Covalently Closed Circular DNA Is the Predominant Form of Duck Hepatitis B Virus DNA That Persists following Transient Infection

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Residual hepatitis B virus (HBV) DNA can be detected in serum and liver after apparent recovery from transient infection. However, it is not known if this residual HBV DNA represents ongoing viral replication and antigen expression. In the current study, ducks inoculated with duck hepatitis B virus (DHBV) were monitored for residual DHBV DNA following recovery from transient infection until 9 months postinoculation (p.i.). Resolution of DHBV infection occurred in 13 out of 15 ducks by 1-month p.i., defined as clearance of DHBV surface antigen-positive hepatocytes from the liver and development of anti-DHBV surface antibodies. At 9 months p.i., residual DHBV DNA was detected using nested PCR in 10/11 liver, 7/11 spleen, 2/11 kidney, 1/11 heart, and 1/11 adrenal samples. Residual DHBV DNA was not detected in serum or peripheral blood mononuclear cells. Within the liver, levels of residual DHBV DNA were 0.0024 to 0.016 copies per cell, 40 to 80% of which were identified as covalently closed viral DNA by quantitative PCR assay. This result, which was confirmed by Southern blot hybridization, is consistent with suppressed viral replication or inactive infection. Samples of liver and spleen cells from recovered animals did not transmit DHBV infection when inoculated into 1- to 2-day-old ducklings, and immunosuppressive treatment of ducks with cyclosporine and dexamethasone for 4 weeks did not alter levels of residual DHBV DNA in the liver. These findings further characterize a second form of hepadnavirus persistence in a suppressed or inactive state, quite distinct from the classical chronic carrier state.

Hepatitis B virus (HBV) infections in adult humans are typically characterized by recovery and the development of anti-surface antibodies (anti-HBs) and immunity to reinfection. Despite the apparent clearance of HBV infection, a number of studies of patient sera have demonstrated persistence of viral DNA for months or years after resolution of transient HBV infection (2, 19, 24, 41). Cytotoxic T-lymphocyte responses also persist, and reactivation of infection can occur following immunosuppression or after liver transplantation (5, 6, 16, 17, 38).

Yotsuyanagi et al. found traces of residual HBV DNA in sera collected from 10 out of 11 patients up to 15 months after the diagnosis of transient HBV infection (41). Michalak et al. reported residual HBV DNA in the serum and peripheral blood mononuclear cells (PBMC) collected from four out of five patients up to 70 months after the resolution of HBV infection (24). Ultracentrifugation and PCR were used to determine that the viral DNA-positive fraction in serum sedimented at the same rate as HBV virions, suggesting that the residual HBV DNA was present within viral particles (24). In another study, evidence for HBV closed circular viral DNA (cccDNA) in the liver almost 4 years after resolution of HBV infection was obtained using PCR techniques (20). However, this result is not totally unambiguous inasmuch as HBV DNA may integrate into host DNA during infection and integrated DNA might produce a positive result in a PCR assay even in the absence of cccDNA.

The mechanism of viral persistence was not defined in these studies, and it remains unclear whether residual virus present in the serum reflects active replication, and whether liver cells are infected or other sites are involved. Mason et al. (20) also reported finding HBV RNA transcripts in the livers of 4/7 patients with resolved chronic HBV infection, particularly in those who had cleared serum HBsAg more recently, but whether antigen expression or virus replication was a usual feature of the cells harboring residual virus DNA was not clear. Some studies suggest that residual virus DNA may be responsible for continuing or intermittent antigen expression. Rehermann et al. detected cytotoxic T-lymphocyte responses to a range of HBV epitopes, which persisted for more than a year after the resolution of transient HBV infection (32). This could be explained by either ongoing infection and antigen expression leading to antigenic stimulation or immunological memory. Stronger evidence for ongoing antigenic stimulation is the presence of major histocompatibility complex class II-restricted induction of T-cell proliferation 2.2 to 13 years after serological resolution of HBV infection (30). In this study, Penna et al. showed that this in vitro proliferative response was abrogated by depletion of T cells with markers of recent activation (i.e., HLA-DR, CD-69, and CD-25), suggesting ongoing antigenic stimulation rather than T-cell memory (30).
Irrespective of the nature of the residual virus/cell relationship, some residual HBV DNA is replication competent, as revealed by the reactivation of HBV replication following immunosuppression or liver transplantation (5, 6, 16, 17, 38). Immunosuppressive drug treatment has been associated with reactivation of HBV in patients initially positive for anti-HBs. The frequency of reactivation is not known but is probably less than 5% (16, 17). Several series from transplant centers have documented the risk of HBV infection acquired by transplantation of livers from donors who had serum anti-HBV core antibodies and were HBsAg negative (5, 6, 38). Many of these cases involved donors who also had anti-HBs, indicating serological resolution of HBV infection.

Similarly, in woodchucks that had resolved transient infection with the related woodchuck hepatitis virus (WHV), traces of residual WHV DNA were detected by PCR in a range of sites including liver, PBMC, and serum for almost 6 years (23) and eight of nine woodchucks had mild histological hepatitis (23). These studies showed infectivity of the residual virus by inoculating PBMC-derived virus into WHV-naive woodchucks, which then developed typical WHV infection (23). Hepatocellular carcinoma, which is virtually unknown in woodchucks that have never been infected with WHV, was also detected in two of the nine woodchucks, implying that residual WHV DNA may play a role in the development of hepatocellular carcinoma.

In the present study, we demonstrated for the first time that residual duck HBV (DHBV) infection is common in the liver of ducks following recovery from transient infection, and used Southern blot hybridization as an unambiguous assay to confirm the persistence of cccDNA. Residual cccDNA persisted at ~0.1% of the level seen at the peak of a transient infection. However, little or no DNA replicative intermediates were detected by this stage, suggesting that virus replication, if present, was at a very low level. Analysis of liver tissue by immunoperoxidase staining for DHBV surface antigen (DHBsAg) did not conclusively reveal the identity of the residually DHBV-infected cells. Furthermore, samples of liver and spleen cells from recovered animals did not transmit DHBV infection when inoculated into 1- to 2-day-old ducklings, and immunosuppressive treatment of ducks with cyclosporine and dexamethasone for 4 weeks did not alter levels of residual DHBV DNA in the liver. These observations suggest that residual DHBV DNA is largely inactive, possibly in a subset of long-lived liver cells with a low capacity for hepadnavirus replication, and that this state represents a second form of persistent hepatitis B virus infection.

