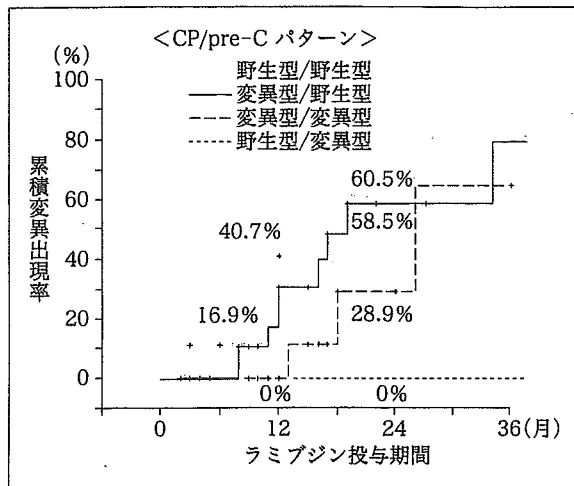

図4　ラミブジン投与後の耐性株出現

は，CP領域変異型では16.9%で，さらに，CP領域野生型では40.7%と高率であった．pre-C領域が変異型の場合には，投与開始1年以内の耐性出現例を認めなかった．そのなかで，CP領域変異型の症例では，開始1年後を過ぎてから耐性が出現し始め，3年後にはpre-C野生型の症例と同等の耐性出現率となったが，CP領域野生型の症例からは，観察期間中には変異は生じなかった．

おわりに

pre-C領域およびCP領域の変異測定により，従来からB型肝炎ウイルスの変化の指標とされているHBe抗原/抗体とは異なった情報が得られる．本稿で示したように，B型肝炎患者の経過・予後の推定の参考になるほか，ラミブジン治療において，治療効果および耐性出現のリスクに関する情報が得られ，治療方針を決定する際に有用であると考えられる．

文　献

1) Carman, W. F., Jacyna, M. R., Hadziyannis, S., et al.：Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet 2；588-591, 1989
2) Tacke, F., Gehrke, C., Luedde, T., et al.：Basal core promoter and precore mutations in the hepatitis B virus genome enhance replication efficacy of lamivudine-resistant mutants. J. Virol. 78；8524-8535, 2004
3) Asahina, Y., Izumi, N., Uchihara, M., et al.：Core promoter/pre-core mutations are associated with lamivudine-induced HBeAg loss in chronic hepatitis B with genotype C. J. Hepatol. 39；1063-1069, 2003
4) Okamoto, H., Tsuda, F., Akahane, Y., et al.：Hepatitis B virus with mutations in the core promoter for e antigen-negative phenotype in carriers with antibody to e antigen. J. Virol. 68；8102-8110, 1994
5) Nagasaka, A., Hige, S., Marutani, M., et al.：Prevalence of mutations in core promoter/precore region in Japanese patients with chronic hepatitis B virus infection. Dig. Dis. Sci. 43；2473-2478, 1998

臨牀消化器内科 2005 Vol.20 No.13
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C型慢性肝炎に対するインターフェロン治療と2′-5′オリゴアデニル酸合成酵素(2-5 AS)の変動

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はじめに

C型慢性肝炎に対する治療として，わが国では1992年からインターフェロン(IFN)が使用可能となり，さらに，2001年にIFNα2bとリバビリンの併用療法，2003年にPEG-IFNα2aの48週間投与，2004年にPEG-IFNα2bとリバビリンの併用療法が，それぞれ可能となり，治療成績の向上が認められてきている．とくにPEG-IFN製剤は，週1回の投与が可能となり，治療のコンプライアンスを高めるうえでも有用であるが，2種類の製剤の投与量設定方法には違いがある．

投与したIFNの抗ウイルス機序に関して多くの作用が報告されているが，抗ウイルス活性の臨床的評価が可能で従来からよく検討されているものに2′-5′オリゴアデニル酸合成酵素(2-5 AS)がある．われわれは，2-5 AS活性とHCV-RNAの減少度の関連性について報告[1]してきたが，本稿では，とくにC型慢性肝炎に対するPEG-IFNによる治療における2-5 ASの変動，および投与量による差異を，われわれの治療成績から検討し，IFNの抗ウイルス効果や用法･用量の決定について，考察する．

I．IFNの作用機序と2-5 AS

投与されたIFNは，肝細胞表面のIFNレセプターに結合し，シグナルが伝達されて多くの遺伝子発現が誘導される[2]．IFNαのシグナル伝達には1型IFNレセプターであるIFNAR 1とIFNAR 2の二つのサブユニットが必要で，IFNαの結合により2種類のチロシンキナーゼ(Jak 1，Tyk 2)が活性化され，さらに，細胞質にある転写因子群(STAT 1およびSTAT 2)がリン酸化される．活性化したSTAT 1，STAT 2はIFR 9(IFN regulatory factor 9)/p 48とともにISGF 3(IFN-stimulated genes factor 3)を形成し，核内に移行してIFN誘導遺伝子の上流に存在するISRE(IFN-stimulated response element)に結合する．IFNによ

Key words：C型慢性肝炎，2′-5′オリゴアデニル酸合成酵素，比体重インターフェロン投与量，PEGインターフェロン

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0911-601X/05/JCLS

り誘導される遺伝子には，2-5 ASのほかに，MxA，RNA依存性プロテインキナーゼ(PKR)などが知られている．

2-5 ASは，ウイルス増殖過程で形成される2本鎖RNAと結合して活性化され，ATPを基質としたアデニル酸のオリゴマー(2-5 A)を合成する．合成された2-5 AがRNaseを活性化しウイルスmRNAを分解することにより，ウイルスの増殖が阻害される．

C型肝炎ウイルス(HCV)と2-5 AS活性の関連について，Murashimaら[3]は，HCVのNS 5 A領域のインターフェロン感受性決定領域(ISDR；interferon sensitivity determining region：NS 5 A 2209-2248)にアミノ酸変異を有する例で2-5 AS活性が有意に高いことを報告している．また，Taguchiら[4]は，ISDRより前の部分のNS 5 A蛋白が2-5 ASと結合してIFNの抗ウイルス活性を抑制することを報告し，HCVと2-5 ASの直接的な関連性について示している．

2-5 ASの測定に関しては，以前は末梢血リンパ球を検体としていたが，現在は血清中酵素を測定している．正常値は100 pmol/dl以下とされている．

II．C型慢性肝炎に対するIFNの用法・用量

従来のIFNの1回投与量は種類により異なるが，多くは600〜1,000万単位が投与されていた．最近，週1回の投与が可能となったPEG-IFN製剤には2種類の製剤があるが，1回投与量の設定方法は異なっている．PEG-IFNα 2 aは，通常，成人には1回180 μgを週1回，皮下に投与する．体重が50 kg未満の場合には慎重投与とするが，基本的には体重にかかわらず一定量を投与する．PEG-IFNα 2 bは，リバビリンと併用のうえ，通常，成人には1回1.5 μg/kgを週1回皮下投与する．PEG-IFNα 2 b投与量の目安は，体重35〜45 kg，46〜60 kg，61〜75 kg，76〜90 kg，91〜120 kgに対して，それぞれ，60 μg，80 μg，100 μg，120 μg，150 μgの投与となっている．両剤とも減量基準が定められており，白血球数・好中球数・血小板数などの減少程度に応じてPEG-IFN投与量を半量に減量する．

