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Persistence of Extrahepatic Hepatitis B Virus DNA in the Absence of Detectable Hepatic Replication in Patients With Baboon Liver Transplants

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The presence of hepatitis B virus (HBV) DNA in extrahepatic tissues has been well documented. Whether HBV DNA can persist in extrahepatic tissues for long periods of time in the absence of replication in the liver has not been determined previously. Recently, two patients with end-stage liver disease secondary to chronic active HBV were treated with baboon liver xenotransplants as these animals are felt to be resistant to HBV infection. Multiple tissues from these two patients were examined for HBV DNA using polymerase chain reaction (PCR). HBV DNA was not detectable in four of five samples of the liver xenografts. A positive signal was observed in a single assay for one sample, but this sample was not positive in subsequent assays. HBV DNA was detected in peripheral blood lymphocytes, spleen, kidney, bone marrow, pancreas, lymph node, heart and small intestine. The level of HBV DNA in these tissues was too low for the detection of HBV DNA replicative intermediates by Southern hybridization; thus, it could not be determined whether the HBV DNA in these tissues represented actively replicating HBV in extrahepatic sites, integrated HBV sequences, HBV in infiltrating lymphocytes, or deposition of HBV immune complexes originating from the plasma. However, it is clear from this study that HBV DNA persisted in multiple tissues for 70 days after replication in the liver had ceased or at least was below the level of detection by PCR.

KEY WORDS: HBV DNA, PCR, extrahepatic HBV, xenotransplantation

INTRODUCTION

The Hepadnaviridae family is comprised of human hepatitis B virus (HBV) and several closely related animal viruses including duck hepatitis B virus (DHBV), woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV). All members of Hepadnaviridae have a primary tropism for the liver. Extrahepatic replication of hepadnaviruses is well documented in animal models, but characterization of extrahepatic replication in HBV-infected humans has been more problematic. The detection of viral DNA in a tissue is insufficient evidence of replication, and replicative forms of viral DNA and viral RNA have not been consistently observed, perhaps due to the low levels of nucleic acids present in these organs.

Recently, two baboon to human liver xenotransplantations were undertaken on patients suffering from end-stage liver disease secondary to chronic HBV infections [Starzl et al., 1993]. Xenotransplantation was undertaken, since allotransplantation of liver in HBV patients is associated with a poor prognosis largely because residual HBV in the recipient can infect the transplanted graft resulting in rapid liver destruction [Lake, 1991; Perrillo and Mason, 1993; Todo et al., 1991].

The mechanism of liver disease before transplantation is immunologically related, whereas the often rapid destruction of the transplanted organ is believed to result from the direct cytopathic effect of high levels of viral replication. Presumably, viral replication is elevated in transplant recipients due to immunosuppression. The use of HBV hyperimmune globulin to prevent infection of the transplanted organ has been only partially successful and may only delay infection of the transplanted liver. Whether infection of the transplanted organ is initiated by residual circulating virus derived originally from the liver or whether infectious virus from extrahepatic tissues is the source of infection has not been determined. The use of xenotransplants from a species not susceptible to HBV infection may overcome this obstacle.

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In the present study, tissues from the two recipients of baboon liver transplants were examined to ascertain that HBV would not replicate in the baboon livers and to determine whether HBV DNA would persist in extrahepatic tissues in the absence of replication in the liver. The results indicate that HBV DNA can persist for long periods of time in the absence of detectable replication in the liver. Longer periods of observation will be required to determine whether this procedure will eventually lead to the elimination of extrahepatic HBV DNA. However, the preliminary data from these short-term studies suggest that the procedure does overcome reinfection of the donor organ.

MATERIALS AND METHODS

Patients

Patient 1 was a 35-year-old male with end-stage liver failure secondary to chronic HBV infection. The patient was also infected with human immunodeficiency virus (HIV) [Starzl et al., 1993] and had undergone a splenectomy due to trauma several years prior to the transplant. Patient 2 was a 62-year-old male with chronic active HBV infection. The HBV serology of the patients was as follows: HBV DNA positive by PCR (Fig. 2), HBsAg-positive, anti-HBcAg-positive, and anti-HBsAg-negative. Patient 1 was anti-HBeAg- and HBeAg-negative, while patient 2 was anti-HBeAg-negative and HBeAg-positive. Both patients were anti-HCV- and delta virus-negative. Patient 1 was anti-HAV-negative, while patient 2 was anti-HAV IgG-positive, IgM-negative. The second patient received donor baboon bone marrow perioperatively [Starzl et al., 1994]. Patients were maintained on a four-drug immunosuppression therapy including FK506, prednisone, prostaglandin and cyclophosphamide. Serum samples were obtained weekly, lymph node and liver biopsies were obtained at various times and the spleen of patient 2 was removed on day 15 post-transplantation. All other tissues were obtained at autopsy.

