV (Table 4-1). Several primate species may be infected with distinct strains of HBV, and a number of mammalian and avian species have their own hepadnavirus infections. IARC reviewed the carcinogenicity of HBV and concluded that there was *inadequate evidence* in experimental animals for the carcinogenicity of HBV. However, some hepadnaviruses closely related to HBV produce hepatocellular carcinoma in susceptible species (IARC 1994).

The current classification of hepadnaviruses is shown in Table 4-1. A recent review of animal hepadnaviruses and tumor induction in hepadnavirus-infected animals or transgenic mice is available (Tennant 2001, Tennant and Gerin 2001).

4.1 Human HBV strains in animals

4.1.1 Primates

The great apes (gorillas, chimpanzees, orangutans) and gibbons are the only primates reliably susceptible to infection with HBV. Chimpanzees have served as a valuable animal model of HBV for many years. Infected chimpanzees can develop chronic infections with associated chronic hepatic inflammation that may be somewhat milder than that in typical human infections (Shouval *et al.* 1980). Yet, despite the ability to sustain chronic HBV infections, hepatocellular carcinomas are very uncommon in chimpanzees. Only two hepatocellular carcinomas have been described in HBV-infected animals, and neither of those animals had a chronic infection (Muchmore *et al.* 1990). One animal that recovered from its HBV infection also had been infected with non-A, non-B hepatitis. Another animal with hepatocellular carcinoma was in the acute phase of HBV infection with coinfection of delta hepatitis. Little evidence is available to suggest an association between HBV infection and development of hepatocellular carcinoma in chimpanzees, but the reasons that chimpanzees do not develop the disease during chronic infection are unclear. It may be attributable to the relatively mild inflammation in HBV-infected chimpanzees compared to that in humans.

Table 4-1. Classification of human and animal hepadnaviruses

Genus	Virus	Natural host	
		Scientific name	Common name
Orthohepadnavirus	Hepatitis B virus (HBV)	*Homo sapiens* (strains A–G)	Humans
		(Distinct strains) *Pan troglodytes* *Gorilla gorilla* *Hylobates sp.* *Pongo pygmaeus*	 Chimpanzee Gorilla Gibbon Orangutan
	Woolly monkey hepatitis B virus (WMHBV)	*Lagotrix lagotricha*	Woolly monkey
	Woodchuck hepatitis virus (WHV)	*Marmota monax*	Woodchuck
	Ground squirrel hepatitis virus (GSHV)	*Spermophilus beecheyi*	California ground squirrel
	Arctic ground squirrel virus (ASHV)	*Spermophilus parryi*	Arctic ground squirrel
Avihepadnavirus	Duck hepatitis B virus (DHBV)	*Anus domesticus*	Duck
	Heron hepatitis B virus (HHBV)	*Ardea cineria*	Heron
	Stork hepatitis B virus	*Ciconia ciconia*	Stork
	Snow goose hepatitis virus	*Anser caerulescens*	Snow goose
	Ross's goose virus	*Anser rossii*	Ross's goose

Adapted from: Tennant 2001.

4.1.2 Transgenic mice

Transgenic technology has allowed the insertion of selected genes into fertilized mouse ova to facilitate studies of the effects of expression of the selected genes during the lifetime of the mice. In HBV research, transgenic mice have been used to bypass the lack of susceptibility of standard laboratory rodents to infection with HBV. A number of mouse lines have been bred that express individual HBV proteins such as envelope (Babinet *et al.* 1985, Chisari *et al.* 1985), core (Milich *et al.* 1994, Guidotti *et al.* 1994), pre-core or e-antigen (Milich *et al.* 1990, Guidotti *et al.* 1996), and X protein (Lee *et al.* 1990, Kim *et al.* 1991, Koike *et al.* 1994). Additional lines have been bred that express the entire genome of HBV (Araki *et al.* 1989, Farza *et al.* 1988, Guidotti *et al.* 1995, Marion *et al.* In press) High levels of expression of both HBs and the HBx can lead to hepatocellular carcinoma; however, expression of the entire HBV genome is not associated with either liver injury or hepatocellular carcinoma. Other studies show that transgenic expression of HBV genes can facilitate chemical-induced hepatic

carcinogenesis (Dragani *et al.* 1989, Sell *et al.* 1991, Slagle *et al.* 1996, Dandri *et al.* 1996, Madden *et al.* 2001).

4.1.2.1 Expression of HBV proteins

HBx, a 17-kDa protein found in all mammalian hepadnaviruses, has been implicated in the pathogenesis of HBV-induced hepatic carcinogenesis due to its ability to transactivate a variety of genes *in vitro*; but the role of HBx in liver cancer is currently unclear. In initial studies of three lines of mice that expressed HBx under the control of human alpha-1-antitrypsin, no hepatocellular carcinomas were observed in any of 80 ICR × $B6C3F_1$ (founder strain) mice studied, some up to two years of age (Lee *et al.* 1990). Similarly, no signs of hepatic pathology or hepatocellular carcinoma were detected in several lines of transgenic C57Bl/6 × DBA2 mice that contained HBx gene constructs (Billet *et al.* 1995). The authors demonstrated that hepatic HBx gene expression diminished early in the lifetime of the mice; thus, the level of expression of HBx and possibly the strain of mouse used might be important determinants in the development of hepatocellular carcinoma. In contrast, Kim *et al.* (1991) demonstrated a carcinogenic effect of HBx expression in lines of outbred CD-1 transgenic mice that expressed HBx under its own regulatory elements. More than 90% of male mice of one line and 80% of male mice of another line developed liver tumors. Approximately 10% of the control mice developed liver tumors (Table 4-2). In another study of transgenic CD-1 mice, which expressed HBx under the authentic controlling elements, hepatocellular carcinomas developed in one line with a relatively high level of HBx but not in the line with the lower level of expression (Koike *et al.* 1994). In the higher expressing line, hepatocellular carcinoma developed in 31 of 37 (84%) male mice studied for two years, but the line with the lower level of transgene expression had a tumor incidence similar to that of the nontransgenic controls. Similarly, hepatocellular carcinoma developed in 9 of 14 (64%) transgenic C57Bl/6 × DBA mice that expressed HBx under its own controlling elements (Yu *et al.* 1999) (Table 4-2). However, in that study only a single line developed hepatocellular carcinoma out of several hundred embryos injected, raising the issue of positional effects of the transgene on tumor development. HBx can facilitate tumorigenesis in mice that contain other constructs. Bitransgenic mice that coexpress HBx and activated c-*myc* under the control of WHV regulatory elements have shorter tumor latency than those that express HBx gene or c-*myc* alone (Terradillos *et al.* 1997).

Table 4-2. Incidence of hepatocellular carcinoma in transgenic mice that express HBx

Strain and line	Age examined (mo.)	Sex	Number examined	Tumor incidence (%)	Reference
Outbred F/C: CD-1					Kim *et al.* 1991
C11	4–18	M	21	19 (91)	
	4–21	F	20	12 (60)	
H9	4–18	M	10	8 (80)	
	4–21	F	6	4 (67)	
Controls	Up to 24	F	nr	nr (< 10)	
Outbred F/C:CD-1					Koike *et al.* 1994
H9	1–24	M	37[a]	31 (84)	
E1	1–24	M	21[b]	9 (43)	
E1	1–24	M	16[c]	1 (6)	
Outbred F: C57Bl/6 × DBA					Yu *et al.* 1999
HEX-3	11–18	M/F	14	9 (64)	

[a]Includes both heterozygous and homozygous mice (average age at tumor development, 16.7 mo.).
[b]Homozygous only (average age at tumor development, 23 mo.).
[c]Heterozygous only (average age at tumor development, 24 mo.).
F = founding strain.
C = crossing strain.
nr = not reported.

Several lines of transgenic mice that express the HBV envelope proteins were developed in the 1980s (Babinet *et al.* 1985, Chisari *et al.* 1985, Burk *et al.* 1988). Liver tumors did not develop in mouse lines that had no apparent hepatocellular injury. However, two lines (50-4, [current designation Tg (alb-1 HBV) Bri44] and 45-2, [current designation Tg (alb-1 HBV) Bri43]) of mice that overexpressed the HBV large envelope polypeptide due to the presence of a constitutively active albumin promoter did develop hepatocellular injury and hepatocellular carcinoma (Chisari *et al.* 1986, 1987, 1989). Extensive accumulation and retention of large envelope polypeptide in the hepatocellular endoplasmic reticulum led to hepatocellular necrosis with subsequent inflammation and hepatocellular regeneration, Kupffer cell activation and proliferation, oxidative DNA injury, and aneuploidy (Chisari *et al.* 1987, 1989, Chisari 1996, Hagen *et al.* 1994, Huang and Chisari 1995). Mice from the 50-4 line developed preneoplastic lesions and eventually fatal hepatocellular carcinomas. Virtually all mice from the 50-4 line that produced the greatest amount of the HBV large envelope polypeptide and had the greatest amount of hepatic inflammation developed hepatocellular carcinoma before 2 years of age. Hepatocellular carcinoma was not observed in a related line (45-3) that produced lower amounts of the large envelope polypeptide and did not develop

inflammation. Viral surface antigen proteins can accumulate in chronic human HBV infections, but it is not known if this accumulation produces hepatocellular injury or has a role in human hepatocellular carcinoma. The mechanism of hepatocellular carcinoma formation in these transgenic mice is most likely attributable to chronic liver necrosis, proliferation, and inflammation (Dunsford *et al.* 1990).

One significant difference between chronic human HBV infection and life-long expression of HBV by transgenic mice is the lack of an immune response in transgenic mice that are presumably tolerant to the viral proteins. Nakamoto *et al.* (1998) developed an elegant adoptive transfer experiment to assess the impact of an active immune response on the pathogenesis of hepatocellular carcinoma in transgenic mice. HBV large envelope polypeptide transgenic mouse lines were used. These lines are immunologically tolerant to HBs antigens, express low levels of the HBV large envelope polypeptide, and have no significant liver lesions other than the accumulation of ground glass cells. Transgenic mice were thymectomized, irradiated, and then immunologically reconstituted by engraftment with bone marrow cells and splenocytes from nontransgenic syngeneic mice that had been immunized with HBsAg. Fulminant hepatitis resulted when high numbers of cells were transferred; however, when lower numbers of cells were transferred, chronic hepatitis resulted. Eight of nine transgenic mice with the adoptive transfer developed hepatocellular carcinoma within 20 months, and the ninth mouse developed a hepatic adenoma. Two hepatic neoplasms, one adenoma, and one carcinoma, were observed in the nine transgenic mice used as controls for irradiation treatment. The control mice were thymectomized, irradiated, and engrafted with marrow and splenocytes from immunologically tolerant inbred transgenic littermates that had not been immunized. No neoplasms were found in any of the nine unmanipulated transgenic mice. No evidence of random HBV integration or HBx gene expression was noted in any of the mice. The authors concluded that chronic immune-mediated inflammation and hepatocellular necrosis play an important, and perhaps central, role in development of hepatocellular carcinoma.

The complicating effects of whole-body irradiation and thymectomy on tumor studies have been bypassed in a new adoptive transfer mouse model that uses transgenic nude mice (Larkin *et al.* 1999). Because nude mice are immunologically deficient, they can be immunologically reconstituted without the use of irradiation or thymectomy. This model should enable the assessment of various components of the immune system in the immunopathogenesis of hepatic inflammation and hepatic carcinogenesis.

4.1.2.2 Interaction with other carcinogens

Several groups have investigated the interactive effects of chemical carcinogens and HBsAg expression by transgenic mice. Dragani *et al.* (1989) exposed HBsAg-expressing C3H/He transgenic mice containing one copy of HBV without the core gene to either diethylnitrosamine (DEN) (10 mg/kg) or *p*-dimethylaminoazobenzene (DAB) (150 mg/kg) once at seven days of age. After 30 weeks of observation, hepatocellular carcinomas and hepatic adenomas were more found frequently in DEN-treated HBsAg-expressing male transgenic mice (12 of 23) than in nontransgenic, similarly treated male mice (6 of 19), but the incidences of hepatocellular carcinoma and hepatic adenomas

were not statistically different. Male mice had more neoplasms than female mice. Female DEN-treated mice had only one hepatocellular carcinoma, in a nontransgenic mouse. DAB-treated male mice had similar incidences of hepatocellular carcinomas and hepatic adenomas in transgenic (6 of 12) and nontransgenic mice (7 of 12). The C3H/He strain of mice has a relatively high susceptibility to chemical carcinogens and promoters that affect the liver.

Sell *et al.* (1991) demonstrated the synergistic effect of HBsAg transgene expression with two chemical carcinogens, aflatoxin B_1 (AFB_1) and DEN. A total of 26 female C57Bl/6 mice treated with one of three doses of AFB_1 developed 20 adenomas and two hepatocellular carcinomas; eight mice dosed with DEN developed eight adenomas and two hepatocellular carcinomas. No tumors were found by 15 months of age in any of the 10 transgenic mice not treated with carcinogens.