Preparation and measurement of viral and cellular DNA. DNA was extracted from liver and other organs for Southern blot hybridization using a phenolchloroform method as previously described (14). DNA was extracted from serum for PCR analysis using a High Pure viral nucleic acid kit (Roche Molecular Biochemicals) and from tissue and PBMC using a QIAGEN DNeasy tissue kit (QIAGEN, Australia). To measure the concentration of DNA, 1 μl of each of five standards containing 0, 10, 20, 30, and 40 ng/ml of lambda DNA in water was mixed with 2 ml of a dye solution consisting of 10 ng/ml of Hoechst 33258 in 2 M NaCl, 50 mM Tris-HCl, pH 7.5. The fluorescence was measured using a Perkin-Elmer MFP-3L fluorimeter to define a standard curve, and the DNA concentration of unknown samples prepared similarly was then determined by comparison with the standard curve.

PCR procedures for analysis of residual DHBV DNA. Strict measures were used to minimize the risk of contamination of PCRs with amplified DNA, cloned DHBV DNA, or extraneous DHBV DNA. To monitor for contamination, DNA was extracted from samples of DHBV-negative normal duck liver in parallel with other samples and tested by PCR.

Materials and Methods

Animals. Commercially available DHBV-infected and uninfected Pekin-Aylesbury ducks (Anas domesticus platyrhynchos) were acquired and housed separately at the Institute of Medical and Veterinary Science, Adelaide, South Australia, in accordance with guidelines set by the National Health and Medical Research Council (NHMRC) of Australia. The Animal Ethics Committees of the University of Adelaide and the Institute of Medical and Veterinary Science approved all research involving ducks.

Transient DHBV infection experiments. DHBV-negative Pekin Aylesbury ducks aged 39 to 40 days were inoculated intravenously with aliquots of a pooled DHBV-positive serum from congenitally DHBV-infected ducks that contained 9.5 × 10⁷ DHBV genomes/ml (13). DHBV-positive serum was serially diluted in normal duck serum to concentrations of 10⁻¹⁰, 10⁻⁹, and 10⁻⁸ genomes/ml and 1-ml aliquots were administered intravenously to ducks in groups A (10⁻⁹), B (10⁻⁸), and C (10⁻⁷) (11). Serum was collected from all ducks at regular intervals for detection of DHBsAg and anti-DHBV surface (anti-DHBs) and anti-DHBV core antibodies (anti-DHBc) by enzyme-linked immunosorbent assay as previously described (8, 11, 25).

Collection and analysis of liver and extraphepatic tissues. Liver biopsy was performed at 3 or 4 days, and 1, 3, and 6 months postinoculation (p.i.) by laparotomy under general anesthesia (4) with induction with 5% isoflurane (Forthane, Abbott) in oxygen and maintenance by ventilation with 3 to 4% isoflurane via an endotracheal tube (size 2.0, Contour Mallinckrodt Medical, Ireland). At 9 months p.i. ducks were administered a lethal dose of phenobarbitone and autopsy was performed using sterile, clean instruments to avoid cross contamination. Tissues were collected in the following order: adrenal gland, lymph node/aorta, kidney, pancreas, spleen, heart, skeletal muscle, and finally liver. Blood samples were also collected for preparation of serum and PBMC as previously described (25) and then stored frozen before analysis of DHBV DNA.

Biopsy and autopsy tissue samples (usually 600 to 800 mg) were divided into three portions; one was snap frozen in liquid nitrogen and stored at ~70°C, another was placed in formalin for histological analysis after hematoxylin and eosin staining, and the remainder was fixed in ethano/acetic acid (3:1) for antigen detection, using microwave-assisted antigen retrieval and immunoperoxidase staining of DHBsAg with anti-DHBV pre-S monoclonal antibodies (25, 31). Anti-duck thrombocyte monoclonal antibodies (BA3) (1) were used as a negative control. Sections of DHBV-infected and uninfected duck liver were also used as positive and negative controls and were stained in parallel. DHBsAg-positive cells were counted at 200× magnification with the aid of an eyepiece graticule. The percentage of DHBsAg-positive hepatocytes was determined by counting up to 250 grid fields (250 by 250 μm) containing ~125,000 hepatocytes and expressing the number of DHBsAg-positive cells as a percentage of the total hepatocyte nuclei. The sensitivity of detection in liver biopsy tissue was 0.0008%. In autopsy liver specimens, 2,000 grid fields (250 by 250 μm) containing ~1,000,000 hepatocytes were counted for each duck, resulting in a sensitivity of detection of 0.0001%.

PCR procedures for analysis of residual DHBV DNA. Nested PCR amplification of DHBV DNA was performed using primers C1 and C2 (Table 1) targeting the DHBV core open reading frame (ORF). A 50-μl reaction containing 40 μl of reaction mix and 10 μl of template was used for the first round of nested PCR. The template contained 100 ng of DNA extracted from skeletal muscle or PBMC, 200 to 300 ng of DNA extracted from heart, kidney, spleen, or pancreas, 600 ng of DNA extracted from liver, 1,000 ng of DNA extracted from adrenal, or DNA extracted from 10 μl of serum. Final concentrations were 0.4 μM each of primers C1 and C2, 200 μM deoxynucleoside triphosphates, 2 mM MgCl₂, 5 μl of reaction buffer (670 mM Tris-Cl, pH 8.8, 166 mM [NH₄]₂SO₄, 2 mg/ml gelatin, 4.5% Triton X-100), and 1 unit of Taq polymerase (Generewks, Adelaide, Australia). Nested PCR was performed using a Perkin-Elmer GeneAmp 2400 and comprised four steps: step 1, denaturation, 4.5 min at 94°C; step 2, annealing, 30 seconds at 60°C; step 3, extension, 30 seconds at 72°C; and step 4, denaturation, 30 seconds at 94°C. Steps 2 to 4 were repeated for 35 cycles and then a final 7-min extension step was performed at 72°C. For the second round of nested PCR, 1 μl
of the first-round reaction was added to a 50-μl reaction containing 1 μM each of primers C3 and C4 (Table 1), 200 nM deoxynucleoside triphosphates, 2 mM MgCl₂, 5 μl of reaction buffer, and 1 unit of Taq polymerase. PCR cycles were as outlined above with 20 cycles of steps 2 to 4. Detection of products of nested PCR was by agarose gel electrophoresis followed by Southern blot hybridization using a genome-length 82P-labeled DHBV DNA probe to confirm the identity of the bands. The sensitivity of detection of DHBV DNA using nested PCR followed by Southern blot hybridization was 1 copy of DHBV DNA in 350,000 cells or 0.000003 copies per cell for liver and other tissues, and 100 copies per ml for serum.