Nucleic Acid Purification

Serum samples (50 μl) were mixed with an equal volume of TNES buffer (20 mM Tris, pH 7.4, 20 mM NaCl, 20 mM EDTA, 1% SDS), and tissue samples (~200 mg) were homogenized by grinding in 200 μl of TNES. Samples were digested with 10 μl of 5 mg/ml proteinase K for 2 hr at 65°C, were mixed with 1 μl of 10 mg/ml tRNA and 10 μl of 3 M sodium acetate, and were extracted with phenol/chloroform/isoamyl alcohol and then chloroform/isoamyl alcohol. DNA was precipitated with ethanol and resuspended in 50 μl of water. DNA concentrations were estimated by the formula OD@260 × 50 = mg/ml and by examination of each sample by gel electrophoresis and ethidium bromide staining.

Polymerase Chain Reaction and Southern Hybridization

Primers for PCR amplification were chosen using the OLIGO program and were selected from a highly conserved domain overlapping the core gene such that all sequenced HBV isolates could be amplified with this set of primers. The forward primer, 5′-CCTTGGGTG-GCTTTGGGGCA-3′, spanned nucleotides 1884–1904, using the EcoR1 site of the ayw sequence as nucleotide 1, and the reverse primer, 5′-GGGCATTTGGTGGTC-TATA-3′, spanned nucleotides 2295–2274. DNA from either 10 μl of serum or 500 ng of DNA from tissue samples was analyzed by PCR amplification with 3.6 min at 94°C for an initial denaturation step followed by 35 cycles of 1.3 min at 94°C, 2 min at 55°C, and 3 min at 72°C and a final step of 7 min at 72°C. Samples were analyzed by agarose gel electrophoresis and Southern hybridization with a ^{32}P-labeled, random-primed HBV DNA probe.

RESULTS

Xenotransplant Patients

Two patients have recently received baboon liver xenotransplants. Both patients had end-stage liver disease associated with chronic HBV infections and were not considered to be good candidates for human liver transplants. Baboon liver transplants were attempted, because the baboon liver is considered nonpermissive for HBV infection, and the use of the immunosuppressive drug FK506 was expected to prevent rejection. In addition, HBIG was administered at the time of transplants for both patients and was continued through the first week for patient 2 to facilitate clearance of residual virus. As a result, both patients were anti-HBsAg-positive and HBsAg-negative throughout the course of the study. Patient 1 survived for 70 days, and the clinical details of this case have been published previously [Starzl et al., 1993]. Patient 2 survived 27 days. Neither patient died due to rejection of the baboon liver, and no evidence of HBV replication in the baboon liver was observed by immunohistochemical staining for HBsAg or HBcAg [Starzl et al., 1993]. An analysis of tissue samples from these individuals by PCR for HBV DNA permitted an examination of several issues. Presumably, HBV would not replicate in the transplanted baboon livers, but the issue of whether the HBV infection would resolve in the absence of replication in the liver was unknown.

To optimize the detection of HBV DNA by polymerase chain reaction (PCR), primers were chosen, from the highly conserved core gene region (Fig. 1A), that were capable of detecting all sequenced isolates of HBV. PCR conditions were optimized using cloned HBV DNA. The level of sensitivity varied between 10 and 100 molecules following one round of 35 cycles of amplification and detection by Southern hybridization (Fig. 1B). Purification of HBV DNA from an infectious chimpanzee plasma (X328) using the same protocol as to be used for clinical samples showed a positive signal at a 10^{-6} dilution (Fig. 1C). Extrapolation of Southern blot data with this serum sample and cloned HBV DNA standards of known concentration estimated the 10^{-6} dilution to contain four molecules of DNA. Thus, this method generally has a sensitivity ranging from 4 to