HBx may have a promotional effect on hepatic carcinogenesis because it enhances the sensitivity of transgenic mice to the effects of the chemical carcinogen DEN and stimulates both the production of preneoplastic hepatic lesions and hepatocellular proliferation (Slagle *et al.* 1996). ICR × B6C3 male mice carrying an HBx transgene under the control of human alpha-1-antitrypsin regulatory elements (ATX) were bred with ICR female mice. F1 males (both transgenic and nontransgenic littermates) were used in this study. Transgenic mice dosed with DEN (2 µg/g body weight) developed a 2-fold increase in hepatocellular carcinomas and preneoplastic lesions compared to nontransgenic littermates (Table 4-3). In another study, double transgenic ATX mice, expressing HBx under the control of human alpha-1-antitrypsin inhibitor regulatory region and a bacteriophage lambda transgene used to monitor mutation frequency, were given a single intraperitoneal (i.p.) injection of DEN (2 µg/g) at 12 days of age. Nontransgenic controls were left untreated (Madden *et al.* 2001). Preneoplastic foci were twice as frequent in the DEN-treated transgenic mice as DEN-treated wild-type mice. Hepatocyte proliferation of the ATX mice was elevated 2-fold compared to nontransgenic mice, suggesting a promotional effect for HBx on liver tumor development.

Table 4-3. Incidence of preneoplastic lesions and hepatocellular carcinoma in DEN-treated ATX mice and nontransgenic littermates

Strain	Treatment	Transgene	Age examined (mo.)	Liver foci		HCC
				Incidence (%)[a]	No. foci/cm^3 (± SE)[b]	Incidence (%)[a]
(ICR×B6C3) × ICR	None	None	6–10	0/7 (0)	0	0/7 (0)
		ATX	6–10	0/13 (0)	0	0/13 (0)
(ICR×B6C3) × ICR	DEN	None	6–10	14/16 (88)	21.2 ± 4.0	4/16 (25)
		ATX	6–9	34/34 (100)	36.3 ± 3.7*	20/34 (59)*

Source: Slagle *et al.* 1996.

*$P < 0.05$ compared to nontransgenic DEN-treated mice.

[a]Number of mice with the lesion/number of mice examined.

[b]Mean/group.

HCC = hepatocellular carcinoma.

Ghebranious and Sell (1998) treated *p53* hemizygous knockout, HBsAg-expressing transgenic C57Bl/6 mice with AFB_1 and used appropriate control animals to assess the relative effects of sex, chemical carcinogenesis, and genetic status on liver tumor development. Appropriate animals received AFB_1 one time at one week of age. By 13 months of age, all seven male mice with the HBs transgene, hemizygous for *p53*, and treated with AFB_1 ($p53^{+/-}$, $HBsAg^+$, AFB^+) had hepatocellular carcinoma. When one of the risk factors was eliminated, the tumor incidence dropped. Male mice with intact *p53* genes, but with the other risk factors ($p53^{+/+}$, $HBsAg^+$, AFB^+) had hepatocellular carcinoma in 10 of 16 mice; mice that were not HBs transgenic but had the other risk factors ($p53^{+/-}$, $HBsAg^-$, AFB^+) had only one of seven mice with hepatocellular carcinoma. Male mice that had the other risk factors, but were not treated with AFB_1 ($p53^{+/-}$, $HBsAg^+$, AFB^-) had hepatocellular carcinoma in two of eight animals. Female mice responded in a similar fashion to male mice, but with a lower overall tumor incidence. *p53* allele loss was not detected in the hepatocellular carcinoma of any of the animals. Each of the risk factors studied was considered to have a potential role in hepatocellular carcinoma development.

4.1.3 Tree shrews

Tree shrews (*Tupaia sp.)* are the only species other than great apes and gibbons that are susceptible to infection with HBV (Yan *et al.* 1996a, Walter *et al.* 1996). These animals were once classified as primitive members of the primate group or as insectivores, but have recently been reclassified into their own order (Scandentia).

4.1.3.1 HBV infection

The ability of primary *Tupaia* hepatocytes to support HBV replication has been demonstrated. Kock *et al.* (2001) were able to infect primary *Tupaia* hepatocytes with HBV particles and woolly monkey HBV, but not woodchuck hepatitis virus. Infected hepatocytes synthesized covalently closed circular HBV DNA and single-stranded replicative intermediates. In another study, *Tupaia* hepatocytes, transduced by a

replication deficient adenovirus vector with the HBV genome *in vitro*, produced replication competent core particles, HBeAg, enveloped HBV virions, and the HBV replicative template (covalently closed circular DNA) (Ren and Nassal 2001). One species of tree shrew (*Tupaia belangeri chinensis*) has been infected with human HBV, and infection has been serially passed through five generations (Yan *et al.* 1996a). The initial infection rate exceeded 50%, and infection could be prevented with HBV vaccine. Most infections were self-limiting, but some animals were chronically infected. In one small study, persistent HBV infection in tree shrews was associated with an increased risk of hepatocellular carcinoma (Yan *et al.* 1996b), approximately 11% (1 of 9) of infected animals developing liver tumors in a 2-year study. In that study, some of the tree shrews had serum markers, immunohistochemically detectable viral antigen or liver HBV DNA indicative of chronic infection; but definitive determination of the proportion of chronically infected animals was not established. In a separate study, however, HBV-infected tree shrews did not develop hepatocellular carcinoma during a 160-week study (Li *et al.* 1999); however, the proportion of animals that developed chronic infections in this study was not clear.

4.1.3.2 Interaction with other carcinogens

More than 25 years ago, AFB_1 was shown to be carcinogenic in tree shrews (Reddy *et al.* 1976). A synergistic effect on hepatocellular carcinoma formation was reported in tree shrews infected with HBV and treated with AFB_1 (Yan *et al.* 1996b). In a 100-week study, 53% (9 of 17) of tree shrews infected with HBV and treated with AFB_1 (200 to 400 µg/kg per day for six days/week) developed hepatocellular neoplasms. Liver tumor incidence was 11% (1 of 9) in HBV-infected tree shrews and 13% (3 of 24) in animals treated with AFB_1. The livers from tree shrews that developed hepatocellular carcinoma also had typical preneoplastic lesions such as areas of dysplasia or enzyme-altered foci. Control animals did not develop hepatic neoplasms or histologic evidence of preneoplastic lesions.

Li *et al.* (1999) also demonstrated the combination of HBV infection and AFB_1 exposure and the resultant increase in liver cancer. During a 160-week study, 67% (14 of 21) of tree shrews treated with AFB_1 (150 µg/kg b.w. per day, six days/week for 105 weeks) and infected with HBV developed hepatocellular carcinoma. In animals treated with AFB_1 alone, 30% (3 of 10) developed tumors, but no tumors were observed in animals infected only with HBV or in control animals.

Park (2000a) mentions hepatocellular carcinoma in tree shrews infected with HBV alone or those also exposed to AFB_1, but details of the incidence of hepatocellular carcinoma were not provided.

4.2 Animal hepadnaviruses

4.2.1 Primates

Several species of primates have their own recently recognized indigenous distinct strains of hepadnaviruses (Table 4-1). HBV-infected chimpanzees were reported in zoos many years ago, but more recent sequence analysis of virus from those animals and from wild chimpanzees in Africa has revealed that chimpanzees have their own HBV that is similar

to, but genetically distinct from, human HBV genotypes (Hu *et al.* 2000). However, very little information is available on the pathologic characteristics of infection with naturally occurring HBV and human-derived HBV in chimpanzees. In addition, a captive gorilla was found to be infected with an HBV isolate that was distantly related to the chimpanzee strain of HBV (Grethe *et al.* 2000). Several strains of HBV have been isolated from gibbons, and at least one has been transmitted to a chimpanzee (Norder *et al.* 1996, Grethe *et al.* 2000). Some of the gibbons were believed to be infected with a human strain rather than an endogenous virus (Lanford *et al.* 2000). Another strain has been isolated from orangutans in their native habitat (Warren *et al.* 1999). The most divergent member of the primate HBV viruses, based on host range studies, has been isolated from woolly monkeys (Lanford *et al.* 1998). To date, no information is available on the histopathology, pathogenesis, or carcinogenicity of these viral strains.

4.2.2 Woodchucks

4.2.2.1 Natural infection with woodchuck hepatitis virus

The first animal hepadnavirus was discovered in woodchucks (*Marmota monax*). Tennant and Gerin (2001) recently published a review on this subject. The possibility of a viral hepatic infection in woodchucks surfaced in the late 1960s, following a report of a high incidence of spontaneous hepatocellular carcinoma in a population of wild woodchucks that were caught and kept in captivity at the Philadelphia Zoo. Subsequently, Summers (1978) conducted an investigation into the etiology of woodchuck hepatocellular carcinoma. In a series of 102 woodchuck necropsies collected over a period of 18 years at the Philadelphia Zoo, 23 animals had hepatocellular carcinomas (22.5%). The neoplasms appeared in animals at an approximate mean age of six years. Many of the wild-caught woodchucks had histologic evidence of hepatic inflammation, and approximately 15% of serum samples from the woodchucks had DNA-polymerase activity, typical of hepadnaviruses. Additional characterization of the virus particles revealed that they formed spheres and filaments and possessed physical characteristics similar to those of HBV. On the basis of these findings, the investigators determined that they had identified a novel virus and termed it woodchuck hepatitis virus (WHV).

The potent carcinogenic effect of WHV infection was initially established in a series of studies of wild-caught woodchucks. Summers *et al.* (1981) reported that all 16 woodchucks with hepatocellular carcinoma from the Philadelphia Zoo had persistent WHV infections and evidence of hepatitis, while no hepatocellular carcinomas developed in uninfected animals or in those that had cleared infection.

Popper *et al.* (1981) studied 33 wild-caught woodchucks trapped in the Pennsylvania and Delaware area and maintained in a colony at the National Institute of Allergy and Infectious Disease. The six animals with hepatocellular carcinoma all had markers of WHV infection, including DNA polymerase, woodchuck hepatitis virus surface antigen (WHsAg), and WHV DNA, in their serum. Four additional animals had all three of these markers, four had antibodies to WHsAg (anti-WHsAg), indicative of recovery from infection, three had no evidence of infection, and 16 had one marker or inconsistent results. Additional studies in this colony (Mitamura *et al.* 1982) found that death from hepatocellular carcinoma occurred in 11 of 13 (85%) chronic carriers of WHV. The study

also noted that woodchucks that recovered from WHV infection still had a risk of hepatocellular carcinoma, although the tumor incidence was significantly lower (2/33 animals, or 6%) than in chronically infected animals. None of the 16 uninfected woodchucks developed hepatocellular carcinoma.

The association between WHV infection and hepatocellular carcinoma in woodchucks was supported by a study conducted by Millman *et al.* (1984). Eight woodchucks from a colony of 113 animals trapped in Pennsylvania, Maryland, and Delaware developed hepatocellular carcinoma within 18 months of captivity, and seven of these were infected at the time of capture. The last animal, initially seronegative, seroconverted to WHsAg seropositivity after 33 weeks in captivity. There were no tumors in uninfected animals.

Hepatocellular carcinoma developed in 13 of 16 persistently WHV-infected (WHsAg positive) wild-caught woodchucks from Pennsylvania and Maryland that were kept at Cornell University for two years (Roth *et al.* 1985). Pulmonary metastasis occurred in one of these animals. Mild to moderately extensive hepatitis was found in all of the animals with hepatocellular carcinoma. Hepatocellular carcinoma in uninfected woodchucks is rare. Only one hepatocellular carcinoma was found in 149 WHsAg seronegative animals trapped in New York and kept in captivity for four weeks or more.

4.2.2.2 Experimental infection with woodchuck hepatitis virus

To study the effects of WHV infection in a controlled population of woodchucks, a breeding colony was established at Cornell University in 1979. Because the susceptibility to chronic infection versus acute self-limiting infection is much greater in neonatal woodchucks, as it is in human neonates, infections were induced in woodchuck pups in the first few days of life, using a standardized pool of infectious virus. Experimental infection of neonate woodchucks reared in captivity and free of possible cocarcinogenic influences that could promote tumor development helped to isolate the effects of WHV. Factors that could have affected wild-caught animals include naturally occurring dietary carcinogens such as AFB_1 and chronic infections with hepatic parasites such as *Ackertia* and *Capillaria* species (Cohn *et al.* 1986). Recently, *Helicobacter marmotae* has been discovered in both captive-born and wild-caught woodchucks (Fox *et al.* 2002). Since some strains of mice infected with *Helicobacter* species have an increased risk of liver cancer, the pathogenesis of this bacterium in woodchucks requires further investigation.

Long-term studies of WHV-carrier woodchucks that had been inoculated as neonates have repeatedly demonstrated the potent carcinogenic effects of WHV. In the first study of captive-born woodchucks, all eight chronic WHV-carriers developed hepatocellular carcinoma within three years of infection, but no tumors were found in 19 animals that had cleared their initial infection or in 15 uninfected control animals that were followed for up to 57 months (Popper *et al.* 1987) (Table 4-4).

In another study carried out by the researchers from Cornell, two groups of 43 woodchucks were injected with WHV at birth or eight weeks of age (Tennant *et al.* 1988). Thirteen of 43 woodchucks (32%) infected at birth became carriers, and hepatocellular carcinoma developed in 11 of the 13 WHV-carriers within three years. Three in the group of 43 that were injected at eight weeks of age were chronic carriers.