Quantitative PCR to detect total DHBV DNA was performed using primers P3 and P4 (Table 1) targeting the polymerase open reading frame, using a Roche LightCycler. Quantitative PCR detection of cccDNA was performed using primers CC2 and R2 spanning the cohesive overlap region of the DHBV genome (Table 1). The reaction contained 2 μl of Roche FastStart Master SYBR Green 1 (Roche Molecular Biochemicals), 4 mM MgCl₂, and 0.5 μM of primers (Table 1) in a total volume of 10 μl. The PCR template consisted of 120 or 200 ng of extracted DNA diluted with water to 10 μl, or plasmid DNA standards containing 120 or 200 ng of extracted normal duck liver DNA to which was added a known number of copies of plasmid DHBV DNA genomes.

A working stock of plasmid DHBV DNA (pBL4.8x2) containing a head-to-tail dimer of the Australian DHBV strain (AusDHBV) (35) was prepared by diluting plasmid DNA to a concentration of 90 ng/μl (1010 DHBV genomes/μl) as determined by spectrophotometry. A stock of 12 or 22 ng/ml of normal duck liver DNA in water was also prepared and a series of 10-fold dilutions of 2 μl of plasmid DNA in 18 μl of normal duck liver DNA were prepared to produce standards containing 2 × 10², 2 × 10³, 2 × 10⁴, 2 × 10⁵ and 2 × 10⁶ copies of the DHBV genome.

Each quantitative PCR run included plasmid DNA standards to generate a standard curve, a normal duck liver DNA negative control sample, and a number of test samples. The reaction mix and template were loaded into glass capillary tubes (Roche Molecular Biochemicals) and centrifuged briefly at 3,000 rpm. The protocol to detect total DHBV DNA included an initial denaturation step of 10 min at 95°C to activate the FastStart Taq polymerase and then 40 cycles of amplification. These consisted of 5 seconds at 95°C, 10 seconds at 55°C, and 15 seconds at 72°C before the samples were cooled to 40°C. The conditions for detection of cccDNA were slightly different with each of the 40 cycles consisting of 5 seconds at 95°C, 10 seconds at 60°C, and 24 seconds at 72°C.

Detection of PCR products was by measurement of SYBR Green. As well as quantification of fluorescence, analysis of the melting temperature curves of samples was used as in the manufacturer's recommendations to determine the specificity of the PCR products. A DHBV-specific product was defined as having a melting temperature at the same temperature as that of the PCR products amplified from the plasmid DHBV DNA standards. The sensitivity of the quantitative PCR for both total DHBV DNA and cccDNA was 10 copies in 80,000 cells, or 0.00012 copies per cell.
RESULTS

Establishment of DHBV infection. Three groups of five 39- to 40-day-old DHBV-negative ducks were inoculated intravenously with either 10^7 (group A), 10^8 (group B), or 10^9 (group C) virions. Sections of liver biopsy tissue from all 15 inoculated ducks collected at day 3 to 4 p.i. were stained using anti-pre-S monoclonal antibodies (1H1) (23, 27) and showed a proportional relationship between the inoculum size and frequency of DHBsAg-positive cells. Ducks from group A showed a mean of 0.00085% DHBsAg-positive total liver cells (0.00038 to 0.0015%), group B showed 0.011% (0.0066 to 0.013%), and group C showed 2.64% (1.5 to 3.8%) (Fig. 1, Table 2). In each case, the single and occasional pairs of DHBsAg-positive cells were evenly and widely distributed throughout the lobules of the liver (Fig. 1).

Non-protein-bound and total cellular DNA was extracted from frozen liver samples collected at day 3 or 4 p.i. and day 31 p.i. and examined for cccDNA and for total DHBV DNA, respectively, by Southern blot hybridization. cccDNA and total DHBV DNA were undetectable at days 3 to 4 p.i. in the livers of the group A and B ducks (data not shown), but were detected in liver tissue from five out of five group C ducks (Fig. 2A and B, respectively) and quantitated by phosphorimager analysis (Table 2). Levels of cccDNA ranged from 0.035 to 0.096 copies per liver cell or 1.6 to 2.6 copies per DHBsAg-positive cell, while levels of total DHBV DNA ranged from 2.7 to 5.2 copies per liver cell or 78 to 347 copies per DHBsAg-positive cell (Table 2). The average ratio of cccDNA to total DHBV DNA in the samples collected at day 3 p.i. from the group C ducks was 1.2%, in contrast to the much higher percentage (40 to 80%) found in liver samples collected from the same ducks with residual DHBV infection at 9 months p.i. as described below.

As can be seen in Fig. 2, two of the group C ducks, 3435 and 4243, had high levels of cccDNA (Fig. 2A, lanes 6 and 10) and total DHBV DNA (Fig. 2B, lanes 6 and 10) at day 31 p.i. These ducks went on to develop persistent DHBV infection (see below) while the three remaining ducks in group C had low levels of cccDNA (Fig. 2A, lanes 7, 8, and 9) and undetectable levels of total DHBV DNA at day 31 (Fig. 2B, lanes 7, 8, and 9) and resolved their transient DHBV infection.

Outcome of infection. Eleven out of 15 inoculated ducks from groups A, B, and C had transient DHBV infection as shown by the clearance of DHBsAg-positive cells from the liver by 31 days p.i. (data not shown) which was maintained until autopsy at 9 months p.i. Autopsy liver tissues were examined by immunoperoxidase staining for DHBsAg in an attempt to identify the cell type harboring residual DHBV DNA. However, only one DHBsAg-positive hepatocyte was detected in the liver of one duck with residual infection (duck 158; Fig. 1, panel D). The duck had residual DHBV DNA detected 6.5 months after inoculation with 10^9 DHBV genomes at 41 days of age. The specificity of DHBsAg detection was confirmed by staining the same cell of a consecutive liver section and by the use of negative control monoclonal antibodies (21). This result suggested that residual DHBV DNA is present in hepatocytes. However, the biological significance of such a low rate of positive cells is not clear, and we are not confident to conclude that hepatocytes are the only cell type containing residual DHBV DNA.