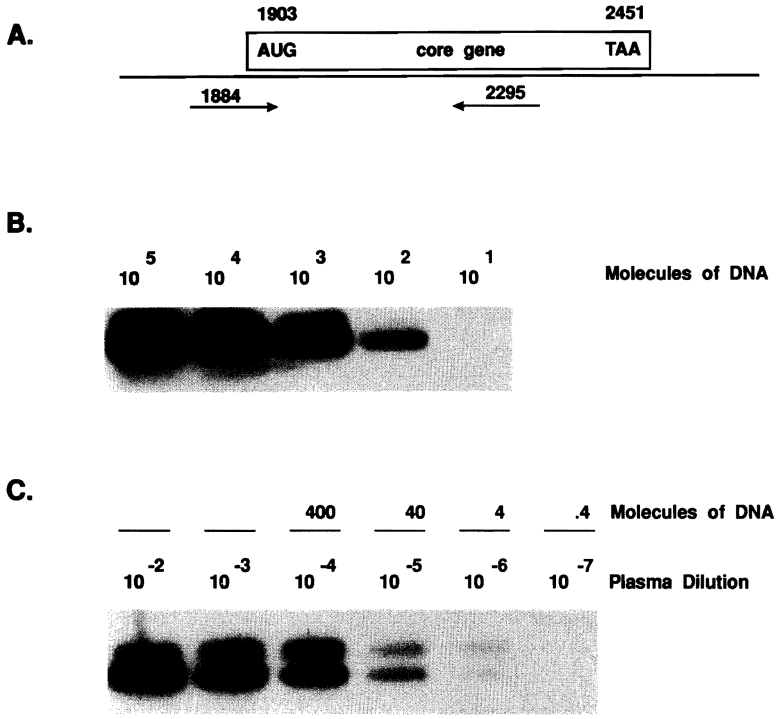

Fig. 1. Standardization of PCR for HBV DNA. **A:** The region of the HBV genome being amplified is depicted including the nucleotide numbers spanning the open reading frame for the core gene. The forward and reverse primers are indicated by arrows below the core gene and the numbers indicate the 5′ nucleotide of each primer. Primers were selected to amplify all sequenced strains of HBV. **B:** The sensitivity of the PCR amplification was evaluated with HBV plasmid DNA. Tenfold dilutions of plasmid DNA were amplified and PCR products were detected by agarose gel electrophoresis and Southern hybridization. **C:** The sensitivity of the extraction, purification and amplification of an HBV-positive chimpanzee (×328) plasma was examined. The number of molecules of HBV DNA in the plasma was estimated by comparison to HBV plasmid DNA of known concentration using Southern hybridization. The sensitivity of amplification ranges from 4 to 100 molecules.

100 molecules in different experiments. The 10^{-4} and 10^{-5} dilutions of the chimpanzee sera were included in all experiments as sensitivity controls.

Examination of serial serum samples from patients 1 and 2 revealed positive signals prior to transplant and intermittent signals thereafter. Of nine samples taken after transplant for patient 1, only the sample on day 31 was positive (Fig. 2). Patient 2 was positive on day 3 and 22 post-transplantation, but was negative on days 9 and 17 (Fig. 2). All positives were confirmed in at least two assays. Some positive samples were not positive in all assays, presumably because the level of HBV DNA in all of the samples was near the limits of detection in our assay, and thus stochastic variation would result in some samples being positive intermittently.

Analysis of DNA from multiple tissues revealed that most were strongly positive by PCR. Each sample was run at 500 ng of DNA such that the level of HBV DNA in each tissue could be directly compared. Peripheral blood lymphocytes (PBLs) from patient 1 were negative 26 days prior to transplantation and positive 24 and 25 days after transplantation (Fig. 3). PBLs for patient 2 were only available 25 days post-transplantation and were negative at that time. For patient 1, a lymph node biopsy on day 11 was positive, while a liver biopsy sample on day 16 was negative. The DNA from liver tissue obtained from patient 1 at autopsy was negative, but due to extensive degradation of the cellular DNA, interpretation of the analysis of this sample must be made with caution. Two kidney samples, a lymph node and heart tissue, taken at autopsy, were all positive for patient 1. A more extensive set of tissues was available from patient 2. Spleen tissue obtained at the time of splenectomy (day 15 after transplantation) was highly positive. A liver biopsy on day 12 and autopsy liver tissue (day 27) were negative. A liver biopsy from day 24 was positive in a single assay, but was not positive in subsequent assays. Since we have demonstrated that baboons cannot be infected with HBV (unpublished data), and 4 of 5 liver samples from patients 1 and 2 were PCR-negative including a sample 3 days later at the time of autopsy, we assume that the day 24 liver biopsy may have been positive due to the presence of positive PBLs in that sample. Alternatively, since this sample was not positive in subsequent assays, it could represent PCR contamination despite the extensive precautions to prevent contamination and the absence of contamination in the negative controls. Of tissue samples obtained from patient 2 at autopsy, lymph node, pancreas, kidney, bone marrow, and small intestine were positive, while the liver was negative.