III．IFN投与開始後の2-5 AS活性の上昇

治療開始4週間の2-5 AS活性の上昇程度を，IFNの投与法により比較した(図1)．IFNの投与量は，IFNα・IFNα 2 bは1回600万単位，IFNβは1回300万単位を1日2回，PEG-IFNα 2 aは180 μg，PEG-IFNα 2 bは1.5 μg/kgの症例で検討した．

検討対象は，①IFNαの2週間連日，以後週3回投与群26例，②IFNαの4週間連日，以後週3回投与群19例，③リバビリン併用下にIFNα 2 bの2週間連日，以後週3回投与群20例，④IFNβの4週間1日2分割投与後，週3回投与群10例，⑤PEG-IFNα 2 aの週1回投与群10例，⑥リバビリン併用下にPEG-IFNα 2 bの週1回投与群11例とした．

いずれの投与法でも2週間後には有意な2-5 AS活性の上昇を認めたが，PEG-IFN製剤投与例やIFNβ 1日2分割投与例で，4週後も高値を維持する傾向を認めた．従来型IFNと比較してPEG-IFN製剤投与時に有意に4週後の2-5 AS活性値の上昇を認めており，週1回の投与ながら従来のIFN以上の抗ウイルス活性が期待できることが示唆された．

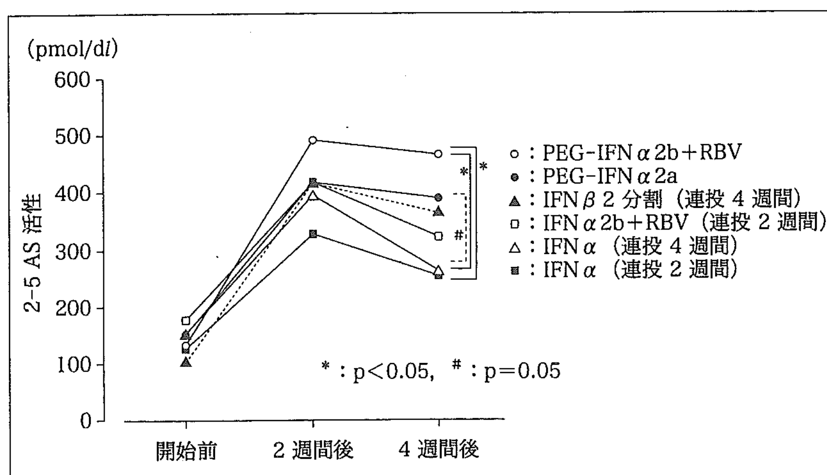

図1 IFN 投与開始 4 週間の 2-5 AS 活性の上昇

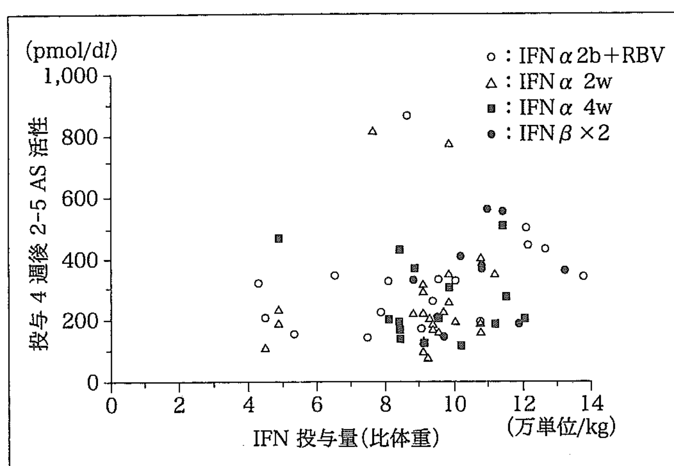

図2 体重当りの IFN 投与量・種類と治療開始 4 週間後の 2-5 AS 活性

IV. 体重を考慮した場合の IFN 投与量と 2-5 AS 活性

従来の IFN や PEG-IFNα 2 a は，通常は患者体重によらず一定の投与量で治療されることが多かったが，2-5 AS 活性上昇に対する体重の影響を検討した．従来型の IFN 治療の場合，減量例を含め，体重当りの IFN 投与量や投与 IFN の種類と治療開始 4 週間目の 2-5 AS 活性には一定の傾向は認められなかった(図 2)．2-5 AS 活性の測定値は，IFN 投与量・種類よりも，個体差が大きい結果であると考えられた．

PEG-IFNα 2 a の場合，基本的には体重にかかわらず一定量の投与であるため，比体重の IFN 投与量は症例差が大きい(図 3)．減量例も含めた，治療開始 4 週間以後の IFN 投与量と 2-5 AS 活性には，全体としては有意な相関関係がみられた．しかし，基本的な PEG-IFNα 2 a 投与量である 2.0〜3.5(μg/kg/

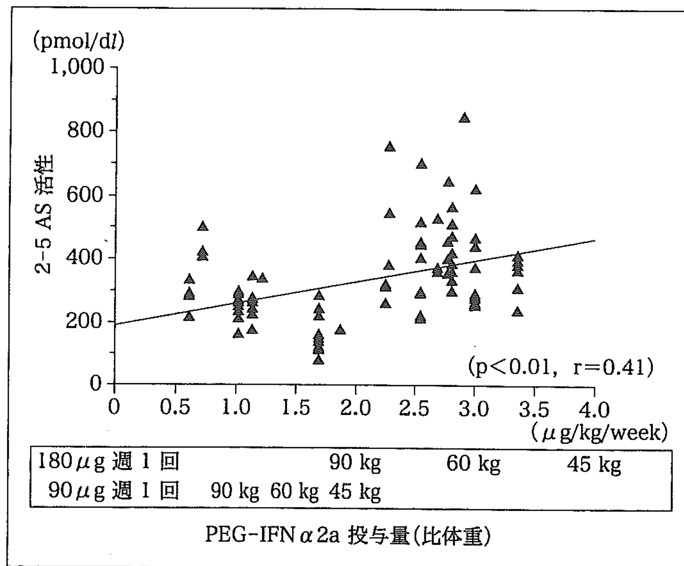

図3　PEG-IFNα 2 a 投与量と 2-5 AS 活性
(治療開始 4 週以降)
下段枠内は，1 回投与量を 180 μg あるいは 90 μg とした場合の比体重投与量と対応する体重を示している．