Of the remaining 4 out of 15 ducks, two of the group C ducks, 3435 and 4243, developed persistent DHBV infection with high levels of virus cccDNA and total DHBV DNA in liver, widespread DHBsAg staining in liver, and detectable DHBsAg in serum from days 15 and 21 p.i. Another two ducks (one each from groups A and B) died prematurely and were unavailable for autopsy. A single series of serum samples from duck 3637 in group C with transient infection were assayed up to day 45 p.i. for viral DNA by quantitative PCR. Viral DNA extracts corresponding to 40 μl of serum were tested by PCR with primers P3 and P4. Samples from days 3, 8, 15, and 30 p.i. were DHBV DNA positive, while those from days 11, 21, and 45 p.i. and samples collected at autopsy at 9 months p.i. were DHBV DNA negative. At day 3 p.i. the estimated copy number of DHBV DNA in serum was 1,525 copies/ml, while copy numbers in the other positive samples were lower. Thus, during transient DHBV infection (days 3 to 30 p.i.) this duck showed very low levels of serum DHBV DNA that fluctuated around the limit of detection by quantitative PCR before becoming undetectable (<25 copies per ml) at day 45 and 9 months p.i.

Anti-DHBs became detectable by day 11 p.i. in four out of five ducks in each of groups A and B (data not shown), while in group C, anti-DHBs antibodies were consistently detected in the three ducks with transient infection (3637, 3839, and 4041), from day 7 p.i. until at least day 90 p.i. (data not shown). In contrast, ducks 3435 and 4243 (the two group C ducks that developed persistent infection) had serum anti-surface antibody bodies only transiently, before, in one duck, and coinciding, in the second duck, with the onset of detectable DHBsAg (data not shown).

Studies of residual DHBV DNA. Tissue samples were collected at autopsy from four group A, four group B, and three group C ducks at 9 months p.i., with the exception of duck 910, which died and was autopsied early, at 5 months p.i. Residual DHBV DNA was assayed by nested PCR followed by Southern blot hybridization (sensitivity of 0.00003 copies per cell) on DNA extracted from liver, spleen, kidney, pancreas, adrenal, heart, PBMC, skeletal muscle, and serum (sensitivity, 100 copies per ml), and was found in at least one site in all ducks. Most liver (10/11) and spleen (7/11) samples were positive for DHBV DNA, whereas DHBV DNA was infrequently detected in other sites, two kidney samples, and one adrenal and one heart sample only. DHBV DNA was not detected in pancreas, skeletal muscle, PBMC, or serum in any residually infected ducks at 9 months p.i.

Quantitative PCR showed that as the DHBV inoculum was increased, the amount of residual DHBV DNA in the liver at 9 months p.i. also increased. One out of four liver samples from the ducks in group A (1516), three out of four samples from the group B ducks (525, 910, and 1718), and all three samples from the ducks in group C (3637, 3839, and 4041) showed detectable levels of DHBV DNA, using an assay which had a minimum sensitivity of 10 copies of DHBV DNA in 200 ng total DNA or 80,000 cells (0.00012 copies per cell). Although residual DHBV DNA was detected in the group A and
B ducks, the levels were too low to be accurately quantified but were estimated to be between 0.00012 and 0.00025 copies per cell. The three group C ducks that cleared their transient DHBV infection had 190, 200, and 1,260 copies of DHBV DNA in $8 \times 10^4$ cell equivalents at 9 months p.i., corresponding to 0.0024 to 0.016 copies/cell or a mean of 0.007 copies/cell (Table 3). Liver samples collected at 9 months p.i. from the group C ducks with residual (3637, 3839, and 4041), and persistent (3435 and 4243), DHBV infection were examined under code by a histopathologist. No evidence was found for ongoing liver inflammation in the three group C ducks with residual DHBV infection compared to uninfected, age-matched control ducks. In contrast, the two group C ducks with persistent DHBV infection had mild to moderate liver inflammation.

Autopsy spleen samples collected at 9 months p.i. were similarly examined for residual DHBV DNA, and three out of four, two out of four, and three out of three samples from groups A, B, and C, respectively, had detectable DHBV DNA (Table 3). Overall, the levels of virus DNA were lower in spleen than in liver, and a clear effect of inoculum dose on the amount of residual virus DNA was not seen in the spleen samples. The samples of kidney, heart, and adrenal which were positive for DHBV DNA by nested PCR were also positive in the quantitative assay, but at levels insufficient for accurate quantification, i.e., between 0.00012 and 0.00025 copies/cell (data not shown).

Sequential liver samples taken at day 3 to 4 and 1, 3, 6, and 9 months p.i. were next analyzed by quantitative PCR for total DHBV DNA to assess fluctuation in DHBV DNA levels over time. For two of the time points for the group C ducks, results of Southern blot hybridization were used because insufficient remaining biopsy material was available. Virus DNA in the group C ducks showed a fall to low levels of during the first month p.i., after which the levels of residual DHBV DNA remained relatively constant, ranging from 0.0044 to 0.007 copies/cell (Table 4).

**Contribution of cccDNA to the total residual DHBV DNA.** The above samples of autopsy liver DNA were next assayed by quantitative PCR for cccDNA using primers spanning the cohesive overlap region. Liver samples from one out of four ducks in group A (duck 20) and one out of four ducks in group B (duck 525) contained detectable cccDNA. However, the levels of residual DHBV cccDNA were insufficient for accurate quantitation (data not shown). Liver samples from all three group C ducks (3637, 3839, and 4041) contained cccDNA at 0.014, 0.0010, and 0.0019 copies/cell, respectively (Table 3), compared with 0.016, 0.0024, and 0.0025 copies of total DHBV DNA per cell, respectively (Table 3). The levels of cccDNA were 40 to 80% of the levels of total DHBV DNA, making cccDNA the predominant form of residual DHBV DNA in ducks with residual DHBV infection. This is in marked contrast to what was seen in the same ducks during the early phase of their transient infection, when cccDNA in liver tissue at day 3 p.i. represented only 1.2% of total DHBV DNA (Table 2).