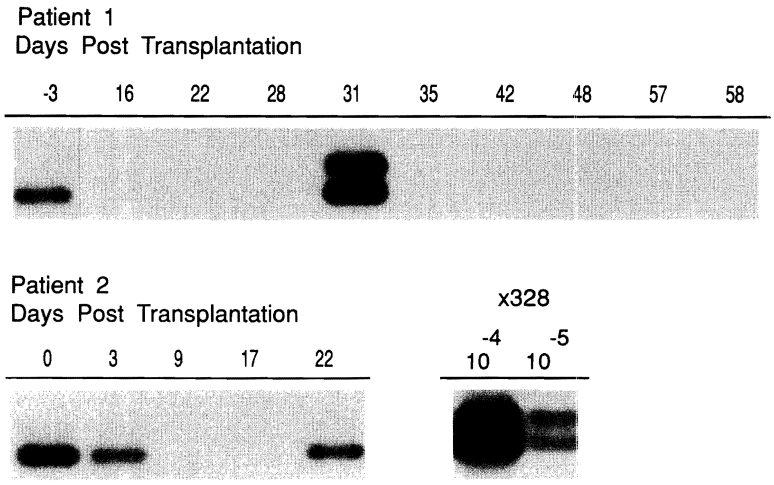

Fig. 2. PCR analysis of HBV DNA in serial serum samples from the two baboon liver recipients HBV DNA from serum samples was extracted, purified and amplified as described in Materials and Methods. PCR products were detected by agarose gel electrophoresis and Southern hybridization. The 10^{-4} and 10^{-5} dilutions of plasma from chimpanzee ×328 were included as sensitivity controls and are the same dilutions as analyzed in Figure 1C.

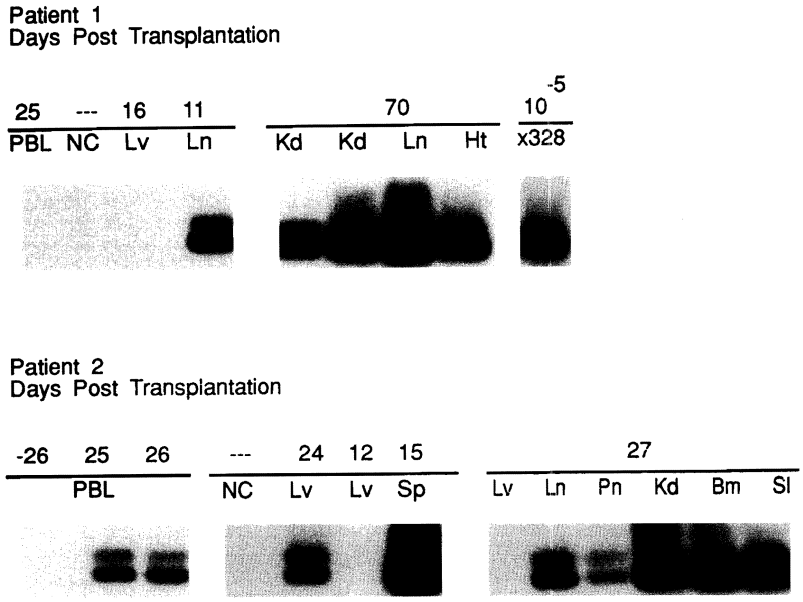

Fig. 3. PCR analysis of HBV DNA in tissue samples from the two baboon liver recipients. DNA was purified from various tissues as described in Materials and Methods and 500 ng of purified DNA was amplified by PCR. The PCR products were detected by agarose gel electrophoresis and Southern hybridization. The days post-transplantation that PBL and biopsy tissues were obtained are indicated as well as the day post-transplantation that tissues were obtained at autopsy, days 70 and 27 for patient 1 and 2, respectively. Two different kidney (Kd) samples were analyzed from patient 1. Multiple tissues were positive for HBV DNA with one of the five baboon liver samples being positive presumably from infiltrating lymphocytes. NC, negative control; PBL, peripheral blood lymphocytes; Lv, liver; Ln, lymph node; Kd, kidney; Ht, heart; Sp, spleen; Pn, pancreas; Bm, bone marrow and SI, small intestine.