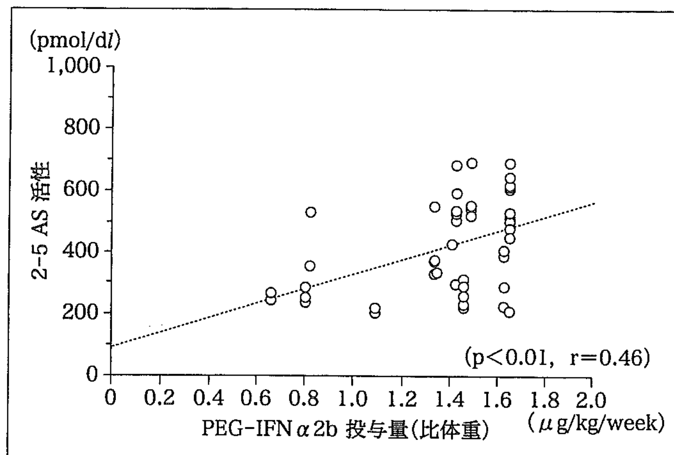

図4　PEG-IFNα 2 b 投与量と 2-5 AS 活性
(治療開始 4 週以降)

week)の範囲では，比体重の IFN 投与量と 2-5 AS 活性には，相関はみられなかった．したがって，2-5 AS 活性の上昇程度を臨床的に評価した場合には，PEG-IFNα 2 a の投与量が画一的であっても支障は認められなかった．

PEG-IFNα 2 b の場合は，減量例を除き，基準となる投与量(1.5 μg/kg)前後に集中して分布している(図 4)．減量例を含めた全体の傾向では有意な相関が認められるが，ほかの IFN と同様に，症例間の差が大きい結果であった．

V．IFN の減量と 2-5 AS 活性

PEG-IFNα 2 a の治療経過中の 2-5 AS 活性の変動を，経過中の投与量減量，あるいは，投与間隔延長の有無別に検討した(図 5)．投与量の減量や投与間隔の延長などを行った症例では，2-5 AS 活性の低下が示された．2-5 AS 活性は症例間の差は大きいが，単一症例を経時的に見た場合には，投与 IFN 量と相関した変動を認める．したがって，個々の症例の IFN 治療効果のレベルの変動を評価するのに有用なマーカーと考えられた．

多くの場合，白血球，好中球，血小板などの血球減少のため，IFN 投与量の減量を余儀なくされるが，2-5 AS 活性低下の結果からは，抗ウイルス効果に関して不利になると思われる．しかし，図 3 に示されるように，比体重の IFN 投与量は少なくても比較的高い 2-5 AS 活性が示される症例もあり，このような症例では無理に IFN 投与量を増やさずに治療を継続するほうが望ましいともいえる．

VI．PEG-IFN 投与量と体重

従来の IFN 治療では，体重は考慮されずに，一定量が投与されている場合が多かった．個体差が大きく，また，通常の投与期間であった 6 カ月では，体重別に抗ウイルス効果の有意差はみられなかった．

最近使用可能となった PEG-IFN は，標準治療期間が 48 週間に延長されたが，2 種類の製剤が使用可能となっている．PEG-IFNα 2 a は，IFNα 2 a に分子量 20 kDa の PEG 鎖 2 本をウレタン結合させたもので，分子量が比較的大きい．そのため，細胞間を容易に通り抜けられずに，血管内や血液供給の多い臓器(肝，腎，膵など)に分布するとされている．したがって，体重の影響が少なく，投与量は一定に設定されている．一方，PEG-IFNα 2 b は，IFNα 2 b に 1 本の PEG 鎖をアミド結合させたもので，PEG-IFNα 2 a に比べて，分子量は小さく，半減期も短縮される．基本的には体重当り一定の投与量に設定されている．

図 3 および図 4 で示したように，比体重の IFN 投与量は，PEG-IFNα 2 a では範囲が広

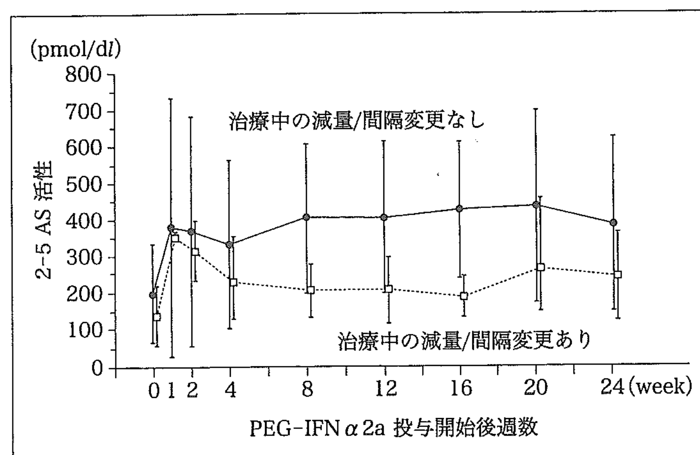

図 5　PEG-IFNα 2 a 投与量変更と 2-5 AS 活性の変動

く，PEG-IFNα 2 b ではほぼ一定であった．また，標準的な PEG-IFNα 2 a 治療の場合には，比体重 IFN 投与量と 2-5 AS 活性には相関は認められなかった．しかし，今回の検討対象である 2-5 AS 活性は個体差が大きい因子のために，このような評価には適当でない可能性も残される．

現在までの，われわれの症例における PEG-IFN 治療開始時体重は，PEG-IFNα 2 a(92例)では，平均 59.3 kg，標準偏差 11.1 kg(40 kg～91 kg)，PEG-IFNα 2 b 投与例(87例)では，平均 64.2 kg，標準偏差 11.5 kg(42 kg～95 kg)である．実際に，症例間の体重差は最大で 2 倍以上に達しており，体重差の影響が少ないとされる PEG-IFNα 2 a においても，比体重での IFN 投与量を意識することは有意義であると思われる．この点は，治療期間の長期化により，さらに重要になると思われる．

まとめ

C 型慢性肝炎の抗ウイルス治療の反応を 2-5 AS を指標に検討した．2-5 AS 活性は個体差が大きいため，治療効果と必ずしも強い相関を示さないものの，初期値と比較した際の治療反応性や減量時の有効性などを経時的に確認する場合には評価の参考になる．

2-5 AS を指標とした，比体重 IFN 投与量と抗ウイルス活性程度の検討では明解な関連性は得られなかったが，今後，使用する IFN の薬物動態を考慮のうえ，他の指標を用いて同様の検討を行う必要があると思われる．

文　献

1) Karino, Y., Hige, S., Saga, A., et al. : Relationship between serum 2-5 AS activity and the reduction of HCV-RNA during interferon therapy. Viral Hepatitis and Liver Disease (1993 ISVHLD) ; 631-634, 1994
2) Gao, B., Hong, F. and Radaeva, S. : Host factors and failure of interferon-treatment in hepatitis C virus. Hepatology 39 ; 880-890, 2004
3) Murashima, S., Kumashiro, R., Ide, T., et al. : HCV NS 5 A 2209-2248 amino acid substitutions affect serum 2,5 AS activities. J. Hepatol. 31 ; 382, 1999
4) Taguchi, T., Nagano-Fujii, M., Akutsu, M., et al. : Hepatitis C virus NS 5 A protein interacts with 2′,5′-oligoadenylate synthetase and inhibits antiviral activity of IFN in an IFN sensitivity-determining region-independent manner. J. Gen. Virol. 85 ; 959-969, 2004