All 11 spleen samples and the remaining samples from other referring to FIG. 1. Expression of DHBsAg in liver at day 3 to 4 p.i. (A, B, and C) and 6.5 months p.i. (D). Immunohistochemical detection of DHBsAg was performed using monoclonal anti-DHBV pre-S antibodies (1H1) (31). DHBsAg-positive hepatocytes (indicated with arrows in panels A, B, and D) in liver tissue from (A) duck 20 from group A (with a group average of 0.0085% DHBsAg-positive cells); (B) duck 910 from group B (average of 0.11%), and (C) duck 3435 from group C (average of 2.64%). A single DHBsAg-hepatocyte was also detected at 6.5 months p.i. in duck 158 with residual DHBV infection following inoculation with $10^9$ DHBV genomes at 41 days of age. DHBsAg was detected in the cytoplasm of hepatocytes as shown (A, B, C, and D). Sections were counterstained with hematoxylin. Bar, 50 μm.
organs which were positive by quantitative PCR for total DHBV DNA (two kidney, one heart, and one adrenal sample) were also tested by quantitative PCR for cccDNA. All samples tested were negative for cccDNA by this assay (i.e., <10 copies/80,000 cells) (data not shown). Thus, within the limits of this methodology for detecting very small amounts of DNA, cccDNA was not shown to be a significant contributor to the residual DHBV DNA in these other organs.

Further analysis of residual DHBV DNA genomes. Further experiments were performed (i) to examine whether full-length DHBV genomes were present and (ii) to confirm, using Southern blot hybridization, that the residual DHBV DNA detected using the cccDNA PCR was indeed in the characteristic cccDNA form.

In the first experiment, PCR amplification of full-length DHBV DNA was performed as previously described (9, 27) using 1 μg of liver DNA extracted from the three group C ducks at autopsy at 9 months p.i. Extracted DNA was amplified using primers FL1 and FL2 (Table 1), which were designed to amplify full-length DHBV DNA and should amplify a cccDNA template with a greater efficiency than relaxed circular DNA, since relaxed circular DNA is not continuous between the primers. The PCR products were analyzed by gel electrophoresis on a 1% agarose gel stained with ethidium bromide (Fig. 3A). A 3-kb product was seen in all three autopsy samples (Fig. 3A, lanes 4, 5, and 6), as well as in a positive control sample that consisted of extracted liver DNA from a duck with high-level DHBV infection (Fig. 3A, lane 2). Southern blot hybridization using a genome-length32P-labeled DHBV DNA probe demonstrated that the 3-kbp product contained DHBV sequences (Fig. 3B, lanes 4, 5, and 6). Also seen in all three samples were discrete smaller than genome-length products, which also hybridized to the DHBV DNA probe and may represent PCR products amplified from defective cccDNA molecules with large deletions.

In the second experiment, DNA was extracted as described above to enrich for non-protein-bound DNA (hence cccDNA), from sequential samples of liver from the three ducks in group C (3637, 3839, and 4041) collected up to 9 months p.i. In order to increase the sensitivity of the Southern blot hybridization a larger than usual quantity of cccDNA extract was loaded onto the agarose gel (38 μl containing cccDNA extracted from 5 × 10⁷ cells compared with the usual volume of 18 μl containing cccDNA from 7 × 10⁶ cells). Samples showed cccDNA at all time points.
points in two out of three ducks analyzed (Fig. 4) and up to 3 months p.i. in the remaining duck (number 3637). The sensitivity of Southern blot hybridization based on the plasmid DNA marker (M) was estimated as equivalent to 0.003 genomes per cell, allowing approximate quantitation of the DNA signal. The residual DHBV cccDNA in liver at 9 months p.i. in ducks 3637, 3839, and 4041 was estimated as 0.003 (not detected), 0.008, and 0.009 copies per cell by Southern blot hybridization, compared with estimates of 0.014, 0.0010, and 0.0019 copies per cell by quantitative PCR for cccDNA (Table 3). Thus, Southern blot hybridization provided independent confirmation of the PCR finding of cccDNA in residual infection.

**Studies of infectivity of liver, spleen, and serum from ducks with residual DHBV infection.** Several strategies were used in an attempt to demonstrate whether infectious virions were present in tissues and serum from ducks with residual hepadnavirus infection. A total of six experiments were performed involving inoculation into 1- to 2-day-old ducklings of material derived from ducks that had recovered from transient DHBV infection; 1- to 2-day-old ducklings can be infected with a single virion when diluted serum from ducks with congenital and experimental DHBV infection is used (13, 21, 22).

Fresh and frozen liver cell homogenates, cultured spleen cells and the culture supernatant, and a sucrose cushion-purified fraction of serum from ducks with residual infection were tested for infectivity in 1- to 2-day-old ducklings by either intraperitoneal or intravenous inoculation. DHBV infection (defined by detectable serum DHBsAg) was not transmitted by infection; 1- to 2-day-old ducklings can be infected with a single virion when diluted serum from ducks with congenital and experimental DHBV infection is used (13, 21, 22).

**TABLE 3. Quantitation of residual DHBV DNA in autopsy liver and spleen in samples collected at 9 months p.i.**

<table>
<thead>
<tr>
<th>Group and dose (virions)</th>
<th>Duck no.</th>
<th>Total DHBV DNA copies/cell in liver&lt;sup&gt;a&lt;/sup&gt; (cccDNA copies/cell)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Total DHBV DNA copies/cell in spleen&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (10&lt;sup&gt;7&lt;/sup&gt;)</td>
<td>322</td>
<td>Not detected</td>
<td>0.00012–0.00025</td>
</tr>
<tr>
<td></td>
<td>1112</td>
<td>Not detected</td>
<td>0.00012–0.00025</td>
</tr>
<tr>
<td></td>
<td>1516</td>
<td>0.00012–0.00025</td>
<td>0.00012–0.00025</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>B (10&lt;sup&gt;8&lt;/sup&gt;)</td>
<td>525</td>
<td>0.00012–0.00025</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>821</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>910</td>
<td>0.00012–0.00025</td>
<td>0.00012–0.00025</td>
</tr>
<tr>
<td></td>
<td>1718</td>
<td>0.00012–0.00025</td>
<td>0.00012–0.00025</td>
</tr>
<tr>
<td>C (10&lt;sup&gt;10&lt;/sup&gt;)</td>
<td>3637</td>
<td>0.016 (0.014)</td>
<td>0.00025</td>
</tr>
<tr>
<td></td>
<td>3839</td>
<td>0.0024 (0.0010)</td>
<td>0.0009</td>
</tr>
<tr>
<td></td>
<td>4041</td>
<td>0.0025 (0.0019)</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

<sup>a</sup> Quantitative PCR for total DHBV DNA used primers P3 and P4 (Table 1) and had a sensitivity of 10 copies in 80,000 cells or 0.00012 copies per cell.