DISCUSSION

Due to the limited duration of the current study, it is not possible to determine conclusively whether baboon livers transplanted into humans infected chronically with HBV are resistant to HBV infection. One of five liver biopsies from humans that received baboon liver transplants was PCR-positive in a single assay. Although it could be argued that the positive PCR signal represents possible infection of the liver, it is more likely that the HBV DNA detected was present in lymphocytes. Examination of this liver at autopsy by immunohistochemical staining failed to detect HBsAg or HBcAg in hepatocytes [Starzl et al., 1993, 1994]. The level of DNA detected in this liver biopsy by PCR was

too low to be detected by Southern hybridization (data not shown), and thus it is not possible to determine whether replication of HBV was occurring. The extreme sensitivity of PCR to monitor infection should have allowed early detection of infection of the baboons livers. The PCR negativity of four of five liver samples is suggestive that the baboon livers were resistant to infection, but an increased survival time for the patients would be required to answer conclusively this question.

HBV DNA persisted in multiple tissues in humans in the absence of detectable replication in the liver. The level of HBV DNA in these tissues was also insufficient to detect by Southern hybridization; thus it was again impossible to determine whether HBV replication was occurring. Viral DNA has been demonstrated in multiple tissues in hepadnavirus animal models and in HBV-infected humans. In the duck, extrahepatic DHBV replication has been detected in the pancreas, spleen and kidney [Halpern et al., 1986; Jilbert et al., 1987; Hosoda et al., 1990] with some evidence for replication in brain, lung, heart and intestine [Hosoda et al., 1990]. In the woodchuck, extrahepatic replication of WHV is detected primarily in the spleen [Korba et al., 1987]. WHV DNA present in peripheral blood lymphocytes is mostly nonreplicative forms; however, in vitro stimulation of PBLs from chronically infected woodchucks with lipopolysaccharide results in the appearance of replicative forms of viral DNA [Korba et al., 1988].

There are many reports on the detection of HBV nucleic acids in PBLs using a variety of techniques including Southern hybridization, in situ hybridization and polymerase chain reaction [Pontisso et al., 1984; Davidson et al., 1987; Lamelin and Trepo, 1990; Leung et al., 1994; Mason et al., 1992; Hadchouel et al., 1988]. In many instances, replicative forms of HBV DNA were either absent [Pontisso et al., 1984; Davidson et al., 1987] or not definitively demonstrated. The HBV DNA in PBLs is presumably not derived from adherence of circulating virus, since HBV DNA can be detected in the PBLs of patients that have cleared serum HBV DNA or HBsAg [Pontisso et al., 1984; Leung et al., 1994; Mason et al., 1992; Hadchouel et al., 1988]. In other studies, HBV nucleic acids have been detected in multiple tissues [Mason et al., 1993; Dejean et al., 1984; Yoffe et al., 1990] with some reports detecting such widespread distribution as to include lymph nodes, bone marrow, spleen, kidney, skin, colon, stomach, testes and periadrenal ganglia [Mason et al., 1993]. It is not known whether these tissues are capable of supporting the complete replication cycle and the production of infectious virus and whether extrahepatic DNA would persist in the absence of hepatic replication.

The PCR signal observed in extrahepatic tissues in this study could originate from several sources. It may represent replication of HBV in cells of that organ. The DNA may reside within lymphocytes present in these organs, whether it is active in replication or not. The HBV DNA could be due to sequestered immune complexes derived from preexisting plasma virus and the injection of HBIG. Inoculation of rhesus monkeys with a high dose of HBV apparently resulted in detection of carry over of the inoculum for a period of 3 months [Lazizi and Pillot, 1993]. Finally, the signal observed in various organs may not represent free viral DNA, but may be from integrated HBV DNA sequences as a consequence of the long-term chronic infections. Thus, in the absence of definitive markers of replication, it is not possible to determine the origin of the HBV DNA in the various tissues.

This study confirms the lack of detectable HBV DNA replication in the baboon liver. The lack of detectable HBV replication in the donor liver and the resistance of baboons to HBV infection (unpublished data) suggests that a maintenance regimen with HBIG is probably not required. The potential for detrimental effects accompanying the use of HBIG and the immune complexes derived from residual HBsAg and hepatitis B virions at the time of transplantation may be greater than any benefit derived from neutralization of the virus. Residual virus in the plasma should eventually be cleared, unless extrahepatic tissues are actually capable of sustaining an active HBV infection. The answer to this question will require longer-term survival for future patients receiving xenotransplants.

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