Two of these carrier animals were followed for three years, and both animals developed hepatocellular carcinoma. This study also demonstrated that animals that have recovered from infection still have a risk of hepatocellular carcinoma, albeit at a reduced level. Two of 28 animals at birth but that cleared their infection developed hepatocellular carcinoma by three years of age. None of eight recovered animals inoculated at eight weeks developed hepatocellular carcinoma within three years. No tumors were found in the 46 uninfected control animals (Table 4-4).

A detailed analysis of the hepatocellular carcinoma incidence in woodchucks from the Cornell breeding facility by Gerin *et al.* (1989) confirmed the hepatocarcinogenic potency of WHV infection. Almost all (61 of 63) chronic carriers developed hepatocellular carcinoma by 56 months. The median time to death from hepatocellular carcinoma in WHV carriers was 29 months. Of 63 neonatally infected animals that had recovered from their infection, 11 developed hepatocellular carcinoma (17%). Hepatocellular carcinoma was not observed in any of the 108 uninfected captive-reared woodchucks (Table 4-4). The incidence of hepatocellular carcinoma in the captive-reared, WHV-infected woodchucks and in wild-caught, WHV-infected woodchucks is similar (Popper *et al.* 1981, Popper *et al.* 1987, Snyder and Summers 1980, Tennant 1999).

Table 4-4. Incidence of hepatocellular carcinoma in experimentally WHV-infected captive-reared woodchucks

WHV status	Age examined (mo.)	Number examined	Tumor Incidence (%)	Reference
Chronic carrier	17–36	8	8 (100)[a]	Popper *et al.* 1987
Recovered	17–36	19	0 (0)	
Uninfected	17–36	15	0 (0)	
Chronic carrier: infected at birth	36	13	11 (85)[a]	Tennant *et al.* 1988
Recovered: infected at birth	36	28	2 (7)[a]	
Chronic carrier: infected at 8 wks.	36	2	2 (100)[a]	
Recovered: infected at 8 wks.	36	8	0 (0)	
Uninfected	36	46	0 (0)	
Chronic carrier	36	63	61 (97)***	Gerin *et al.* 1989
Recovered	36	63	11 (17)***	
Uninfected	36	108	0 (0)	

***$P < 0.001$ compared to uninfected controls

[a]P value not reported.

No gender-related difference exists in the risk of hepatocellular carcinoma in WHV carrier woodchucks, unlike the situation in humans in which a considerably greater risk exists for men than for women. A possible explanation proposed by Tennant (2001) is that the lack of testosterone production in woodchucks for most of the year may be an important factor. The testicles in woodchucks are descended and functional for only approximately four months of the year (Baldwin *et al.* 1985). Therefore, the effects of

testosterone are limited to only about one-third of the year in woodchucks versus continual testosterone production in human males.

Histologically, WHV-induced hepatocellular carcinoma in woodchucks resembles hepatocellular carcinoma in other species (Popper *et al.* 1981, Roth *et al.* 1985). The tumors tend to have a well-differentiated trabecular and pseudoglandular appearance. Moderate variation in nucleus size and cell size is typical. Nuclei are often hyperchromatic, and tend to be prominent. Infiltration of the tumor with hematopoietic elements is common. Areas of necrosis, sometimes large, are scattered throughout most tumors. Chronically infected animals typically have mild to moderate chronic hepatitis characterized by lymphocytic and plasmacytic infiltration in portal areas and occasionally within the hepatic parenchyma. Biliary hyperplasia is often seen. Fibrosis may occur. Enhanced inflammatory activity is noted with foci of necrosis at the margin of the neoplasm and the parenchyma. Aneuploidy is frequent in the hepatocellular carcinoma (Cullen *et al.* 1994, Mi *et al.* 1994). The biologic behavior of woodchuck hepatocellular carcinoma is less aggressive than the human counterpart, and metastasis is relatively uncommon (Roth *et al.* 1985).

Like the more standard laboratory rodents (i.e., mice and rats), woodchucks develop preneoplastic lesions, termed foci, of altered hepatocytes in their livers during the course of chronic infection. These foci were classified by Bannasch *et al.* (1995) as glycogen storage, fat storage, tigroid, amphophilic and basophilic, and mixed cell types. Similarities between the enzyme histochemical staining reactions of the rat, mouse, and woodchuck were noted by Abe (1988) who showed an increase in gamma glutamyl transpeptidase and a decrease in glucose-6 phosphatase in woodchuck liver foci. The enzyme histochemistry and electron microscopic appearance of woodchuck liver foci was characterized in more detail by several investigators (Bannasch *et al.* 1995, Toshkov *et al.* 1990, Radaeva *et al.* 2000).

4.2.2.3 Transgenic mice expressing WHV proteins

Several lines of transgenic mice that express WHV proteins or regulatory sequences have an increased risk of hepatocellular carcinoma. WHV/c-*myc* transgenic mice that express WHV regulatory sequences and a mutated c-*myc* have a high incidence of hepatocellular carcinoma that develops by 8 to 12 months of age (Etiemble *et al.* 1994). Another line that contains the woodchuck N-*myc2* gene under the control of WHV-regulatory sequences also has an increased tumor risk (Renard *et al.* 2000). Transgenic mice (C57Bl/6) that express WHx do not have a spontaneous increased incidence of hepatocellular carcinoma, but they do have an increased risk of preneoplastic lesions when treated with DEN compared to nontransgenic controls (Dandri *et al.* 1996).

4.2.2.4 Interaction of woodchuck hepatitis virus and aflatoxins

The first study of the interaction of AFB_1 and WHV was reported by Tennant *et al.* (1991). Fifty-two neonatal woodchucks were infected with WHV. Twenty-five of these animals and 27 uninoculated animals were given 0.25 to 1.0 µg AFB_1 per kg in their diet for six months from beginning at three months of age, or a similar total dose by i.p. injection of AFB_1 in dimethyl sulfoxide (125 µg/kg, three times/week) for three to four

months beginning at one to four months of age). Twenty-seven animals were WHV infected only and 23 were untreated controls. AFB_1 treatment did not affect the proportion of injected animals that became chronic carriers; 73% for AFB_1-treated animals and 70% for WHV-injected only. The dose of AFB_1 selected produced significant histologic evidence of hepatic injury, and the survival of AFB_1-treated animals was relatively low, interfering with interpretation of the results. At 36 months, only 15 WHV-infected, AFB_1-treated animals survived; six of these had hepatocellular carcinoma (40%) compared to 21 of 27 WHV-carrier animals (78%). Two of 21 AFB_1-treated animals and none of 22 surviving untreated control animals had hepatic tumors.

A synergistic interaction between AFB_1 and chronic WHV infection in woodchucks has been reported by Bannasch *et al.* (1995). WHV-carriers infected as neonates, and uninfected animals were dosed with 40 µg/kg of AFB_1 daily for four months starting at one year of age and then 20 µg/kg five days/week for life. Preneoplastic lesions and neoplasms developed in WHV-carrier animals. When hepatocellular adenomas and carcinomas were combined for statistical analysis, AFB_1-treated, WHV-carrier animals had a significantly earlier onset of tumor development than WHV-infected animals. AFB_1-treated, WHV-carrier woodchucks had higher incidence of liver tumors (75%) than animals that were only WHV-infected (33%), although this difference was not statistically significant ($P < 0.1$) due to the small number of animals in the study. However, after excluding animals that recovered from their WHV infections during the study (3 of 12 animals with AFB_1 treatment and 1 of 12 without), the incidence of hepatocellular carcinoma only was significantly different, as was the time of appearance for combined adenomas and carcinomas. AFB_1-treated animals developed preneoplastic foci, but no tumors; uninfected animals not exposed to AFB_1 did not develop any hepatic lesions.

4.2.2.5 Woodchucks infected with ground squirrel hepatitis virus

Seeger (1991) designed an experiment to determine if the observed differences in tumor incidence and onset were attributable to the features of the infecting viruses or the host animal's response to infection. Accordingly, woodchuck neonates were infected with either WHV or ground squirrel hepatitis virus (GSHV), and the subsequent tumor development was followed for two years. Seventeen of 29 (59%) GSHV-infected woodchucks became carriers, and 27 of 36 (75%) WHV-infected animals developed chronic infections. By the completion of the study, 13 of 16 WHV-infected woodchucks developed hepatic neoplasms, but only 1 of 16 GSHV-carriers had developed a small (5-mm) hepatic adenoma. The median time to tumors was 32 months for the WHV-infected animals and 55 months for those infected with GSHV. Based on this study, it was concluded that viral factors were more likely to be responsible for the greater oncogenic potency of WHV compared to GSHV. Attempts to infect ground squirrels with WHV have not been successful, so the reciprocal experiment cannot be conducted and host factors cannot be completely eliminated. However, experiments with chimeric WHV/GSHV viruses have been undertaken (Tennant 2001).

4.2.3 Ground squirrels

Following the discovery of WHV, a search for related viruses in other members of the Scuridae (squirrel) family revealed a novel virus infecting a population of Beechey ground squirrels in the area around Palo Alto, California (Marion *et al.* 1980). The new hepadnavirus, named ground squirrel hepatitis virus, produced chronic infection and was associated with liver tumor development. Chronic GSHV infection produces a milder hepatic inflammation in its host than WHV infection does in woodchucks (Cullen and Marion 1996). Other hepadnaviruses have been confirmed in arctic ground squirrels (see Section 1.2.3.2) and Richardson's ground squirrels (see Section 1.2.3.3). Although hepadnaviruses in Gray tree squirrels and Indian palm tree squirrels have been reported, they have not been confirmed by subsequent research (Feitelson *et al.* 1986, Mehrotra and Srivastava 1987).

4.2.3.1 Beechey ground squirrels

In a 9-year study of GSHV-induced hepatocarcinogenicity, the tumor incidence in chronically GSHV-infected ground squirrels was lower than that observed in WHV-infected woodchucks, and the age of tumor onset was higher (Marion *et al.* 1986, Sherker and Marion 1991, Marion and Cullen 1992). This series included 24 GSHV carriers, 20 animals that had recovered from past infection, and 26 uninfected ground squirrels more than four years of age. Hepatocellular carcinoma was found in 11 of the carriers, 5 of those who had recovered and 2 of the uninfected ground squirrels (Table 4-5). GSHV infection was significantly associated with development of hepatocellular carcinoma (P = 0.0016). The average age of animals with hepatocellular carcinoma was 6.5 years for GSHV-carrier ground squirrels and 6.8 years for animals that had recovered from GSHV infection. The average age of the two uninfected ground squirrels that developed hepatocellular carcinoma was 8.5 years of age and was close to the average life expectancy of 9.5 years. No relationship was apparent between sex and tumor incidence, similar to the findings in WHV-carrier woodchucks.

Table 4-5. Incidence of hepatocellular carcinoma in naturally GSHV-infected, captive-reared ground squirrels

GSHV status	Age examined (mo.)	Number examined	Tumor incidence (%)
Chronic carrier	108	24	11 (46)**
Past infection	108	20	5 (25)
Uninfected	108	26	2 (8)

Source: Marion and Cullen 1992.

**P < 0.01 compared to uninfected.

The histologic appearance of ground squirrel hepatocellular carcinoma is similar to that reported for woodchucks. Most neoplasms are trabecular with a well-differentiated appearance, although one anaplastic medullary carcinoma has been described (Marion *et al.* 1986).

4.2.3.2 Arctic ground squirrels

A search for a hepadnavirus in arctic ground squirrels (*Spermophilus parryi*) from Alaska was prompted by the observation of a high incidence of liver disease and hepatic neoplasms in these animals (Testut *et al.* 1996). The virus isolated has similarities to WHV and GSHV with approximately 16% base and 19% amino acid exchanges among the three viruses. Phylogenetic analysis indicates that the virus named arctic ground squirrel virus (ASHV) is more closely related to GSHV than to WHV. ASHV appears to be less potent than WHV as a hepatic carcinogen, based on the analysis of a small number of tumors. Preliminary studies identified hepatic masses in four of eight DNA-positive animals compared to 4 of 48 DNA-negative animals, suggesting that infection may play a role in tumor development. However, adenomas and carcinomas have been found in ASHV carrier and noncarrier animals, and no clear evidence is available that ASHV is oncogenic. Unlike WHV-infected woodchucks, only older arctic ground squirrels have developed liver tumors. Additional studies are needed to better characterize the pathogenesis of ASHV infection.

4.2.3.3 Richardson's ground squirrels

A high frequency of hepatocellular carcinoma noted in Richardson's ground squirrels (*Spermophilus richardsonii*) from Alberta, Canada, led to an investigation for the presence of hepadnavirus infection in these animals (Tennant *et al.* 1991). Of 12 animals examined, there was evidence of mild or moderate hepatic inflammation in seven individuals, and six animals had hepatocellular carcinoma. Serologic tests specific for GSHV surface antigen or anticore antibodies did not detect either virus-specific proteins or antibody responses by these animals. However, in six of ten animals, an appropriately sized, 3.2-kb DNA band that hybridized to a GSHV probe was extracted from nontumorous hepatocytes.DNA polymerase was not detected. Similar results were found in samples from two of five hepatocellular carcinomas. The inability to detect anti-GSHV core protein antibodies in animals with DNA evidence of infection suggests that the Richardson's ground squirrel virus is antigenically distinct from GSHV. Additional studies are needed to characterize the virus found in the squirrels and its pathogenesis.