<sup>b</sup> Quantitative PCR for cccDNA used primers CC2 and R2 (Table 1). The assay had a sensitivity of 10 copies in 80,000 cells, or 0.00012 copies per cell.

<sup>c</sup> Levels of residual DHBV DNA in the group A and B ducks were too low to be accurately quantified but were estimated to be between 0.00012 and 0.00025 copies per cell.

**FIG. 3. Detection of full-length DHBV DNA by PCR using primers FL1 and FL2 (Table 1) and liver DNA extracted from group C ducks at 9 months p.i. (A) An ethidium bromide-stained gel of the products of a full-length PCR. (B) Southern blot hybridization of the same samples using a genome-length 32P-labeled DHBV DNA probe, with autoradiographic exposure for 30 min. Lane 1, λ DNA digested with PstI showing bands of 2.8, 1.7, and 1.2 kbp, indicated by arrows; lane 2, PCR product amplified using total DNA from the liver of a congenitally DHBV-infected duck with high-level persistent infection; lane 3, empty; lanes 4 to 6, products of full-length PCR using autopsy liver from ducks with residual DHBV infection (ducks 4041, 3839, and 3637, respectively). The positive control (lane 2) and the three group C samples show a ~3-kbp product (indicated by an asterisk) and several smaller products from 1 to 1.8 kbp in length.

**TABLE 4. Time course of total DHBV DNA levels in the liver measured by quantitative PCR or Southern blot hybridization**

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>Day 3–4</th>
<th>1 mo</th>
<th>3 mo</th>
<th>6 mo</th>
<th>9 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>No. PCR positive/no. tested&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3/3</td>
<td>3/4</td>
<td>0/1</td>
<td>1/3</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>Mean copies/cell&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0034</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>B</td>
<td>No. PCR positive/no. tested</td>
<td>1/1</td>
<td>5/5</td>
<td>3/3</td>
<td>4/4</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td>Mean copies/cell</td>
<td>0.017</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>C</td>
<td>No. PCR positive/no. tested</td>
<td>5/5</td>
<td>3/3</td>
<td>1/1</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>Mean copies/cell</td>
<td>3.75&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.013&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0044</td>
<td>0.0042</td>
<td>0.007</td>
</tr>
</tbody>
</table>

<sup>a</sup> Samples were not available for all ducks at every time point.

<sup>b</sup> Total DHBV DNA was detected by quantitative PCR using primers P3 and P4 (Table 1). The assay had a sensitivity of 10 copies in 80,000 cells, or 0.00012 copies per cell.

<sup>c</sup> Determined by Southern blot hybridization using a 32P-labeled DHBV DNA probe.
FIG. 4. Southern blot hybridization to detect DHBV cccDNA in non-protein-bound DNA extracted from autopsy liver of the three group C ducks with residual DHBV infection. Samples from day 3 and 31 p.i. (3D and 31D, respectively) and 3, 6, and 9 months p.i. (3M, 6M, and 9M, respectively) show cccDNA detected in liver from duck 3637 until 3 months p.i., and in ducks 3839 and 4041 until 9 months p.i. Lane M, 80 pg of 3-kbp plasmid DHBV DNA. Non-protein-bound DNA extracted from 20 mg of liver was used for days 3 and 31 p.i. and from 150 mg of liver at the later time points. After Southern transfer the membrane was hybridized to a genome-length 32P-labeled DHBV DNA probe with autoradiography for 64 h. Copy numbers of cccDNA per cell are shown for the 9-month samples (<0.003, 0.008, and 0.009).

any inocula prepared from material from ducks with residual DHBV DNA. Infection was, however, transmitted by a liver homogenate prepared from a congenitally DHBV-infected duck, confirming the validity of the assay. The results of a total of six infection experiments are summarized in Table 5.

**TABLE 5. Attempts to demonstrate the presence of infectious DHBV in samples collected from ducks with residual DHBV infection**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Material and quantity inoculated into 1–2-day-old DHBV-negative ducklingsa</th>
<th>Route of inoculationa</th>
<th>No. of ducks inoculated</th>
<th>No. of ducks serum DHBsAg-positive/no. tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 mg liver (7 × 10⁷ cells) from a congenitally DHBV-infected duck</td>
<td>IP</td>
<td>2</td>
<td>2/2 (from day 6 p.i.)</td>
</tr>
<tr>
<td>2–4</td>
<td>100 mg liver (7 × 10⁷ cells) from ducks with residual DHBV DNA</td>
<td>IP</td>
<td>9</td>
<td>0/9</td>
</tr>
<tr>
<td>5</td>
<td>100 mg liver (7 × 10⁷ cells) from immunosuppressed ducks (group D)</td>
<td>IP</td>
<td>3</td>
<td>0/3</td>
</tr>
<tr>
<td>6</td>
<td>50 mg liver (3.5 × 10⁷ cells) from a duck with residual DHBV DNA</td>
<td>IV</td>
<td>2</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>6 ml of serum (suicide cushion purified)ab</td>
<td>IV</td>
<td>1</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>6 ml of spleen cell culture supernatant (suicide</td>
<td>IV</td>
<td>1</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>cushion purified)c</td>
<td>IV</td>
<td>3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

a One- to 2-day-old ducklings are highly susceptible to the development of persistent DHBV infection and can be infected with diluted duck serum containing the equivalent of 1 DHBV particle (13, 21, 22). IP, intraperitoneal; IV, intravenous.

b Ultracentrifugation onto a sucrose cushion was used to concentrate any virus present before inoculation into ducklings.

c Spleen cells were cultured for 3 days as previously described (25).