Journal of Medical Virology 69:41–49 (2003)

Histological Improvement of Chronic Liver Disease After Spontaneous Serum Hepatitis C Virus Clearance

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The long-term histological and virological outcomes of spontaneous circulating hepatitis C virus (HCV) clearance were studied in chronic liver disease. Between 1979 and 1984, three patients underwent laparoscopy for chronic non-A, non-B liver disease, and two were found to have cirrhosis and one with chronic active hepatitis. After HCV assays became available in 1990, they were positive persistently for HCV antibody without serum HCV RNA. Reductions of antibody levels to HCV core and/or nonstructural proteins were observed, and liver biopsies were undertaken between 1995 and 2000. Liver biopsies at 11–19 years after laparoscopy disclosed marked alleviation of liver inflammation and fibrosis in each case although a low grade of inflammation remained. The two patients with cirrhosis no longer showed histological features of cirrhosis, and the poor liver function in one patient had been ameliorated. Liver specimens from two patients were subjected to polymerase chain reaction to detect positive and negative HCV RNA strands and hepatitis B virus DNA. Only the positive HCV RNA strand was detected for one patient who had previously cirrhosis. Liver specimens were examined from another six nonviremic HCV-seropositive individuals without chronic liver disease. Five patients displayed low-grade liver inflammation without evident fibrosis, but none had any viral genome in the liver. These findings suggest that spontaneous circulating HCV clearance in chronic liver disease confers favorable liver histological outcome, although occult HCV infection persists. ***J. Med. Virol. 69:41–49, 2003.*** © 2003 Wiley-Liss, Inc.

KEY WORDS: HCV antibody; HCV RNA; hepatitis C

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection is the major cause of liver cirrhosis and hepatocellular carcinoma worldwide. However, spontaneous circulating HCV clearance is not a very rare event. An unexpectedly high incidence of HCV clearance from the serum has been reported for children [Vogt et al., 1999] and drug users [Beld et al., 1999]. Consequently, there are individuals who have circulating HCV antibodies but are nonviremic, indicating possible recovery from infection. Recently, several groups have reported that low levels of ongoing intrahepatic HCV replication can be present in such individuals [Haydon et al., 1998; Dries et al., 1999]. Thus, the clinical relevance of nonviremic HCV-seropositive states, which occurs in the natural course, has not been fully elucidated. Sustained HCV clearance from sera after interferon (IFN) therapy is usually accompanied by intrahepatic HCV clearance [Marcellin et al., 1997; Lau et al., 1998], decrease in HCV antibody levels [Saracco et al., 1993; Yuki et al., 1993a,b; Diodati et al., 1994], and histological improvement [Marcellin et al., 1997; Lau et al., 1998]. The clinical implications of HCV antibody-positive but serum HCV RNA-negative states in the natural course may differ from those observed after successful IFN therapy. To address these issues, long-term histological and virological outcomes of nonviremic HCV-seropositive states were investigated in patients not given IFN therapy.

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Accepted 19 February 2002
DOI 10.1002/jmv.10250
Published online in Wiley InterScience (www.interscience.wiley.com)

MATERIALS AND METHODS

Patients

Between 1979 and 1984, three patients with chronic non-A, non-B liver disease (2 males and 1 female, age range = 50 to 55 years) underwent diagnostic laparoscopy. They had been negative persistently for hepatitis B surface antigen (HBsAg). They had had no history of administration of hepatotoxic drugs or alcohol abuse (>80 g/day) and showed no evidence of autoimmune liver disease. Thus, there was no apparent cause of hepatocellular injury. Laparoscopic and histological findings of two patients showed the features of liver cirrhosis and chronic active hepatitis, respectively. Laparoscopic findings in another patient showed typical features of established cirrhosis although liver biopsy was not carried out. He also had esophageal varices and other symptoms of liver cirrhosis. After HCV was discovered, these three patients were found to be positive for HCV antibody without viremia between November 1990 and January 1991. They were positive for HCV antibody by a first-generation enzyme-linked immunosorbent assay (ELISA) (Ortho Diagnostic Systems Co., Ltd., Tokyo, Japan). These serum samples were retested later with a second-generation assay, and the results were confirmed. Despite the presence of circulating HCV antibody, all three patients were negative for serum HCV RNA by reverse transcription-polymerase chain reaction (RT-PCR). Thereafter, HCV antibody profiles, serum HCV RNA, and routine liver function tests were monitored for 8–9 years. Between 1995 and 2000, these three patients underwent ultrasound-guided liver biopsies again, and the biopsy specimens were reevaluated histologically and virologically. All liver biopsies were performed using 14-gauge Tru-Cut needles to obtain biopsy specimens sufficient for histological evaluation.

Another six nonviremic HCV-seropositive individuals without history of chronic liver disease (3 males and 3 females, age range = 51 to 70 years) underwent liver biopsies in 1999 and were also subjected to histological and virological evaluation. One patient had an episode of acute hepatitis C in February 1998. The diagnosis was based on marked elevation of serum alanine aminotransferase (ALT) activity (peak value = 1,448 U/L) and detection of serum HCV antibody and HCV RNA. The disease resolved spontaneously in one month with disappearance of serum HCV RNA. Thereafter, circulating HCV antibody remained detectable by third-generation ELISA. However, HCV antibody levels decreased, and liver biopsy was done in July 1999. The remaining five patients had had no symptomatic episode of acute hepatitis. Two of these five patients had histories of blood transfusions 46 and 49 years earlier. None had any cause of liver disease other than HCV except that fatty liver was revealed by ultrasonography in one patient. The biopsy study was approved by the local Research Ethics Committee in accordance with the 1975 Declaration of Helsinki, and all patients provided written informed consent.

Laboratory Tests

HCV antibody profiles were tested with a third-generation recombinant immunoblot assay (RIBA-3) (Ortho Diagnostic Systems Co., Ltd.). The accuracy of changes in antibody levels was validated by repeating the assay. Quantitation of the HCV core antibody was also undertaken using a commercially available kit (HCV Core-Ab IRMA, Ortho Diagnostic Systems Co., Ltd.). Serum HCV RNA was detected by RT-PCR. HCV RNA was extracted from 100 μL of serum samples, copied into complementary DNA (cDNA) by RT, and amplified by PCR as described elsewhere [Hagiwara et al., 1993]. Primers were derived from the 5′-noncoding region of the published sequence [Takamizawa et al., 1991]; antisense primer 5′ATGGTGCACGG TCTACGAGACCTCC3′ and sense primer 5′CACTCCCCTGTG AGGAACTACTGTC3′.