4.2.4 Avian hepadnaviruses

Unlike the mammalian hepadnaviruses (Orthohepadnaviruses), no direct evidence exists that avian hepadnaviruses can cause hepatocellular carcinoma. Duck HBV (DHBV), first described in 1980 (Mason *et al.* 1980), is the best characterized of the avian hepadnaviruses (Avihepadnaviruses). Several related viruses have been isolated from wild birds, including snow geese (Chang *et al.* 1999), herons (Sprengel *et al.* 1988), and storks (Pult *et al.* 2001) (Table 4-1). Few data are available on the natural history of infection with these other avian hepadnaviruses, and hepatocellular carcinoma has not been reported in affected species. Consequently, this section will focus on DHBV.

Hepatocellular carcinoma in ducks is very uncommon (Rigdon 1972), with the exception of domestic Chinese ducks in the Qidong region of China (Omata *et al.* 1983, Marion *et al.* 1984). The role of DHBV infection in the pathogenesis of these naturally occurring duck hepatocellular carcinomas has been difficult to resolve because of the likelihood of dietary AFB_1 contamination of affected ducks.

Hepatocellular carcinomas in DHBV-infected ducks raised in controlled experimental conditions have been rare. Several studies have been conducted of congenitally infected or DHBV-injected ducks from laboratories around the world (Freiman and Cossart 1986, Lambert *et al.* 1991, Cova *et al.* 1990, Cullen *et al.* 1990, Omata *et al.* 1984), and only one hepatocellular carcinoma has been described (Cullen *et al.* 1989) (Table 4-6). Integration of DHBV into hepatocellular carcinoma DNA has been shown in a minority of cases (Yokosuka *et al.* 1985, Cullen *et al.* 1990, Cova *et al.* 1994). These carcinogenicity studies, however, were concluded after less than 2.5 years. Given the 10- to 12-year lifespan of domestic ducks, it is possible that DHBV infection, like GSHV infection, could lead to tumor formation at a later stage of the lifespan. However, long-term studies have not been published and such studies would be difficult given the propensity of ducks to develop fatal hepatic amyloidosis as they age.

Table 4-6. Incidence of hepatocellular carcinoma in experimentally or congenitally DHBV-infected ducks

DHBV status	Infection route	Age examined (mo.)	Number examined	Tumor incidence (%)
Chronic carrier	Experimental	4–21	15	1 (7)
Chronic carrier	congenital infection	4–21	8	0 (0)
Uninfected		4–21	14	0 (0)

Source: Cullen *et al.* 1989.

Several laboratory-conducted studies of the interaction of AFB_1 and chronic DHBV infections have been performed in Pekin ducks and were reviewed in the IARC (1994) monograph. The conclusion from these studies was that AFB_1 exposure was responsible for hepatocellular carcinoma formation in ducks and that DHBV infection did not contribute to an increased liver cancer risk. AFB_1 treatment produces characteristic liver lesions, including biliary proliferation, variably intense lymphoplasmacytic inflammation, fibrosis, hepatocellular necrosis, and regenerative nodule formation, in addition to hepatocellular carcinoma formation. The livers from ducks infected by injection with DHBV were characterized by minimal to mild lymphoplasmacytic infiltrates of the portal regions. Congenitally infected ducks did not have significant histologic lesions in the liver.

Numerous field studies of hepatocellular carcinoma in domestic ducks have been conducted in the Qidong region of China because of the high tumor incidence in that region. Dietary contamination with AFB_1 is likely to be a significant factor for tumor development. Ducks are quite sensitive to the carcinogenic effects of AFB_1 compared to other species, and small levels of exposure can lead to liver cancer (Carnaghan 1965). The incidence of human hepatocellular carcinoma and AFB_1 contamination of the diet is high in this region of China (Sun *et al.* 1986), and the ducks' diet contains a high proportion of corn, which can be a source of AFB_1. In two recent studies of domestic Chinese ducks collected from the Qidong region, DHBV infection was detected by PCR in four of eight (50%) (Duflot *et al.* 1995) and in 23 of 34 (68%) formalin-fixed

hepatocellular carcinomas (Cova *et al.* 1993). However, an association between tumor formation and DHBV infection cannot be clearly made since the overall rate of infection of ducks in the region was not determined, and the rate of infection in the tumor-bearing ducks is similar to that in the general population of ducks from this region in previous surveys (Marion *et al.* 1984, Omata *et al.* 1983). Livers from ducks with hepatocellular carcinoma had histological changes typical of those seen with experimental AFB_1 treatment, such as biliary proliferation, fibrosis, and cellular atypia. Such lesions are not specific for AFB_1, however, and other environmental carcinogens could produce similar lesions. None of these lesions has been observed in ducks with chronic DHBV infection and raised in controlled conditions (Cullen *et al.* 1990, Cova *et al.* 1990), suggesting that etiologic agents other than DHBV are likely to be responsible for liver cancer in ducks.

4.3 Summary

The 1994 IARC monograph on HBV concluded that there was *insufficient evidence* for the carcinogenicity of HBV in experimental animals. Although a variety of experimental animal models of hepadnavirus infection are available, great apes (chimpanzees, gorillas, and orangutans), lesser apes (gibbons), and tree shrews are the only animals that can reliably be infected with human HBV. In addition, transgenic mice may be used to bypass the lack of susceptibility of laboratory rodents to infection with HBV. This section reviewed the available data on infection of experimental animals with human HBV, including interactions with other carcinogens, and animal hepadnaviruses in primates, woodchucks, ground squirrels, and birds.

Chronic hepadnavirus infection poses a considerable risk for hepatocellular carcinoma in some of the mammalian animal models of HBV infection. However, despite many years of observation and significant numbers of animals infected, the risk of hepatocellular carcinoma does not appear to be increased in HBV-infected chimpanzees. Additional studies will be needed to clarify the potential for hepatocellular carcinoma development in tree shrews, a relatively new animal model of HBV infection.

Transgenic mice that express the entire HBV genome do not have an increased risk of hepatocellular carcinoma; however, some lines of transgenic mice that express high levels of HBs gene or the HBx gene do. The level of expression of these gene products may have an important influence on their ability to produce tumors.

Compelling evidence is available for the hepatic carcinogenicity of HBV to the mammalian animal models. The risk of hepatocellular carcinoma in WHV-infected woodchucks is very high, approaching 100%. GSHV-infected ground squirrels also have an elevated risk of hepatocellular carcinoma but with a longer latency period and a lower proportion of infected animals that develop tumors. More information is needed to characterize the pathogenesis of hepadnavirus infection with the arctic ground squirrel virus. Infection of Richardson’s ground squirrels and Palm tree squirrels also needs more investigation.

Although several new avian hepadnaviruses, in addition to DHBV, have been discovered, none has been shown thus far to produce hepatocellular carcinomas in infected hosts. However, chronic infections have been studied only for DHBV-infected birds, and

lifetime studies are needed to definitively establish the potential of chronic infection to produce hepatocellular carcinoma.

AFB_1 appears to have some effect on hepatocellular carcinoma development in woodchucks and transgenic mice, but similar experiments in ground squirrels have not been reported. Synergy between AFB_1 treatment and WHV-infection in woodchucks has been demonstrated by higher tumor incidences in chronically WHV-infected animals that received AFB_1. AFB_1 treatment contributes to hepatocellular carcinoma development in male transgenic HBs gene-expressing mice. AFB_1 is a potent hepatic carcinogen in ducks, and concurrent DHBV infection has not been shown to affect tumor development.

5 Genotoxicity

Studies on viral integration are discussed in Section 6. No other genotoxic studies were identified.

6 Other Relevant Data

The pathology of HBV infection was recently summarized by IARC (1994). The chronic form of HBV infection is associated with a significant risk of hepatocellular carcinoma. IARC reviewed data on the mechanisms and the role of HBV in the development of cancer (IARC 1994). It was concluded that although the data in hepadnavirus-infected woodchucks presented a unifying mechanism regarding the role of WHV in hepatocellular carcinoma, the data to explain a mechanism for human HBV-associated hepatocellular carcinoma were less clear. The data on human hepatocellular carcinoma come from three main areas of research: the contribution of viral integration, the expression of viral genes, and the identification of genetic changes in HBV-positive versus HBV-negative hepatocellular carcinoma. These findings are summarized in Sections 6.2 and 6.3.

This section summarizes the current knowledge of the mechanisms of HBV-associated carcinogenesis. Recent studies provide evidence that integration-mediated activation of growth-promoting genes may occur more commonly than previously thought, although different genes are activated in different tumors. Other studies in transgenic mice carrying all or parts of the HBV genome have led to the conclusion that, under certain conditions, continued expression of these proteins can contribute to cancer. Finally, technical advances that allow determination of genome-wide chromosome allele status have provided a more comprehensive picture of the genetic changes that occur during hepatocellular carcinoma, with some differences now emerging for HBV-positive versus HBV-negative tumors.

6.1 Pathogenesis of HBV

Inflammation of the liver caused by infection with HBV may involve an acute phase, a chronic phase, or both. Hepatocellular carcinoma usually evolves during the chronic phase of HBV infection and within the context of liver cirrhosis, but in some cases hepatocelluar carcinoma may develop in the absence of cirrhosis.

6.1.1 Acute hepatitis

As summarized by IARC (1994), liver pathology caused by acute infection with HBV is variable and cannot be distinguished from that of other hepatitis viruses. Although replication of HBV in hepatocytes is noncytopathic, the typical acute HBV infection results in several histological changes in the liver, including portal expansion with lymphoid hyperplasia, lobular inflammation with sinusoidal cell proliferation, and acidophilic necrosis (apoptosis). These changes are believed largely mediated by the host immune response because liver pathology is typically (1) very mild in the neonate and other immunocompromised individuals and (2) quite variable among individuals, suggesting the importance of the host immune response.

Recent studies from animal models have revealed specific details on the pathogenesis mediated by the host immune response. It is generally agreed that viral hepatitis is initiated by an antigen-specific antiviral cellular immune response. Experimental support of this idea has now been provided. Adoptive transfer of CD8-positive, MHC Class I-

restricted, anti-HBsAg cytotoxic T lymphocytes (CTLs) into recipient mice normally tolerant to their HBV transgene resulted in acute viral hepatitis, thus indicating a role for CTLs in mediating acute hepatitis (reviewed in Chisari 2000). These same CTLs are thought to be responsible for clearance of the virus from the liver, which may occur by a mechanism that is largely noncytopathic.

6.1.2 Chronic hepatitis

Chronic HBV, defined by circulating HBsAg of greater than six-months duration, is associated with a high risk of liver complications, including hepatocellular carcinoma (Beasley *et al.* 1981). While most adults with acute HBV infection develop immunity and are able to clear the virus, a subset of infected persons (5% to 10% of adults) are not able to do so (Ganem and Schneider 2001). Several possibilities have been offered to explain why some patients appear unable to resolve their acute HBV infection. These include the development of immune escape mutant viruses, differences in viral strains, a weakened immune response to the virus, and other undefined host determinants (Hollinger and Liang 2001).

It is generally accepted that the factors determining whether acute HBV infection progresses to the chronic phase are not entirely known, but that they include the status of the immune system at the time of initial infection (Hyams 1995, Seeger and Mason 2000). The risk of chronic infection is greatest when HBV infection is acquired in childhood; approximately 90% of infants infected before one year of age and 30% to 50% of children infected between one and four years of age develop chronic infection with HBV. In contrast, the risk of chronic infection in adults is 5% to 10% (Hollinger and Liang 2001). WHO (2000) estimated that the risk of death from HBV-related cirrhosis or liver cancer is approximately 25% when chronic infection was acquired during childhood. These observations are supported by data from the woodchuck hepatitis virus model, which shows that an initial robust immune response to WHV infection is more likely to result in resolution of acute hepatitis than is the case for animals showing a weaker immune response to initial infection (Nakamura *et al.* 2001).

During chronic HBV infection, the continued host immune response to virus-infected cells leads to cycles of cell death and regeneration that may ultimately progress to fibrosis of the liver (Hollinger and Liang 2001). Often, the normal architecture of the liver has been replaced by bands of fibrosis surrounding regenerative nodules of liver tissue, a condition known as cirrhosis. There is a strong correlation between cirrhosis and the development of hepatocellular carcinoma (Hollinger and Liang 2001). Cirrhosis related to chronic HBV infection is more likely to progress to hepatocellular carcinoma than cirrhosis from other causes, such as chronic HCV, alcoholic cirrhosis, or autoimmune hepatitis (IARC 1994). The molecular events involved in the progression of acute HBV to chronic HBV or chronic HBV to hepatocellular carcinoma are the subject of current investigation. However, the regenerative nodules that arise following cell death are considered precursors to hepatocellular carcinoma.

Risk factors for the development of cirrhosis in patients with chronic HBV infection have been assessed in prospective studies in Taiwan (Liaw *et al.* 1988, Yu *et al.* 1997c, Huo *et*

al. 2000) and Italy (Fattovich *et al.* 1991). An increased risk for development of cirrhosis with older age has been the most consistent finding in these studies. Observations related to persistence of disease, either elevated serum ALT or HBV DNA in serum, were significant factors in some but not all studies (Yu *et al.* 1997c, Liaw *et al.* 1998, Huo *et al.* 2000). Other factors that correlate with viral persistence remain undefined. The presence of HBeAg or its reappearance in serum also was associated with more frequent development of cirrhosis. The remaining risk factors, such as cigarette smoking, non-A blood types, low education level, male gender, diabetes mellitus, bridging hepatic necrosis, and hepatic decompensation, were each significant in only a single study.