Levels of cyclosporine taken as trough (i.e., immediately before dosing) were 58, 134, and 244 μg/liter in the three treated ducks (1006, 1001, and 1005; the therapeutic range in humans is 80 to 250 μg/liter). Histological changes were assessed in sections of autopsy spleen and liver samples by a histopathologist who was not aware of the treatment status of each duck. Depletion of spleen cells was noted in two out of three treated ducks (1001 and 1005), but not in duck 1006 (which had the lowest serum level of cyclosporine) or in the control ducks (1003 and 1007) (data not shown). Liver tissue from the treated and control ducks was also examined, but no changes in liver inflammation or necroinflammatory activity were noted.

In vitro lymphocyte proliferation assays (22) were also performed using PBMC collected pretreatment and after 4 weeks of immunosuppressive therapy. However, levels of proliferation were inconsistent when PBMC were stimulated with the mitogen phytohemagglutinin, liver-derived DHBcAg, and DHBV particles, making the results difficult to interpret (data not shown).

Levels of residual DHBV DNA were measured in liver DNA extracts by quantitative PCR for total DHBV DNA using primers P3 and P4. No significant change in levels of DHBV DNA was found at 2 or 4 weeks after commencing immunosuppressive treatment compared to the pretreatment samples (Table 6). Similar results were seen in the ducks given placebo. It was concluded that immunosuppressive treatment had no effect on levels of residual DHBV DNA as determined in our assays.
DISCUSSION

The finding of residual DHBV DNA following recovery from transient infection is consistent with studies of HBV (17, 18, 24, 30, 41) and WHV (23) and is the first report of residual DNA in DHBV infection. Both quantitative PCR and Southern blot hybridization show that cccDNA is the predominant form of residual viral DNA in liver, suggesting that cccDNA is the key mediator of hepadnavirus persistence following transient infection.

The presence of residual DHBV DNA in 10 out of 11 liver samples from individual ducks, ~9 months p.i. and 8 months after the disappearance of detectable DHBsAg in the liver, indicates that complete clearance of viral DNA is not the usual outcome of transient infection, or if so, it takes longer than 9 months. Data from studies of HBV are less uniform, probably reflecting the limited tissue available in clinical studies (17, 18, 30, 41). In woodchucks, residual WHV DNA was found in liver and other tissues in all animals studied (23).

Residual DHBV DNA was found chiefly in liver and spleen with only 4 other samples testing positive (two kidney, one heart, and one adrenal). In addition the quantity of viral DNA was highest in liver, followed by spleen. This suggests that the liver is the most important site of residual DHBV DNA. The experience with solid-organ transplantation in humans is consistent with this in that the liver appears to be the only organ that, when transplanted after HBV infection, frequently transmits HBV infection (6). Most other reports are also consistent with this, although the finding of residual WHV predominantly in lymphoid tissue, including PBMC, in some woodchucks following transient infection has led to the proposal that lymphoid tissue is an important site for residual hepadnavirus infection (23). The findings of the current study differ significantly from those of the woodchuck study in that no DHBV DNA was found in PBMC at autopsy; although it must be remembered that the spleen is also a key lymphoid organ in which residual DHBV DNA was found.

Changes in levels of DHBV DNA in liver were studied over time. Although the data are more limited due to insufficient available tissue, levels fell during the first month and then persisted at a fairly constant level from 3 months p.i. until autopsy at 9 months p.i. This observation suggests maintenance of a residual steady state. In a formal sense this could be achieved either (i) by a balance between ongoing replication, the immune response and loss of virus and infected cells or (ii) by persistent inactive infection in long-lived cells. Our study next sought evidence to distinguish between these alternatives.

The presence of cccDNA is a sine qua non of true infection of a cell with a hepadnavirus, since this is the essential template for production of progeny virus (26, 36). The finding of cccDNA in liver is essential for but does not necessarily prove ongoing replication. Quantitation of DHBV DNA forms found in liver suggested that most residual DHBV DNA was cccDNA (range, 40 to 80%). This is in marked contrast to the findings in early infection after inoculation with 1010 virions (average of 1.2%; Table 2) or in the setting of congenital infection, where cccDNA represented less than 2% of the total pool of DHBV DNA (13, 14; Le Mire et al., unpublished).

This study is the first report in studies of hepadnaviruses to confirm unambiguously by Southern blot hybridization that most residual viral DNA is in the form of cccDNA. Previously, evidence of residual HBV cccDNA in human tissue was based on qualitative PCR (18, 20). This finding is consistent with the known relative stability of cccDNA and its place in the viral replication cycle in which it functions as the template for synthesis of pregenomic RNA and mRNA. The lack of viral DNA replication intermediates relative to cccDNA is consistent with the hypothesis of persistent inactive infection, although rapid removal of any cells that do support virus replication or express virus antigens cannot be excluded and would not negate the general hypothesis. Possible explanations for the lack of ongoing replication include (i) defective genomes may be present that do not allow replication or (ii) residual cccDNA may be present in cell types that do not permit replication or antigen expression or (iii) there may be endogenous or exogenous suppression of replication for example by cytokine effects. In each of these cases, antigen expression might not occur, thus assisting in evasion of immune responses.

We next sought to distinguish between these hypotheses using three approaches. Firstly, full-length PCR assays demonstrated that at least some of the residual DNA consisted of full-length DHBV DNA. Second, to examine whether any ongoing replication could be detected, we attempted to rescue replication-competent virus from tissues containing residual DHBV DNA; 1- to 2-day-old ducklings that are highly susceptible to DHBV infection (13, 21, 22) were inoculated with various preparations of liver shown to harbor residual virus DNA. In addition, spleen cells were harvested and cultured before inoculation into ducklings; this approach is similar to that used successfully by Michalak et al. (23) to infect woodchucks, and possibly analogous to the reactivation of herpes simplex virus from latently infected ganglia when these are maintained as explant cultures. Unlike observations with other hepadnaviruses, transmission experiments using inocula from ducks with residual DHBV DNA failed to show the presence of infectious virus.