Serum samples were tested for HBsAg, antibody to HBsAg (anti-HBs) and antibody to hepatitis B core antigen (anti-HBc) by radioimmunoassay (Abbott Laboratories, North Chicago, IL). To exclude occult hepatitis B virus (HBV) infection, serum HBV DNA was detected by real-time detection PCR based on Taq Man chemistry as previously reported [Abe et al., 1999]. A portion of the HBV surface region was amplified using set 2 primers: antisense primer HBSF2 (5′CTTCATCCTGCTGCTATG CCT3′, nucleotide positions [nt] 406-426) and sense primer HBSR2 (5′AAAGCCCAGG ATGATGGGAT3′, nt 627-646). Another portion of the HBV X region was amplified further using set 3 primers: antisense primer HBXF1 (5′ACGTCCTTTGTTTACGT CCCGT3′, nt 1,414–1,435) and sense primer HBXR1 (5′CCCAACTCCTCCCAGTCCT TAA3′, nt 1,723–1,744).

Liver specimens for PCR testing were obtained by percutaneous needle liver biopsy. Two biopsy samples were obtained from each patient, and one sample was used for routine histological evaluation. The other sample was frozen immediately and then stored at −80°C until PCR testing. Positive and negative HCV RNA strands in the liver were amplified independently by specific RT-seminested PCR as described elsewhere [Tomimatsu et al., 1997]. Briefly, after denaturation of the RNA extracted from the liver, synthesis of the positive and negative HCV cDNA strands was done with an antisense primer YCA (5′ACTCGCAAGCACCCTATCAG3′) or a sense primer YCS13 (5′GAGGAACTACTGTCTTCACG3′) derived from the 5′-noncoding region, respectively. The total cDNA obtained in the RT step was subjected to the first PCR by adding the other primer (YCS13 for the positive strand and YCA for the negative strand amplification). The seminested PCR round was carried out further by adding an inner sense primer YCS2 (5′GAGCCATAGTGGTCTGCCGA3′). The specificity of the detection of each HCV RNA strand was confirmed by the lack of amplification of the synthetic positive and negative strands using primers of the same polarity in the RT step. To test the specificity, several controls were run in parallel to all reactions and included reactions without reverse transcriptase in the

RT step (to detect contaminating DNA) and reactions without primers (to check for self-priming). For the detection of HBV DNA, total DNA was extracted from liver tissue using a commercially available kit (SMI test EX R and D, Sumitomo Metal Industries, Tokyo, Japan). Purified total hepatic DNA was resuspended in 500 μL of distilled water. A 25-μL aliquot of DNA solution was subjected to real-time detection PCR using set 2 primers and set 3 primers. To avoid contamination in all PCR assays, the contamination avoidance measures of Kwok and Higuchi [1989] were strictly applied throughout, and positive and negative controls were used.

Histological Evaluation

Liver biopsy specimens were fixed in formalin and embedded in paraffin for routine staining with hematoxylin-eosin. All specimens were examined by the same experienced pathologist (K.K.), who was unaware of the clinical, serological and virological data. Biopsy specimens were evaluated semiquantitatively by the histological activity index (HAI) described by Knodell et al. [1981].

RESULTS

Figure 1 shows the clinical, virological, and histological course of the three chronic liver disease patients (patients 1, 2, and 3) with spontaneous circulating HCV clearance. They had undergone laparoscopy for the diagnosis of chronic non-A, non-B liver disease 6–11 years before their HCV-seropositive states without viremia were found in 1990–1991. After the discovery of HCV, they were positive persistently for HCV antibody by RIBA-3 over 8–9 years but remained negative for serum HCV RNA. Patient 1 showed slight serum ALT elevation (58 U/L, normal value ≤33 U/L) at laparoscopy in 1984. Histological findings demonstrated that she had moderate chronic hepatitis with bridging fibrosis (Fig. 2A). The HAI scores of categories I (piecemeal necrosis), II (lobular necrosis and inflammation), III (portal inflammation), and IV (fibrosis) were 3, 3, 4, and 3, respectively, and portal lymphoid aggregates characteristic for HCV infection were observed. During a 15-year follow-up period, the slight elevation of serum ALT activity was normalized. The levels of serum albumin, cholinesterase activity, and platelet count remained normal over the entire follow-up period. At the second liver biopsy in 1995, the histological findings were improved markedly (Fig. 2B). Although minimal chronic hepatitis remained, no fibrosis was seen. The HAI categories I, II, III, and IV were scored 0, 1, 1, and 0, respectively.

Laparoscopic findings in 1982 showed that patient 2 had established cirrhosis with an obviously nodular liver surface. Although his liver biopsy specimen was not available, he also had symptoms of liver cirrhosis including esophageal varices. Over a 17-year follow-up period, serum ALT levels remained normal or near-normal. The levels of serum albumin and cholinesterase activity were also within the normal range although a slight decrease of platelet count persisted during follow-up. Liver histology both in 1998 and in 2000 showed minimal chronic hepatitis with bridging fibrosis (Fig. 2C). The HAI categories I, II, III, and IV were scored 1, 1, 1, and 3, respectively.

At laparoscopy in 1979, patient 3 showed slight elevation of serum ALT activity (62 U/L) and had low levels of serum albumin (2.8 g/dL, normal value 3.8–5.3 g/dL), cholinesterase activity (64 U/L, normal value 97–249 U/L), and platelet count ($9.6 \times 10^4/\mu L$, normal value $13–35 \times 10^4/\mu L$). The liver biopsy specimen obtained at laparoscopy showed the HAI scores of categories I, II, III, and IV to be 3, 3, 3, and 4, respectively. Thus, this patient had moderate chronic hepatitis with cirrhosis (Fig. 2D). HCV RNA RT-PCR carried out on a stored serum sample revealed that he was already nonviremic at this point. During a 19-year follow-up period after laparoscopy, normalization was observed for serum ALT activity, albumin levels, cholinesterase activity, and platelet count, and the second liver biopsy in 1998 disclosed remarkable amelioration of the liver histology (Fig. 2E). The HAI scores after the follow-up were 0 for category I, 1 for category II, 1 for category III, and 3 for category IV. Only minimal chronic hepatitis was seen, and fibrosis was diminished significantly although bridging fibrosis still remained.

These long-term follow-up studies demonstrated that the stage of liver fibrosis as well as the grade of liver inflammation can improve in nonviremic HCV-seropositive chronic liver disease cases. A decrease in HCV antibody levels to multiple RIBA-3 antigens was observed during follow-up in each case, thus indicating recovery from previous HCV infections. Quantitative analysis of HCV core antibody revealed further reductions of HCV core antibody titers in two patients examined (patients 2 and 3). In patients 1 and 2, HCV RNA was measured using liver biopsy specimens obtained at the end of follow-up in 2000. Patient 1 tested negative for both positive and negative HCV RNA strands. However, patient 2 had the positive HCV RNA strand in the liver while the negative HCV RNA strand was not found. The three patients studied were persistently negative for serum HBsAg with anti-HBc and/or anti-HBs in patients 2 and 3. Each patient did not have serum HBV DNA by PCR during follow-up. At the end of follow-up, HBV DNA was also absent from the liver of Patients 1 and 2.