6.2 Tumor viruses

Tumor-associated viruses have been studied extensively for nearly 100 years, and certain unifying hypotheses on their mechanism(s) of action have emerged (Chow 1993, Hoppe-Seyler and Butz 1999, Butel 2000). It is helpful to consider the general principles of infectious diseases that have been reaffirmed in these and other systems (Christen *et al.* 1999, Cohen 1999) as a backdrop for understanding the underlying molecular basis of HBV in hepatocellular carcinoma.

6.2.1 Principles of viral carcinogenesis

The tenets of tumor virology, gathered from studies of many tumor-associated viruses, include that tumor viruses (1) frequently establish chronic infections in their natural hosts, (2) are seldom complete carcinogens, (3) cause tumors in a subset of infected people, (4) experience a long latency period, (5) may act either directly or indirectly, and (6) frequently modulate growth-control pathways of the cell. Unlike chemical carcinogens, viral carcinogens such as HBV are infectious agents. The virus-host interaction introduces a variability that complicates attempts to establish a unifying mechanism of action in tumor formation.

6.2.2 Importance of virus-host interactions

Viral transformation should be thought of as an accidental outcome of the virus-cell interaction and thus will be influenced by host factors. For example, viruses that replicate well in their host will infect a large number of cells, increasing the likelihood that a critical gene will be mutated. Indeed, two recent studies established that increased HBV load is associated with increased risk of hepatocellular carcinoma (Evans *et al.* 1998, Ishikawa *et al.* 2001). Understanding the molecular mechanism of tumor-associated viruses such as HBV is further complicated by the fact that multiple risk factors are associated with hepatocellular carcinoma (e.g., chronic HBV and HCV, exposure to environmental carcinogens, genetic factors), and these risk factors are present to a variable degree in different geographic locations (see Section 2).

6.2.3 Multistep tumor formation

It is not possible to effectively measure the precise onset of tumors in a population of HBV chronic carriers. However, it seems likely that synergistic exposure to multiple risk factors would decrease latency periods to tumor development. It is accepted that liver cancer develops through multiple steps and arises from the accumulation of mutations

(IARC 1994). This multistep process is reflected in the long latency period of hepatocellular carcinoma formation (usually emerging after 30 years of chronic HBV infection), and the anticipated ability of hepatocellular carcinoma risk factors to act synergistically (Yeh *et al.* 1989). Other data that support the idea of multistep tumor formation include a shared viral integration pattern between some atypical hyperplastic nodules and hepatocellular carcinoma within the same liver (Tsuda *et al.* 1988). Although there are only limited data to support the notion of activated oncogenes in the genesis of hepatocellular carcinoma (Nishida *et al.* 1994, Abou-Elella *et al.* 1996), there is no evidence that this activation is linked to HBV integration. However, abundant data support the idea that tumor suppressor genes are frequently inactivated in hepatocellular carcinoma (Buendia 2000). The chromosome allele loss patterns differ in individual patients, indicating that multiple pathways may lead to hepatocellular carcinoma (Slagle *et al.* 1992, Buendia 1992). It is anticipated that HBV may act at different steps in this multistep process.

6.2.4 Role of host immune response

The role of chronic inflammation in the development of cancer is well established (Christen *et al.* 1999). The chronic inflammation caused by infectious agents such as HBV is thought to contribute to multistep carcinogenesis either by directly damaging the DNA through oxidative damage or by inducing compensatory proliferation; both of these mechanisms increase the chance of a permanent DNA mutation. It is generally agreed that the host immune response to HBV-infected cells contributes to the mechanism(s) by which chronic HBV leads to hepatocellular carcinoma (Chisari 2000). As previously summarized by IARC (1994), the loss of e-antigen by hepatocytes may favor the lysis of virus-infected cells, leading to cycles of cell death and regeneration that provide a cellular environment favoring DNA damage. Alternatively, e-negative virus may be less pathogenic for other reasons, and further studies are needed to understand the pathogenic implications of e-negative virus.

Current experimental evidence supports a contributing role of the host immune response in HBV-associated hepatocellular carcinoma. Transgenic mice that express nontoxic concentrations of all three forms of HBV surface antigen (preS1, preS2, and S) have been developed (Nakamoto *et al.* 1998). These mice are immunologically tolerant to the surface proteins. Adoptive transfer of HBV-specific CTLs leads to the development of acute and chronic immune-mediated liver cell injury that triggers the development of hepatocellular carcinoma (Nakamoto *et al.* 1998). While important differences exist between this model and chronic HBV pathogenesis in humans, including that the transgene provides an endless “incurable” source of antigen target for the CTLs, these studies emphasize the contribution of the immune response to carcinogenesis during chronic HBV in humans.

6.3 HBV-associated hepatocellular carcinoma: mechanism(s) of action

Human tumor-associated viruses display complex interactions with their hosts, and therefore it is not surprising that no single mode of transformation underlies these cancers. For HBV, a variety of mechanistic data support the idea that HBV may contribute differently in the etiologies of individual tumors. These data come from

studies on human tissue specimens and from animal models. The data are organized into the following areas: (1) integration of HBV DNA during chronic HBV infection, (2) potential oncogenic properties of HBV gene products, and (3) genetic alterations in HBV-positive and -negative human hepatocellular carcinoma. Together, these data support the idea that in the process of chronic HBV infection, virus-induced changes in cell growth control likely contribute to the development of hepatocellular carcinoma. In conjunction with the host immune response-induced liver cell regeneration, these subtle virus-induced changes may lead eventually to the development of hepatocellular carcinoma.

6.3.1 Integration of HBV sequences

6.3.1.1 Summary of IARC report

During the decades of chronic HBV infection, portions of the viral genome integrate randomly into the host chromosome (IARC 1994). HBV integration does not occur through a virus-encoded integrase enzyme; integrated sequences are usually fragmented and/or rearranged, and thus unable to serve as template for virus replication. It is not clear whether these rearrangements occurred at the time of integration or during subsequent evolution of the tumor. A highly preferred integration site within the cohesive overlap region was identified. A significant percentage of virus-host junctions result in carboxyl terminal truncation of the viral X gene, but the properties of proposed X-cell fusion proteins are not known. It was concluded that minor rearrangements of cellular DNA are present at the sites of viral integration. Although it is uncertain whether integration occurs during the acute viral infection, multiple integrations are found in chronically infected liver. Hepatocellular carcinomas containing a single viral integration are common in childhood tumors, but tumors in adults typically have a more complicated pattern of viral integration. The identification of viral DNA from HBsAg-negative patient tumors is controversial because of the low estimated copy number. Limited evidence exists for direct activation of cellular genes by HBV integration. Analysis of hepatocellular carcinomas containing single HBV inserts revealed only three instances in which viral integration occurred in cellular genes, leading to the conclusion that insertional mutagenesis by HBV occurs infrequently in hepatocarcinogenesis.

6.3.1.2 Integration of viral DNA

HBV integration occurs at random locations in the hepatocellular genome, and hepatocellular carcinomas arising in HBV-endemic areas usually contain from one to six viral integrations per tumor (Feitelson *et al.* 2002, Brechot *et al.* 2000). The identification of integrated sequences in chronically infected children and adults without hepatocellular carcinoma indicates that integration precedes the development of hepatocellular carcinoma (Brechot *et al.* 2000). Nearly 90% of hepatocellular carcinomas arising in HBV-endemic areas contain integrated viral sequences (Slagle *et al.* 1992, Buendia 1992, Brechot *et al.* 2000, Feitelson *et al.* 2002).

This mechanism was first described in studies of bursal lymphomas arising in chickens chronically infected with an avian leukosis virus (ALV), a retrovirus. ALV by itself is nontransforming. Transformation is facilitated by integration of the provirus adjacent to the c-*myc* gene of the host, leading to its inappropriate activation (Hayward *et al.* 1981,

Payne *et al.* 1982). Unlike retroviruses, integration of viral DNA does not appear to be part of the replication cycle of any hepadnavirus.

6.3.1.2.1 Mechanism of integration

HBV DNA is believed to integrate into chromosomal DNA through nonhomologous recombination. In the duck hepadnavirus model, linear DHBV DNA efficiently integrates into chromosomal DNA in cell culture (Yang and Summers 1995) and during natural infection of animals (Yang and Summers 1999). Relaxed circular viral DNA also is reported to serve as a template for integration (Gong *et al.* 1999). It has been suggested that the eukaryotic topoisomerase I enzyme can linearize circular hepadnaviral DNA, generating DNA ends that subsequently recombine with cellular DNA (Pourquier *et al.* 1999). The possible contribution of other cellular enzymes capable of linearizing circular HBV DNA has not been examined.

The cohesive overlap region frequently serves as one virus-cell junction in the integrated HBV, although the reasons for this finding are not clear. It may be that this is a consequence of a linear HBV template serving as the source of the integrated DNA. Alternatively, it is known that DNA with triple-helix-forming capacity enhances recombination with target DNA (Faruqi *et al.* 1996); and since portions of the HBV cohesive overlap region may transiently be in a triple-stranded state, this arrangement may favor recombination with cellular DNA. There is no sequence specificity at the integration site for either viral or cellular DNA.

An increased frequency of hepadnaviral integration has been observed in the presence of oxidative DNA damage and/or inhibition of DNA repair for both DHBV (Petersen *et al.* 1997) and HBV (Dandri *et al.* 2002). These data predict that the immune response to HBV-infected hepatocytes, as occurs during chronic HBV infection, may create a cellular environment that further enhances viral integration.

6.3.1.2.2 Contribution of integration to hepatocellular carcinoma

The determination of whether a given viral insert contributed to the development of a hepatocellular carcinoma is time consuming and problematic. It is recognized that the analysis of viral inserts from end-stage tumors may provide limited insight into molecular events that occurred 30 years previously. In addition, the insertion of a viral *cis*-acting element may act over a significant distance in the cellular genome (Fourel *et al.* 1994), and consequently, important clues on *cis*-activation of specific cellular genes may not be apparent. Most hepatocellular carcinomas contain multiple inserts, and therefore, each insert (and its flanking cellular DNA) must be carefully analyzed to distinguish viral inserts that contributed mechanistically from those that are simply a marker for the clonal nature of the tumor. A strategy to analyze only tumors that contain a single HBV insertion (Dejean *et al.* 1986, Wang *et al.* 1990, Gozuacik *et al.* 2001) resulted in the analysis of a subset of tumors that often lack cirrhosis; therefore, the molecular pathways revealed in those studies may not extend to the majority of hepatocellular carcinomas, which arise on a background of cirrhosis.

The insertion of viral DNA into the chromosome is proposed to contribute to the development of hepatocellular carcinoma by inducing genetic alterations in the cell.

These alterations would occur by at least three different mechanisms: (1) *cis*-activation of growth promoting genes, (2) inactivation of growth suppressor genes, and (3) virus-mediated genetic instability. In recent years, molecular evidence has been obtained in support of each of these possibilities.

6.3.1.3 Cis-activation of cellular genes by hepadnaviruses

The woodchuck provides a useful model for understanding HBV pathogenesis (Roggendorf and Tolle 1995). At least half of woodchuck hepatocellular carcinomas contain WHV inserts that *cis*-activate the expression of c-*myc* or N-*myc2* proto-oncogenes (Fourel *et al.* 1990, Wei *et al.* 1992, Brechot *et al.* 2000). There is some specificity to WHV insertional activation of *myc* gene family members. GSHV also can cause hepatocellular carcinoma in woodchucks (Seeger *et al.* 1991), but one study found that only 1 of 16 (6%) of GSHV-induced woodchuck hepatocellular carcinomas had rearrangements at the N-*myc* locus (Hansen *et al.* 1993).

The contribution of those WHV/*myc* integrants to hepatocellular carcinoma has now been well established in follow-up studies. Although two WHV enhancers were identified (Flajolet *et al.* 1998), it is enhancer II that is important in the activation of expression of the *myc* family of genes (Wei *et al.* 1998). Expression of a WHV N-*myc1* antisense vector in a highly malignant woodchuck hepatoma cell line reversed the phenotype of those cells, demonstrating that continued N-*myc* expression is required to maintain the malignant phenotype (Wang *et al.* 1998a). In another study, expression of the N-*myc2* gene controlled by WHV regulatory sequences in transgenic mice led to adenomas and hepatocellular carcinomas in more than 70% of the animals; further, introduction of a *p53* null allele into those mice greatly accelerated the onset of hepatocellular carcinoma (Renard *et al.* 2000). This study also revealed molecular changes in common with those changes often detected in human hepatocellular carcinoma. Mutations in the β-catenin gene were found in 25% of the N-*myc2*-induced hepatocellular carcinomas, and most tumors that developed on a *p53*+/- background contained either a mutation or a deletion in the remaining normal *p53* allele. Together, these data provide direct evidence that WHV activation of N-*myc2* and reduction in *p53* act synergistically in liver cell transformation.