HBV has been transmitted by liver transplantation using grafts from donors with past HBV infection (5, 6, 38). In this case the intact liver (over 1,000 g and containing up to 7 × 1011 cells) is transferred to the recipient. The current study used homogenates of 50 or 100 mg of liver representing 3.5 to 7 ×

---

TABLE 6. Levels of residual DHBV DNA under immunosuppressive treatment

<table>
<thead>
<tr>
<th>Duck no.</th>
<th>Pretreatment</th>
<th>2 weeks into treatment</th>
<th>4 weeks into treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1001b</td>
<td>0.0026</td>
<td>0.0016</td>
<td>0.0014</td>
</tr>
<tr>
<td>1005b</td>
<td>0.0025</td>
<td>0.00075</td>
<td>0.0018</td>
</tr>
<tr>
<td>1006b</td>
<td>0.0021</td>
<td>0.0021</td>
<td>0.0019</td>
</tr>
<tr>
<td>1003c</td>
<td>0.0086</td>
<td>0.0036</td>
<td>0.0150</td>
</tr>
<tr>
<td>1007c</td>
<td>0.0061</td>
<td>0.0012</td>
<td>0.0035</td>
</tr>
</tbody>
</table>

a Quantitative PCR for total DHBV DNA using primers P3 and P4 (Table 1) was performed on extracted liver DNA pretreatment and at 2 and 4 weeks of treatment.
b 40-day-old ducks inoculated with 1010 DHBV virions had resolved their infection by 1 month p.i. and were treated from 5 months p.i. with cyclosporin and dexamethasone for 4 weeks.
c 40-day-old ducks inoculated with 1010 DHBV virions had resolved their infection by 1 month p.i. and were treated from 5 months p.i. with placebo.

---
10^7 liver cells, which we estimate to contain 8.4 × 10^4 to 1.12 × 10^5 residual DHBV genomes. Although this did not fully replicate the process of human liver transplantation, which effectively involves placing and maintaining a large number of infected hepatocytes in a new host with inadequate immune responses, our failure to infect ducklings suggests that few or no free virions are available in these tissues. It is still possible that some virions are produced but cleared or inactivated rapidly by neutralizing anti-surface antibodies. However, our findings taken together suggest that any such replication is likely to be minimal.

Third, to examine whether an ongoing immune response was playing a role in containing or modulating residual infection, ducks harboring residual virus were treated for 4 weeks with immunosuppressive drugs and monitored for changes in levels of viral DNA in liver and serum. This was an attempt to replicate the anecdotal reports of reactivation of HBV in humans following immunosuppression (16, 17). No significant change was seen in the levels of virus DNA in three ducks, suggesting that, if immune containment was involved in regulating persistent infection, the effect was outside the parameters of this study.

Outside the liver, residual viral DNA was detected only in the total DNA fraction and not as cccDNA, raising the possibility that the extrahepatic residual DNA does not represent true infection. The presence of DHBV DNA without detectable cccDNA in spleen might reflect (i) binding or phagocytosis of circulatory virus by follicular dendritic cells or other phagocytic cells within the spleen (as previously described in ducks with persistent DHBV infection by Jilbert et al.) (12) or (ii) the presence of circulating virus which is detected in the vascular compartment in tissues, such as spleen and occasionally kidney. However, no residual viral DNA was detected in serum by either nested or quantitative PCR for total DHBV DNA. It is therefore likely that the DHBV DNA detected in extrahepatic tissues represents tissue-associated rather than circulating material, with the absence of detectable cccDNA meaning that true residual infection has not been demonstrated.

Integration of hepadnavirus DNA into cellular DNA is well recognized and might be considered a possible mechanism for persistence of DHBV DNA. Yang and Summers have reported integration of DHBV DNA in vivo at an estimated rate of 1 integration in 10^3 to 10^6 cells (40), and determined that in situ-primed linear and cohesive-end linear forms of DHBV DNA ranging from nucleotides 2485 to 2575 at one end and from nucleotides 2400 to 2537 at the other end are commonly integrated. However, these integrated DHBV DNA sequences would not be detected by our cccDNA assay as the region spanned by the cccDNA PCR primers CC2 and R2 (Table 1) is not continuous in the above integrated DHBV DNA forms. The low percentage of cells initially infected with DHBV in our studies (range, 1.5 to 3.8% in group C) make it unlikely that these cells would also contain integrated DHBV DNA that is being used as a template for expression of DHBV antigens. Hence, it is unlikely that integrated DNA forms a significant part of the pool of the residual DHBV DNA measured by quantitative PCR for cccDNA, although this cannot be ruled out.

The significance of residual hepadnavirus DNA, apart from the problems of transmission with liver transplantation and reactivation with immunosuppression, is not fully understood. In addition, the specific cell type that harbors residual DHBV DNA is not clear. Our failure to detect significant numbers of DHBsAg-positive cells despite the persistence of readily detectable levels of residual DHBV DNA suggests that the residual DHBV DNA is not in a highly active transcriptional state. Hepatocytes, Kupffer cells, and bile duct epithelial cells are among the candidates for the residually DHBV-infected cells. There may be effects of residual infection on the natural history of other liver diseases. There is some evidence that past HBV infection may be linked to a higher rate of cirrhosis from hepatitis C (3) and that hepatocellular carcinoma may be more common in those with hepatitis C who also have markers of past HBV infection and viral DNA sequences in the liver (15, 33). An epidemiological study of sporadic hepatocellular carcinoma, i.e., not related to active hepatitis B or C, showed anti-HBs antibodies were associated with a 4.7-fold increased risk of hepatocellular carcinoma (42).

Probably of greater significance is the fact that the existence of residual hepadnavirus DNA represents a host-parasite relationship in which a noncytopathic virus escapes eradication by an apparently effective immune response but is normally prevented from reactivation. This situation contrasts with the state of suppression of replication induced by clinical antiviral drugs, where reactivation of high-level replication will occur posttherapy unless an augmented immune response can be generated.

In summary, we have further defined a second stable form of persistent hepadnavirus infection, quite distinct from classical chronic infection characterized by extensive intrahepatic virus expression and surface antigenemia. This second form is marked by circulating surface antibodies, undetectable antigen expression within the liver and a preponderance of cccDNA rather than replicative virus DNA forms within the liver. Although under some circumstances virus reactivation or recovery of infectious virus may occur, our studies suggest that the majority of virus DNA present at this stage of infection occurs as inactive, nonreplicating cccDNA. Understanding further the mechanisms of these distinct states would be of great value in developing immune therapies for chronic HBV infection and in understanding the complexities of the virus/host relationship.

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