Liver biopsy specimens were also obtained from six patients (patients 4–9) who were also positive for HCV antibody by RIBA-3 but negative for serum HCV RNA (Table I). In contrast with patients 1–3, these six patients had no history of chronic liver disease. Serum ALT activity was normal in four patients and near-normal in patients 5 and 9. A low grade of liver inflammation was seen in all but patient 8. Liver histology showed persistent lobular necrosis and inflammation in four patients, portal inflammation in four, and piecemeal necrosis in one. Each category of necroinflammation was scored 1 for these cases except that steatosis was observed in patient 5 and his HAI score for category II was 4. No fibrosis was found in all but patient 4. Liver

fibrosis was scored 1 in patient 4. None of the six patients had the positive or negative HCV RNA strand in the liver. The six patients were all negative for serum HBsAg with anti-HBc and/or anti-HBs in patients 5 and 7. They were all negative for serum HBV DNA and liver HBV DNA.

DISCUSSION

A considerable proportion of HCV antibody-positive individuals not treated with IFN are negative for serum HCV RNA. Recently, it has been demonstrated that spontaneous HCV clearance from the serum occurs in an unexpectedly high percentage of some populations [Vogt et al., 1999; Beld et al., 1999]. If serum HCV RNA is undetectable by RT-PCR at the end of IFN therapy and again 6 months after treatment, this has been taken to indicate HCV eradication by IFN, and patients with such sustained HCV RNA clearance from sera have been shown to continue to be liver HCV RNA-negative [Marcellin et al., 1997; Lau et al., 1998] accompanied by

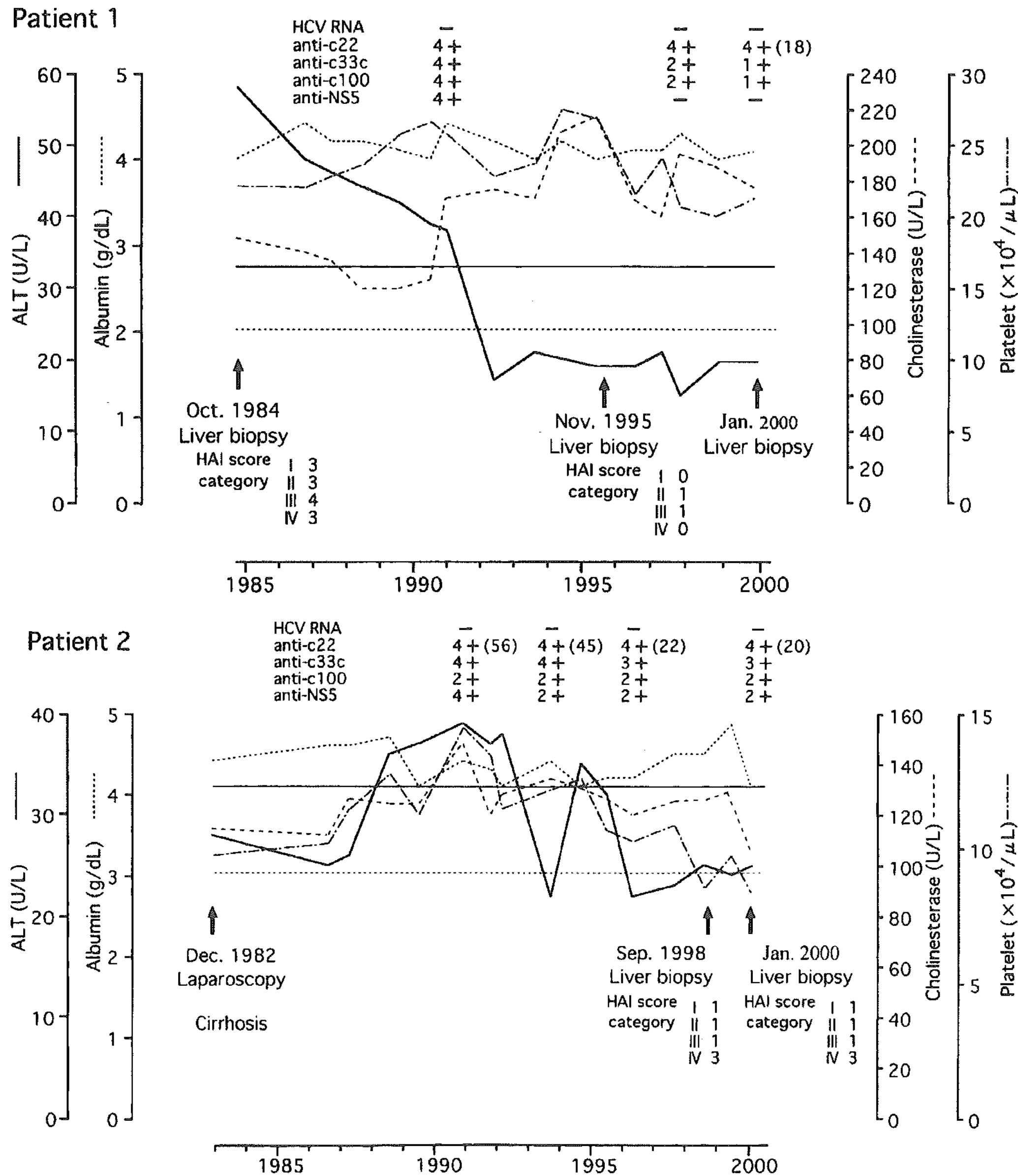


Fig. 1. Clinical, virological, and histological courses of three nonviremic HCV-seropositive patients without IFN therapy (patients 1, 2, and 3). All patients had undergone diagnostic laparoscopy for chronic non-A, non-B liver disease. After the discovery of HCV, they were found to be positive for HCV antibody but negative for serum HCV RNA and enrolled in this study. Solid and dashed horizontal lines represent the upper normal limit of ALT (33 U/L) and the lower normal limit of cholinesterase (97 U/L), respectively. Absolute titers of HCV core (c22) antibody are shown in parentheses.