6.3.1.3.1 Enhancer-mediated cis-activation

Similar analyses of HBV-positive hepatocellular carcinoma did not yield a common insertional activation site in the human genome. Activated oncogenes have been documented in a limited number of studies. One report found that 50% of 20 hepatocellular carcinomas analyzed by differential PCR demonstrated c-*myc* amplification (Abou-Elella *et al.* 1996). In a separate study of 45 hepatocellular carcinomas, 5 (11%) revealed amplification of the cyclin D1 gene (Nishida *et al.* 1994). It is not clear whether HBV was responsible for the elevated oncogene expression in those studies. Therefore, at present there are no data to support a unifying theme of oncogene activation by HBV in human hepatocellular carcinoma as has been observed for WHV in the woodchuck model. One complication in the interpretation of such data is that viral enhancers can function from quite large distances. A study of WHV insertional activation suggested the action of those viral enhancers over a 150-kb to 180-kb

intervening distance (Fourel *et al.* 1994). In addition, viral insert mapping data indicated that HBV inserts map to many different chromosomes (Slagle *et al.* 1992); therefore, if enhancer-mediated activation of cellular genes occurs in HBV-positive hepatocellular carcinoma, there may be multiple cellular targets through which HBV will act.

6.3.1.3.2 HBV integration into growth-regulatory genes

In the absence of *cis*-activation of cellular genes by HBV enhancers, investigators have sought evidence that critical cell regulatory genes might be directly altered by viral integration into a cellular gene. Previous studies of human hepatocellular carcinoma identified three instances of such integration-mediated mutagenesis of a gene, and it was concluded that this mechanism was not a common feature of HBV-positive hepatocellular carcinoma (Slagle *et al.* 1992, Buendia 1992, Ganem and Schneider 2001, IARC 1994, Feitelson *et al.* 2002).

Table 6-1. Cellular targets of HBV integration in hepatocellular carcinoma

Gene[a] (reference)	Normal function of gene	Effect of HBV integration
ErbB-like (Zhang *et al.* 1992)	Growth factor receptor	Unknown[b]
Cyclin A2 (Wang *et al.* 1990)	S, G2/M check-points of cell cycle	Stabilized (elevated) cyclin A (Wang *et al.* 1992)
Retinoic acid receptor β (Dejean *et al.* 1986)	Nuclear hormone receptor	Virus-cell hybrid protein transforms cells (Garcia *et al.* 1993)
Hst-1 (Hatada *et al.* 1988)	Transforms NIH3T3 cells	Unknown[b]
Carboxypeptidase N-like (Pineau *et al.* 1996)	High expression in liver; often deleted in hepatocellular carcinoma	Chimeric transcript with unknown function (Pineau *et al.* 1996)
SERCA1 (Chami *et al.* 2000)	ER calcium regulator; role in cell growth	Virus-cell hybrid protein causes apoptosis (Chami *et al.* 2000)
Thyroid hormone receptor-associated protein (TRAP 150 α) (Gozuacik *et al.* 2001)	Coactivators of nuclear receptors; role in apoptosis	Unknown[b]
Telomerase reverse transcription (TERP) (Gozuacik *et al.* 2001)	Cell immortalization	Overexpressed hTERT (Gozuacik *et al.* 2001)
Minichromosome maintenance protein (MCM)-related gene (Gozuacik *et al.* 2001)	DNA replication	Truncated MCM due to premature stop codon (Gozuacik *et al.* 2001)
Unnamed EST (FR7) (Gozuacik *et al.* 2001)	Unknown function; expressed in human liver and cancer tissues	Chimeric transcript
Nuclear matrix protein p84 gene (Gozuacik *et al.* 2001)	Binds retinoblastoma protein; role in cell cycle	Unknown[b]

[a]Gene, identification of cellular gene containing HBV insertion.
[b]Unknown, effect of integration event on cellular gene expression has not been demonstrated.

It now appears that HBV integration-mediated insertional mutagenesis occurs more frequently than previously thought. A recently developed Alu PCR-based assay that amplifies virus-cell junctions was used to identify 21 virus-cell junctions from 18 hepatocellular carcinoma DNAs. HBV DNA was integrated into different cellular genes

in six (30%) of the 18 independent tumors (Gozuacik *et al.* 2001). Five of the six involved cellular genes were from gene families that normally function in cell proliferation and/or cell viability. Integration-mediated altered expression of those genes was demonstrated in the original hepatocellular carcinoma for four of the six genes, resulting in a total of 11 growth regulatory genes that have been interrupted by viral insertion events (Table 6-1). It is suggested that the improved DNA sequence databases (from the Human Genome Project) may have facilitated the identification of coding sequences in the flanking cellular DNA that had been missed in previous studies. Additional studies are needed to determine whether specific cellular pathways are consistently usurped during the development of hepatocellular carcinoma.

6.3.1.3.3 Viral genes expressed from the integrated template

Although only portions of the HBV genome are retained in the viral inserts, it has been demonstrated that these gene products (truncated preS2, HBx, and truncated HBx; see Section 6.3.3) may retain transactivation function that could contribute to multistep hepatocellular carcinoma, depending on the cellular target of that transactivation. A survey of 26 hepatocellular carcinoma tissues and/or cell lines revealed that 21 (81%) contained coding sequences for either HBx or truncated preS2 transactivator proteins, indicating that HBV-positive hepatocellular carcinomas commonly contain these types of viral sequences. Thus, a high probability exists that viral transactivators are continually expressed from the integrated viral sequences in such tumors and that these retain the potential to contribute to multistep transformation, depending on the specific cellular gene that is transactivated.

Some studies have suggested that a truncated surface protein may be oncogenic (Hildt and Hofschneider 1998, Kekulé *et al.* 1990), raising the possibility that mutagenesis within integrated viral DNA in normal hepatocytes could contribute to their oncogenic transformation. This finding may be mechanistically related to the observation that the large surface protein, L, can accumulate in the ER and can induce an ER-mediated stress response (Xu *et al.* 1997). This event leads to the transcriptional activation of the small surface protein, which, in combination with the large surface protein, forms mixed secretory particles and consequential alteration of the physiology of host cells.

The newly developed Microarray assays may aid in the identification of cellular targets of these transactivators. Two studies have been performed to identify HBx-induced genes. In the first, the gene expression profile of HepG2 cells was compared to a stable HepG2 clone expressing HBx using a chip containing 588 cellular cDNAs (Han *et al.* 2000). HBx expression was associated with the upregulation of nine genes (including *p21*), and the downregulation of three others. The second study expressed HBx via adenoviral vector in primary human adult hepatocytes and SK-Hep-1 hepatoma cells (Wu *et al.* 2001); this study utilized a chip carrying 2,208 human gene cDNAs and found 23 genes up-regulated and 15 down-regulated (including *p21*) in response to HBx expression. These studies identified different expression profiles, likely a result of differences in levels of HBx, cell type, and microchip utilized. Further research is needed to clarify the exact cellular targets of HBx transactivation under carefully controlled experimental conditions.

6.3.1.4 Tumor suppressor gene loss in hepatocellular carcinoma

Tumorigenesis proceeds through the accumulation of mutations in genes that control cell proliferation and/or death. The very limited information on activated oncogenes in human hepatocellular carcinoma (Nishida *et al.* 1994, Abou-Elella *et al.* 1996) has not been linked mechanistically to HBV integration. The results suggest that the loss of negative growth regulatory pathways (i.e., tumor suppressor genes) may be a more common mechanism for the development of hepatocellular carcinoma.

Tumor-specific loss of heterozygosity (LOH) is thought to represent genetic loci harboring tumor suppressor genes whose loss contributes to carcinogenesis. Extensive evidence is available for nonrandom LOH in hepatocellular carcinoma (Slagle *et al.* 1992, Buendia 1992, Feitelson *et al.* 2002, Buendia 2000). Earlier studies were limited by small sample population size and by the labor-intensive nature of restriction fragment length polymorphism (RFLP) analyses. In recent years, the development of microsatellite analysis (MSA) and the molecular cytogenetic method called comparative genomic hybridization (CGH) has allowed a more comprehensive genome-wide analysis that requires a relatively small amount of matched normal and tumor DNA. One study of 44 hepatocellular carcinomas using 216 genome-wide microsatellite markers revealed high rates (> 30%) of LOH on 11 chromosome arms (4q, 6q, 8p, 8q, 9p, 9q, 13q, 16p, 16q, 17p, 19p) (Okabe *et al.* 2000). A second study of 120 hepatocellular carcinomas using 195 genome-wide satellite markers, revealed LOH on seven chromosomal arms (1p, 4q, 6q, 8p, 9p, 16q, 17p) (Buendia 2000). A CGH analysis of 44 hepatocellular carcinomas identified LOH on 1q, 4q, 8p, 8q, 16q, and 17p (Lin *et al.* 1999). LOH on certain chromosomal loci (4q, 8p, 16q, 17p) were identified in common in all studies, while other loci were not confirmed in the different tumor populations.

6.3.1.4.1 Evidence for HBV role in chromosome allele loss

Currently, data are available to support the idea that HBV may contribute, in part, to the high rate of chromosome allele loss in hepatocellular carcinoma. In a survey of Japanese hepatocellular carcinomas, 13q and 16q LOH occurred significantly more frequently in HBV-positive hepatocellular carcinomas, and the LOH on 6q occurred significantly more frequently in HBV-negative tumors (Okabe *et al.* 2000). A second large study of tumors from Europe and China did not identify a specific correlation of LOH with viral infection (HBV or HCV), but noted an overall increased frequency of allelic loss in HBV-positive hepatocellular carcinomas (Buendia 2000). The latter observation was confirmed in a CGH analysis of 44 tumors from Taiwan (Lin *et al.* 1999). A third study concluded that LOH occurs more frequently in HBV-positive hepatocellular carcinomas than in hepatocellular carcinomas associated with HCV (Zondervan *et al.* 2000), a finding consistent with the observation that HCV is not believed to integrate into chromosomal DNA during hepatocarcinogenesis. The mechanism by which HBV integration might contribute to chromosome allele loss is not known, but existing data support several possible mechanisms.

6.3.1.4.2 HBV-mediated genomic instability

HBV integration is frequently accompanied by gross chromosomal rearrangements at the site of viral integration, including large deletions, duplications, and translocations (Slagle *et al.* 1992, Buendia 1992, Brechot *et al.* 2000). It can be argued that these gross

chromosomal changes may have evolved subsequent to the viral integration event, but a more recent study demonstrated that deletions in flanking cellular DNA can be detected very soon following the integration of hepadnaviruses in cell culture (Gong *et al.* 1996).

Attempts to correlate viral integration sites cloned from hepatocellular carcinomas with specific chromosome LOH are limited by the relatively small number of viral inserts mapped to date (fewer than 30). It is intriguing that a higher than expected proportion of these inserts map to chromosome 17p (Slagle *et al.* 1992, Brechot *et al.* 2000), one of the chromosome arms that frequently undergoes LOH (Okabe *et al.* 2000, Buendia 2000, Lin *et al.* 1999).

Further, viral integration sites often are the sites of chromosomal translocations, and it has been estimated that these types of gross changes occur in approximately 25% of viral inserts (Slagle *et al.* 1992). It is clear that in those instances, the alleles lost during the translocation process will contribute to carcinogenesis if they harbored a tumor suppressor gene. Finally, limited evidence has shown that HBV sequences may generate a general instability of chromosomal DNA. Peripheral blood cells of chronic HBV carriers have a higher incidence of chromosomal breaks than blood cells of uninfected persons (Simon *et al.* 1991). HBV sequences from the direct repeat/cohesive overlap region enhances *in vitro* recombination events (Hino *et al.* 1991).

6.3.1.4.3 Functional inactivation by HBx

For many tumor-associated viruses (e.g., SV40, HPV-16, adenovirus), the strategies used to promote virus replication also inadvertently trigger changes leading to cancer (Op De Beeck and Caillet-Fauquet 1997). Tumor suppressor genes *p53* and retinoblastoma (*Rb*) are common targets of these tumor-associated viruses (Op De Beeck and Caillet-Fauquet 1997). One critical function (of many) for *p53* is its role as a G1 checkpoint protein, and this function is abrogated by its binding to SV40 T antigen (Linzer and Levine 1979), adenovirus 5 (Ad5) 55-kDa E1B (Sarnow *et al.* 1982), and human papilloma virus-16 (HPV-16) E6 (Demers *et al.* 1994).

The direct interaction of HBx with *p53* remains controversial. There are reports that HBx can bind *p53* (Feitelson *et al.* 1993), inhibit *p53* sequence-specific DNA binding (Wang *et al.* 1994), repress *p53* promoter activity (Lee and Rho 2000), inhibit *p53* transactivation (Lin *et al.* 1997), and relieve *p53* repression of HBV promoters (Lee *et al.* 1995a, Takada *et al.* 1996). A report that HBx causes functional inactivation of *p53* was based on colocalization of HBx and *p53* in one mouse liver tumor, but the report did not examine the effect of HBx on *p53* function (Ueda *et al.* 1995). Others have been unable to reproduce a direct interaction between HBx and *p53* (Su *et al.* 2000). Furthermore, unlike the interaction of *p53* with SV40 T antigen and HPV-16 E6, there is no evidence that HBx alters the steady-state level of *p53*. While the data for a direct interaction of HBx with *p53* remain inconclusive, it is possible that HBx exerts its effect(s) in hepatocytes through a *p53* pathway even if HBx does not directly bind to *p53*.

Inactivation of $p16^{INK4A}$ in the *Rb* pathway of cell cycle control occurs commonly in hepatocellular carcinoma. An analysis of hepatocellular carcinomas identified p16 inactivation (by a variety of mechanisms) in 34 (70.8%) of 48 hepatocellular carcinomas.

The precise role of HBV in that inactivation is not clear. The ability of HBx to directly modify the expression of p*Rb* has been examined. There is a single report that HBx is able to relieve *Rb*-mediated inhibition of the growth-promoting E2F1 protein (Choi *et al.* 2001). However, attempts to demonstrate a direct interaction between HBx and p*Rb in vitro* were unsuccessful (Farshid *et al.* 1997).

6.3.1.4.4 Evidence for HBV integration hit-and-run mechanism

The presence of highly rearranged viral and cellular DNA sequences in viral inserts cloned from hepatocellular carcinomas raises the possibility that the inserts may be dynamic within the evolving tumor. Indeed, one study demonstrated that the presence of integrated HBV increased the recombination frequency of flanking cellular DNA (Hino *et al.* 1991). More recently, a single-cell cloning approach was used to track viral inserts in chicken LMH-D2 cells that replicate DHBV (Gong *et al.* 1996). Three lines were followed, and analysis of integration sites by Southern blot hybridization revealed a variable frequency of single and multiple new viral integrations, as expected for cells that constitutively express DHBV. However, integrations also were lost upon passage of cells, and the loss was accompanied by loss of flanking cellular DNA (Gong *et al.* 1996). Significant differences are apparent between this model and HBV, including that LMH-D2 cells are actively dividing while hepatocytes are generally nondividing; thus, the acquisition/loss of viral integrants is likely to occur at a lower frequency in nondividing hepatocytes.

This result provides direct evidence for a hit-and-run mechanism by hepadnaviruses and illustrates the limitations of studying viral inserts cloned from hepatocellular carcinomas. It is likely that the conclusions drawn from the analysis of hepatocellular carcinoma viral inserts do not provide a complete story for the role of HBV integration in hepatocellular carcinoma.

6.4 Potential oncogenic properties of HBV gene products

6.4.1 Summary of IARC report

Seven viral proteins are encoded in four ORFs of the HBV genome (IARC 1994). Experimental evidence indicates that expression of three of these proteins may contribute to oncogenesis. When overexpressed in hepatocytes, the large Surface antigen (preS1) can be directly cytotoxic to hepatocytes and initiate events that ultimately progress to the development of hepatocellular carcinoma. A novel truncated form of the middle Surface antigen ($preS2^t$) possesses a new ability to transcriptionally activate cellular promoters. The HBx protein is expressed during chronic HBV infection, and high levels of HBx may induce transformation in 3T3 cells that are already immortalized by the SV40 large tumor antigen. Studies on HBx transgenic mice, however, have yielded conflicting results with some lines developing hepatocellular carcinoma while others do not. Viral integration frequently results in the truncation of the X gene and/or fusion with cellular genes, and the fusion genes may possess enhanced transactivation function.

6.4.2 Background

It is generally accepted that HBV does not encode an oncogene (Ganem and Schneider 2001). Furthermore, as hepatocellular carcinoma typically develops following decades of

chronic infection, it is anticipated that any contribution of viral proteins to the carcinogenesis process will be subtle. Finally, in keeping with the multistep nature of hepatocellular carcinoma development, the contribution of these proteins could occur at different stages during development of the cancer.

6.4.3 HBsAg

During natural HBV infection, and in HBV transgenic mice that replicate the virus, the levels of the three surface antigen proteins are tightly regulated and do not result in liver pathology (IARC 1994). However, the consequence of deregulated surface antigen expression is dramatic (IARC 1994), and the transgenic mice that mimic this condition by overexpression and accumulation of preS1 are considered a relevant model for certain aspects of human liver disease, albeit in accelerated form. In transgenic mice, the overloading of the liver with preS1 results in permanent inflammation and oxidative radical production with resultant DNA damage (Hagen *et al.* 1994). The mice develop regenerative nodules and hepatocellular carcinoma by 12 months of age and are more susceptible to carcinogens (Sell *et al.* 1991). It has been reported that a small portion of total preS1 also may have transactivator function (Hildt *et al.* 1996), and this finding also may contribute to the pathology of these transgenic mice.

Viral integration that results in a truncation of preS2 between nucleotide 221 and 573 leads to a novel protein with transactivator activity (IARC 1994). The potential role for the truncated preS2 in hepatocellular carcinoma has now gained support from studies in cell culture and in transgenic mice. A cloned HBV insert bearing X and truncated preS2 sequences was able to transform murine fetal hepatocyte cells (Luber *et al.* 1996). In transgenic mice, expression of the 3'-truncated preS2 specifically led to the activation of c-raf-1/Erk2 signaling, increased hepatocyte proliferation and, in older animals, an increase in hepatocellular carcinoma (Hildt *et al.* 2002). The latter study also demonstrated that the truncated preS2 was phosphorylated and that a mutation that abolished the phosphorylation site resulted in loss of activator function (Hildt *et al.* 2002). Deletions in the 3' end of preS2 were found in approximately 30% of integrated HBV investigated (Schlüter *et al.* 1994), indicating that this rearrangement of HBV genes occurs commonly during the evolution of individual hepatocellular carcinomas. No studies have been performed using antisense preS2 to confirm the role of its expression in the malignant phenotype.

6.4.4 HBx

All mammalian hepadnaviruses encode the 17-kDa HBx protein, which is required for virus replication *in vivo* (Chen *et al.* 1993, Zoulim *et al.* 1994). The precise role(s) provided by HBx in virus replication has not been defined. An abundance of data demonstrates that HBx can weakly activate viral and cellular promoters (Yen 1996, Rossner 1992), as well as signal transduction pathways (Benn and Schneider 1994, Cross *et al.* 1993, Natoli *et al.* 1994, Wang *et al.* 1998b, Klein and Schneider 1997, Benn *et al.* 1996, Su and Schneider 1997). These results suggest that HBx may promote cellular growth under certain conditions. Since HBx is the sole regulatory protein encoded by HBV, many studies also have examined its potential role in hepatocellular carcinoma.

6.4.4.1 HBx cofactor role in transgenic mice

HBV *X* transgenic mice have been developed in several laboratories, with HBx expression driven by viral and cellular promoters and on different genetic backgrounds. Most HBx transgenic lines do not develop any altered liver pathology during the lifespan of the animal (Lee *et al.* 1990, Billet *et al.* 1995, Dass *et al.* 1999, Guidotti *et al.* 1995, Perfumo *et al.* 1992, Reifenberg *et al.* 1997, Dandri *et al.* 1996). It is assumed that the ability of HBx to cause hepatocellular carcinoma in one line of transgenic mice is associated with a high background incidence of spontaneous hepatocellular carcinoma in that mouse colony (Kim *et al.* 1991). However, other variables may explain the differing pathologies of the HBx mice, including the level and duration of HBx expression and sequence variation among the transgenes. These variables have not been examined.

While the majority of HBx transgenic mice do not reveal liver pathology, several independent studies have now demonstrated a hepatocellular carcinoma cofactor role for the HBx protein. HBx mice are more sensitive than their nontransgenic littermates to carcinogens (Slagle *et al.* 1996, Dandri *et al.* 1996, Madden *et al.* 2001, 2002). HBx expression in mouse livers also potentiates the effect of activated c-*myc* (Terradillos *et al.* 1997). Since HBx is expressed continuously during chronic infection (Dandri *et al.* 1996), these animal studies suggest a potential for synergism of chronic HBV with environmental carcinogens in human hepatocellular carcinoma.

6.4.4.2 Inhibition of DNA repair

Several studies have focused on the identification of cellular proteins that interact with HBx as a means of understanding the function of HBx. Thirteen such proteins have been identified to date (Ganem and Schneider 2001). One interacting protein that has been identified in several independent laboratories is the UV-DDB protein (Lee *et al.* 1995b, Sitterlin *et al.* 1997, Becker *et al.* 1998), which is involved in nucleotide excision repair (Hwang and Chu 1993). Butel *et al.* (1996) hypothesized that this interaction might alter the ability of cells to repair damaged DNA If proven, this putative interaction could provide an explanation for the HBx cofactor role in carcinogenesis studies (Slagle *et al.* 1996, Dandri *et al.* 1996). Several studies utilized functional DNA repair assays in liver cell lines (Becker *et al.* 1998, Sitterlin *et al.* 1997, Jia *et al.* 1999, Groisman *et al.* 1999) and in primary mouse hepatocytes (Prost *et al.* 1998, Madden *et al.* 2000) to demonstrate that cells expressing HBx were unable to efficiently repair DNA damage. A panel of HBx mutants was used to demonstrate that the HBx inhibition of damaged DNA repair appears to depend on its ability to interact with UV-DDB (Becker *et al.* 1998).

A double transgenic mouse model was developed to address the possibility that HBx inhibits DNA repair *in vivo* (Madden *et al.* 2000). It was predicted that this inhibition would result in the increased DNA mutations that led eventually to hepatocellular carcinoma. Analysis of these mice revealed that in the absence of any treatment, HBx expression did not alter the frequency of spontaneous liver DNA mutation (Madden *et al.* 2000). However, in the presence of added liver carcinogens, researchers did observe a modest effect of HBx on DNA mutation frequency (Madden *et al.* 2001, 2002); this effect was similar to that reported for well-known tumor promoters (Miller *et al.* 2000). An altered DNA mutation spectrum was noted for HBx mice in two independent

carcinogen studies (Madden *et al.* 2001, 2002), indicating the hepatocytes expressing HBx respond differently to carcinogens, although the molecular basis of this difference is not understood. These results have implications for a similar outcome in chronic HBV carriers who also are exposed to environmental carcinogens (see Section 6.4.2).

6.4.4.3 Effects on the cell cycle

Many tumor-associated viruses encode nonstructural proteins that induce cell cycle progression as part of the viral strategy to enhance virus replication (Op De Beeck and Caillet-Fauquet 1997). It is precisely this ability to deregulate cell-cycle control that is intimately linked to the mechanism by which most tumor-associated viruses are believed to transform virus-infected cells (Op De Beeck and Caillet-Fauquet 1997). Because HBV replicates in nondividing hepatocytes, it is likely that HBx may provide a similar function in the HBV life cycle. However, this function for HBx has been difficult to establish since most experiments on HBx function are performed in actively dividing cells. When cells are released from quiescence (G0 or G1) by the addition of serum, HBx accelerates their progression through the cell cycle. This cell cycle progression includes pushing cells into S phase under some experimental conditions (Koike *et al.* 1994, Benn and Schneider 1994, 1995). In the absence of serum, HBx causes quiescent cells to move through the cell cycle and to halt at an undefined point in G1 (Bouchard *et al.* 2001b). Another study that also suggested that HBx slows the cell cycle utilized an inducible expression system, but failed to examine HBx expression (Park *et al.* 2000b). *In vivo*, the expression of HBx is associated with a significant increase in S-phase hepatocytes in livers of young animals; however, similar findings were not observed in adult liver (Madden *et al.* 2001).

Definitive interpretation of many current HBx-cell cycle reports is further complicated by the use in many studies of Chang liver cells (Benn and Schneider 1994, 1995, Bouchard *et al.* 2001a); these cells are known to contain the HPV E6 protein that binds to and subsequently inactivates *p53*. Furthermore, although it is clear that HBx has the capacity to deregulate cell cycle checkpoints *in vitro* and *in vivo*, none of the studies utilized markers that distinguish G0 cells from G1, and so the stage of the cell cycle altered by HBx has not been defined. Nevertheless, any HBV-induced cell cycle progression that occurs in the presence of environmental carcinogens is anticipated to lead to enhanced mutagenesis and to contribute to hepatocellular carcinoma.

6.4.4.4 Effect on apoptosis

In vivo, cell proliferation must be balanced with cell death to maintain liver homeostasis. The ability of HBx to deregulate cell cycle checkpoints (see Section 6.3.4.3) suggests that HBx also may alter apoptosis. This function of HBx might then contribute to the development of hepatocellular carcinoma by allowing the survival of cells containing DNA damage. Indeed, other viruses encode proteins that either suppress apoptosis as a strategy to permit virus maturation, or promote apoptosis as a means of spreading progeny virus in the absence of a host immune response (Teodoro and Branton 1997). Accumulating data regarding HBx and apoptosis indicate that in general, HBx sensitizes cells to apoptosis by DNA-damaging agents (Chirillo *et al.* 1997, Kim *et al.* 1998, Bergametti *et al.* 1999), but that it protects against cytokine-mediated apoptosis such as that mediated by fas (Diao *et al.* 2001, Pan *et al.* 2001, Gottlob *et al.* 1998) and TGF-β_1

(Shih *et al.* 2000). A definitive conclusion from most of these studies cannot be made due to the absence of a negative control protein expressed at similarly high levels. Thus, it remains unclear whether the proapoptotic effects reported for HBx are intrinsic to the HBx protein or the result of overexpressed and misfolded protein, which can trigger a stress response in cells that may include apoptosis (Tibbles and Woodgett 1999).