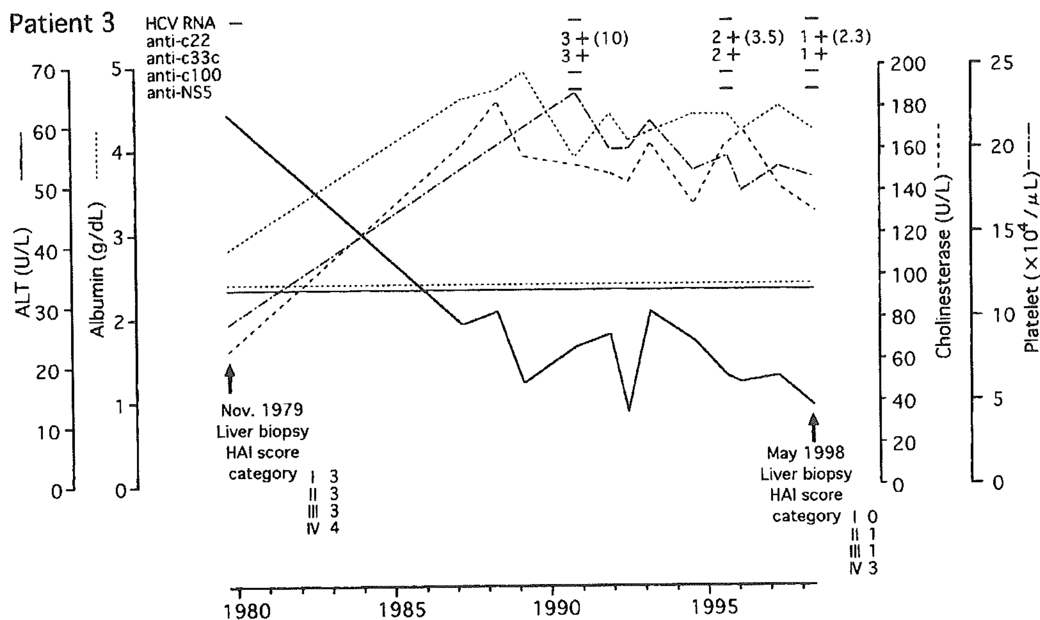


Fig. 1. (*Continued*)

sustained reductions of HCV antibody levels [Saracco et al., 1993;Yuki et al., 1993a,b; Diodati et al., 1994] and continuous improvement in histological findings [Marcellin et al., 1997; Lau et al., 1998]. In contrast, the clinical relevance of spontaneous serum HCV RNA clearance in HCV antibody-positive patients has not been well documented, and controversy remains.

The present study demonstrated that HCV antibody-positive but serum HCV RNA-negative patients without IFN therapy were likely to be negative for HCV

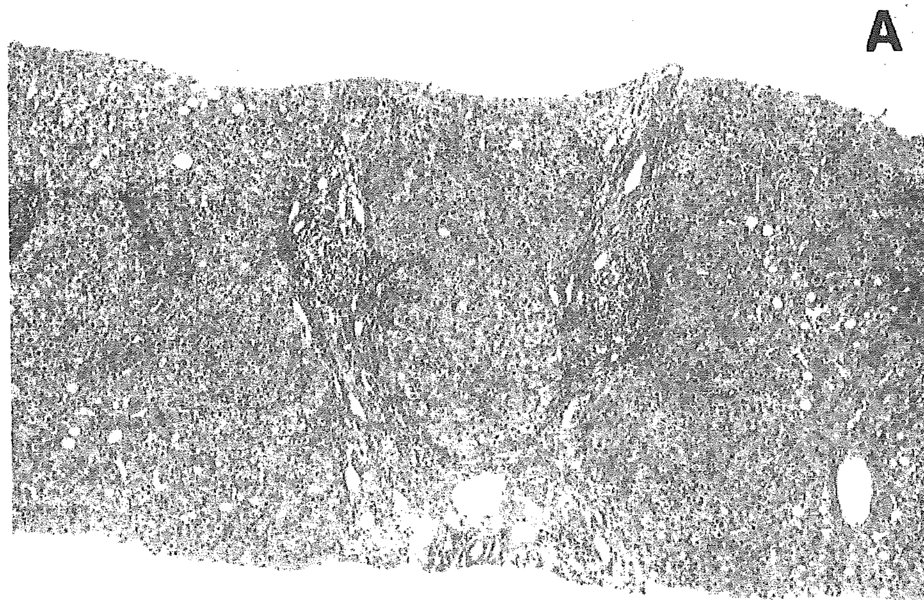
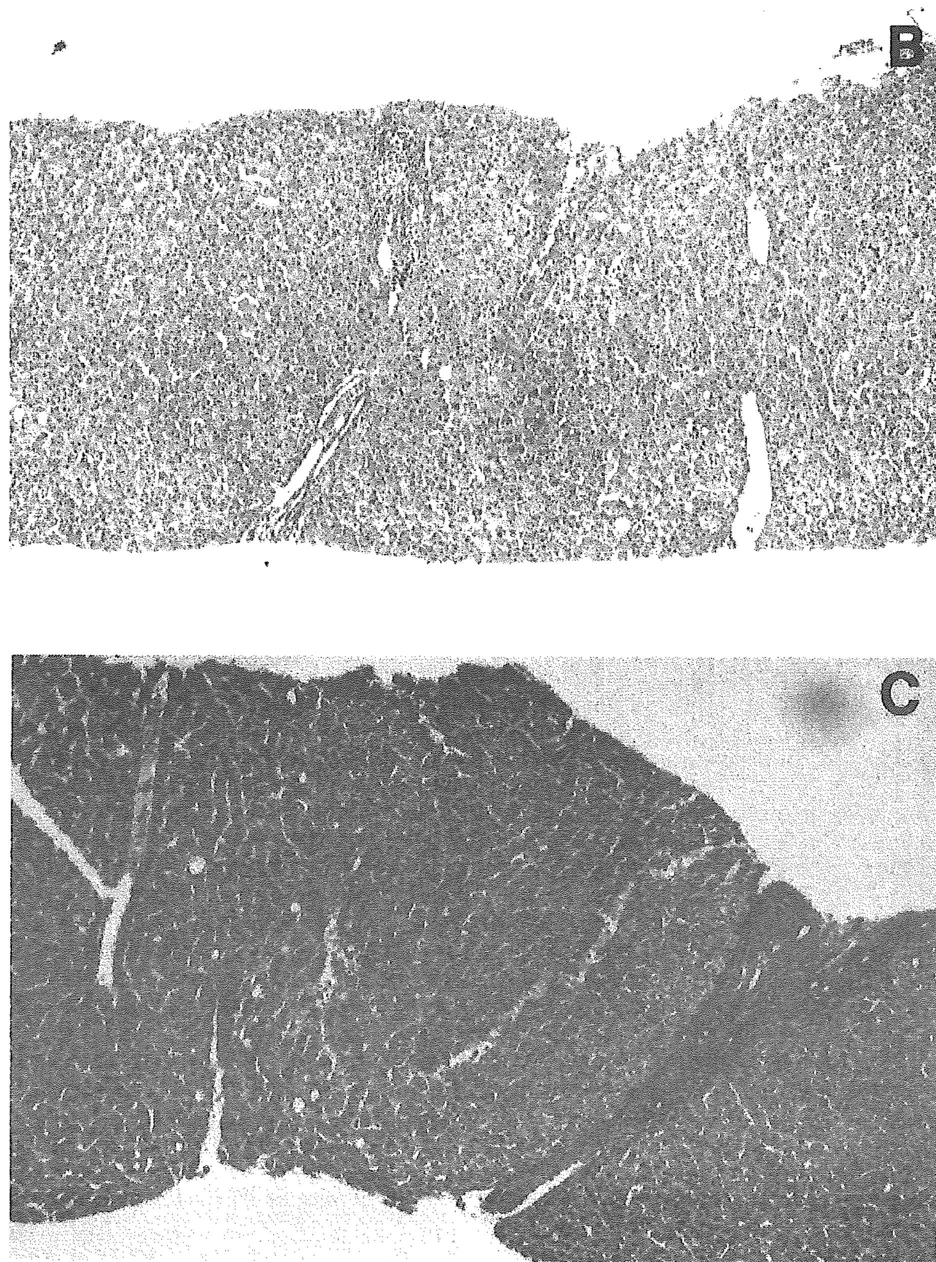