Definitive studies on apoptosis in HBx transgenic mice are extremely limited. Transgeneexpression in most lines of HBx-expressing mice is restricted to the weanling stage (Pollicino *et al.* 1998, Terradillos *et al.* 1997). Increased apoptosis has been reported in HBx mice that develop spontaneous tumors (Kim *et al.* 1991), but that result appears to reflect changes related to tumor progression rather than an intrinsic function of HBx (Koike *et al.* 1998). A second study in double transgenic mice demonstrated that overexpressed Bcl-2 protected livers from fas-mediated apoptosis and that HBx interfered with this protection (Terradillos *et al.* 2002). In neonatal mice that express very high levels of HBx, an increased rate of spontaneous apoptosis was noted (Pollicino *et al.* 1998). Together, these results indicate that HBx has the capacity to alter growth regulatory and apoptotic pathways in the cell, although how these changes may relate to hepatocellular carcinoma is not yet understood.

6.5 Other observations relevant to possible mechanisms of action

6.5.1 Introduction

Hepatocellular carcinoma is multifactoral and develops after decades of chronic HBV infection. Although chronic viral infection is a primary risk factor for hepatocellular carcinoma (Beasley 1988), additional risk factors are often present simultaneously, raising the possibility that chronic HBV infections synergize with other risk factors to further increase the risk of hepatocellular carcinoma (Chen and Chen 2002). These risk factors include coinfection with HCV, exposure to environmental carcinogens, and host genetic factors (Yeh *et al.* 1989).

6.5.2 Interaction with chronic HCV infection

Several studies have examined the possible synergism of HCV and HBV in patients who are chronically infected with both viruses. As summarized previously, there is a synergistic risk (greater than the sum of each infection alone) for hepatocellular carcinoma in patients who are chronically infected with both HBV and HCV compared to patients infected with either virus alone (Donato *et al.* 1998; see Tables 3-5, 3-6 and 3-7). Such synergism is consistent with the idea that the molecular mechanism(s) of HBV- and HCV-associated HCCs develop along distinct molecular pathways. This idea received further support in a recent microarray analysis of 45 HCCs, of which 31 were positive for HCV antibody and 14 were positive for HBsAg. A group of 83 genes were identified whose expression profile differed between HBV- positive HCCs and those that were HCV positive (Iizuka *et al.* 2002), consistent with the separate pathways used in the evolution of HCC. Additional, larger sample populations must still be analyzed to confirm this finding.

The mechanism of this synergism is not clear. There is accumulating evidence that HBV may interfere with HCV replication, and vice versa. Virus titers are lower in patients

coinfected with HBV and HCV than in those infected with either virus alone (Jardi *et al.* 2001, Kazemi-Shirazi *et al.* 2000, Mathurin *et al.* 2000). However, the mechanism by which this event occurs has not been defined. Futhermore, it is unknown how this viral interference might explain the poorer prognosis of HBV/HCV coinfected patients.

6.5.3 Interaction with aflatoxin B_1

Synergistic interactions between AFB_1, (a potent mycotoxin present in aflatoxin), and either infection with hepadnaviruses, including HBV in humans and animal hepadnaviruses, or transgenic mouse models expressing HBV proteins, have been reported. Results from human studies and animal models of hepatocellular carcinogenesis are described below.

6.5.3.1 Human studies

There is evidence that dietary exposure of HBV chronic carriers to AFB_1 greatly increases the risk of hepatocellular carcinoma (summarized in Table 3-7). As discussed above, the molecular mechanism of human hepatocarcinogenesis has not been defined; however, a specific mutation in the *p53* gene has been linked to dietary AFB_1. It is now clear that hepatocellular carcinomas from Qidong, China and sub-Saharan Africa contain a high prevalence of identical inactivating mutations in the *p53* gene (Hsu *et al.* 1991, Bressac *et al.* 1991, Scorsone *et al.* 1992). The identical nature of this mutation among unrelated patients is consistent with a carcinogen as the causative agent of the mutation. Whereas *p53* mutations generally span the highly conserved DNA binding domain of *p53*, the point mutations in the hepatocellular carcinomas were confined to a "hotspot" affecting codon 249. The identical G:C to T:A transversion mutation was reminiscent of a chemical carcinogen; further, it is generally accepted that dietary AFB_1, produced by *Aspergillus flavus* and *Aspergillus parasiticus*, which contaminate stored grains, is the environmental carcinogen.

Although the studies described above support a link between AFB_1 and human hepatocellular carcinoma, they do not explain the synergistic interaction between AFB_1 and HBV. Chen *et al.* (2001) investigated the association between HBV and AFB_1-albumin adducts. The investigators measured HBsAg and AFB_1-albumin adduct levels in 200 adolescents in Taiwan. Adducts were significantly higher ($P < 0.02$, *t*-test) for male and female HBsAg-positive adolescents combined, than for those who were HBsAg negative. The authors hypothesized that exposure to aflatoxin might affect infection from HBV through the immunosuppressive actions of AFB_1.

6.5.3.2 Animal models

The possible synergy between hepadnaviruses and AFB_1 also has been investigated in several animal models. One study reported that hepatocytes from WHV-positive woodchucks demonstrate an enhanced metabolic activation of aflatoxin (De Flora *et al.* 1989), but a second study did not reach the same conclusion (Gemechu-Hatewu *et al.* 1997). The discrepancy is thought to be due to the extreme variability in carcinogen metabolism between individual animals. However, administration of AFB_1 to woodchucks did accelerate the appearance of hepatocellular carcinoma (Bannasch *et al.* 1995).

Ducks from the Qidong region of China, where AFB_1 exposure is high, were examined to determine if they have a mutation analogous to the well-described G to T transversion in codon 249 of the *p53* tumor suppressor gene in humans exposed to aflatoxin. No mutations were detected by sequence analysis of the duck *p53* gene from 11 hepatocellular carcinomas from ducks with or without DHBV infection from Qidong or from four hepatocellular carcinomas from aflatoxin-treated ducks (Duflot *et al.* 1994). It was noted that although the duck and the human codon 249 encode the same amino acid, the codon itself is different in each. The third nucleotide residue, where aflatoxin-adduct formation occurs, is a guanine in humans, but a cytosine in ducks. The experimental design does not exclude the possibility of a mutation elsewhere in the duck *p53* gene.

Transgenic mice that overexpress PreS1 protein develop AFB_1-induced hepatocellular carcinoma at a significantly higher rate than AFB_1-treated nontransgenic control animals (Sell *et al.* 1991). In that model, the increased sensitivity to AFB_1 is believed to be due to immune-mediated induction of carcinogen-metabolizing enzymes (Kirby *et al.* 1994).

The synergy between AFB_1 and HBV also has been investigated in HBx transgenic mice. One property attributed to the HBV X protein is its ability to interfere with nucleotide excision repair, the pathway predominantly responsible for repair of bulky lesions such as those induced by UV light or AFB_1 exposure. When the DNA mutation spectrum was determined for AFB_1-treated HBx and nontransgenic littermate mice, a dramatic shift of DNA mutation spectrum was found in HBx mice (Madden *et al.* 2002). Specifically, while AFB_1-induced mutations in wild-type mice were predominantly DNA transition mutations (and similar to the spectrum found in spontaneous DNA mutations), those found in AFB_1-treated HBx mice were predominantly DNA transversions. This result demonstrates that hepatocytes expressing HBx respond differently to environmental carcinogens such as AFB_1.

The predominant AFB_1-induced mutation detected in the HBx mice was a G:C to T:A transversion in the reporter gene; it is identical to the *p53* “hotspot” mutation found in hepatocellular carcinomas from certain geographical locations. The human *p53* gene mutation is also a G:C to T:A transversion mutation, which results in an amino acid change from arginine to serine. Based on results from AFB_1-treated HBx mice, it is anticipated that HBx expression during chronic HBV infection may influence the frequency of *p53* mutations in areas where chronic exposure to aflatoxin is an additional risk factor. However, the role of HBV in the *p53* mutation profile is not clear. Several studies have detected mutant *p53* significantly more often in HBV-positive hepatocellular carcinomas than in HBV-negative hepatocellular carcinomas (Buendia 2000), suggesting that expression of HBV may somehow enhance the development of *p53* mutations. The *p53*-specific AFB_1 mutation was not detected in treated nonhuman primates (Fujimoto *et al.* 1992), woodchucks (Rivkina *et al.* 1996), ground squirrels (Rivkina *et al.* 1994), or HBx mice (Madden *et al.* 2002), which suggests that other targets of aflatoxin-mediated hepatocellular carcinoma progress through non-*p53* pathways in those species.

6.6 Summary

During the decades of chronic viral infection, many changes are introduced to the cell as a consequence of the ongoing virus replication. Viral DNA becomes incorporated into

cellular DNA through illegitimate recombination, and these sequences may contribute to multistep hepatocarcinogenesis by any of several mechanisms. New methodologies have allowed investigators to identify a significant number of growth regulatory genes whose expression is altered by viral integration. Integration also may lead to a truncation in the 3' end of the *preS2* and *X* genes, leading to novel proteins that possess transactivation function. A majority of tumors contain viral sequences that have the capacity to transactivate cellular genes, although the critical cellular targets of that transactivation are not known. Advances in technology also have led to new genome-wide chromosome allele studies, and it appears that HBV-positive hepatocellular carcinomas contain higher levels of chromosome allele loss than HBV-negative hepatocellular carcinomas. The targets of those allele losses are not known, but the data suggest that several tumor suppressor genes may be lost in the evolution of a tumor. Further, new data from hepadnaviral animal models confirm the synergism between hepadnaviral infections and aflatoxins. All of these genetic changes occur on the background of immune-mediated cell death and regeneration, which facilitates the selection of cells that have a growth advantage during the development of the tumor.

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Glossary of Hepatitis- or Virology-related Terms

Acute hepatitis: Newly acquired symptomatic hepatitis virus infection, usually less than six months in duration.

Adoptive transfer: A procedure in which immune cells from one animal are transferred into an immunologically naïve animal.

AFB_1: Aflatoxin, secondary metabolite produced by *Aspergillus flavus* and *Aspergillus parasiticus*; considered a liver carcinogen.

Antigen: Any substance that, as a result of coming in contact with appropriate cells, induces a state of sensitivity and/or immune responsiveness after a latent period (days to weeks) and which reacts in a demonstrable way with antibodies and/or immune cells of the sensitized subject *in vivo* or *in vitro*.

Australia antigen: Previous designation for hepatitis B surface antigen (HBsAg).

Avihepadnavirus: Genus of viruses phylogenetically related to duck hepatitis B virus, and that that have been isolated from birds.

Carrier: A person or animal that harbors a specific infectious agent without visible symptoms of the disease. A carrier acts as a potential source of infection.

Chronic hepatitis: Any of several types of hepatitis persisting for ≥ 6 months, often progressing to cirrhosis; this condition is characterized by abnormal levels of liver enzymes and inflammatory changes on liver biopsy.

Cirrhosis: Irreversible hepatic fibrosis with regenerative nodule formation.

Clearance: Removal of a substance, such as viral particles, from the blood (e.g., by renal excretion).

DHBV: Duck hepatitis B virus.

DNA virus: A set of viruses that use DNA for the storage of their genetic information.

Flaviviridae: A family of enveloped single-stranded positive sense RNA viruses formerly classified as the “group B” arboviruses.

GSHV: Ground squirrel hepatitis B virus.

HBeAg: Protein secreted by infected cells, similar to the nucleocapsid subunit but lacking carboxy terminal amino acids of the core gene product.

HBsAg: Hepatitis B virus (HBV) surface antigen particles, 22-nm rods and spheres secreted by cells infected with HBV and made up of the viral envelope proteins, but lacking viral nucleic acids.

HBx: HBV X gene protein.

HBV: Hepatitis B virus (human)

HCC: Hepatocellular carcinoma.

Hepadnavirus: A group of animal DNA viruses including viruses of ducks, woodchucks, squirrels, and others as well as the virus causing hepatitis B in humans.

Hepatitis: Inflammation of the liver.

Infectivity: The characteristic of a disease agent that embodies capability of entering, surviving in, and multiplying in a susceptible host.

Nucleocapsid: A unit of viral structure, consisting of a capsid (protein coat) with the enclosed nucleic acid.

Orthohepadnavirus: Genus of viruses phylogenetically related to human HBV that have been isolated from mammals.

Pestivirus: A genus of viruses composed of the classical swine fever virus, bovine viral diarrhea virus, and related viruses; these viruses are animal pathogens and are especially important in livestock.

Productive infection: The result of a virus' ability to yield an infection.

Seroconversion: Development of antibodies in the blood of an individual who previously did not have detectable antibodies.

Viral envelope: The outer structure, composed of two layers of lipids, that encloses the nucleocapsids of some viruses; the envelope may contain host material.

Viral titer: The concentration of infectious viral particles per milliliter of suspension fluid.

Viremia: The presence of a virus in the bloodstream.

Virion: The complete viral particle, found extracellularly and capable of surviving and infecting a living cell.

WHV: Woodchuck hepatitis virus.

WHx: X protein encoded by WHV.