Fig. 2. Histological alterations in liver biopsy specimens years after HCV clearance from sera in the natural course. For the diagnosis of chronic non-A, non-B liver disease, laparoscopy was performed on patient 1 in 1984, patient 2 in 1982 (liver histology not available with laparoscopic diagnosis of established cirrhosis), and patient 3 in 1979. After serological assays for HCV were established in 1990, they remained positive for HCV antibody without viremia. Ultrasound-guided liver biopsies were performed on patient 1 in 1995, patient 2 in 2000, and patient 3 in 1998. (**A**) Patient 1 in Oct. 1984; (**B**) patient 1 in Nov. 1995; (**C**) patient 2 in Jan. 2000; (**D**) patient 3 in Nov. 1979; (**E**) patient 3 in May 1998. Paraffin sections stained with hematoxylin-eosin. (Original magnification ×16.)

nonstructural antibodies and have low titers of HCV core antibody. In addition, when nonviremic HCV-seropositive states of the three chronic liver disease patients were monitored over 8–9 years, reductions of HCV core and nonstructural antibodies occurred. Thus, the data obtained suggest that HCV antibody profiles of our patients were consistent with those observed years after successful IFN therapy and were indicative serologically of HCV eradication although further studies are necessary to confirm these observations. In the current study, the nonviremic HCV-seropositive patients were tested further for occult HCV infection in the liver. HCV RNA was not found in the liver biopsy specimens from any of the six patients who had no history of chronic liver diseases and were considered to have cleared HCV in the early phase of infection. On the other hand, persistence of the HCV genome was found in one of the two chronic liver disease patients examined who had been clear serologically of HCV for many years. Previous reports have suggested that HCV RNA can be detected in the



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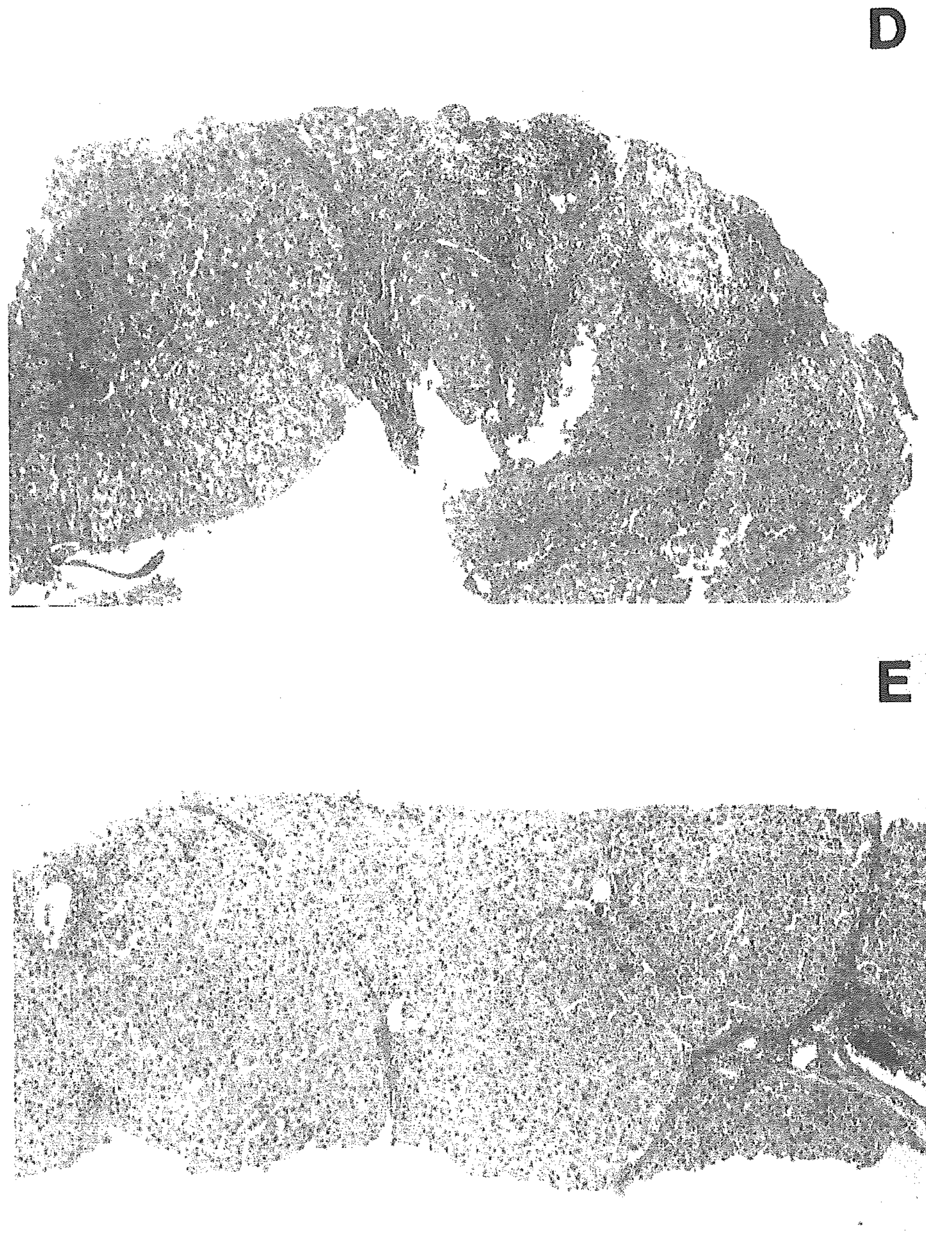

Fig. 2. (*Continued*)

liver, which generally harbors higher viral loads than the serum, in HCV antibody-positive but nonviremic patients without IFN therapy [Haydon et al., 1998; Dries et al., 1999]. In these cross-sectional studies, occult HCV infection was also shown to be associated with the presence of chronic liver disease on liver biopsy. Taken together, these observations raise the possibility that spontaneous serum HCV RNA clearance does not necessarily imply cure of HCV infection in chronic liver disease patients and the results of HCV RNA PCR for serum samples may not always serve as a reliable marker for HCV eradication. This presents a striking contrast to complete HCV eradication from the liver after successful IFN therapy for chronic hepatitis C. However, further studies are necessary to establish the range of such serologically undetectable occult HCV infections in chronic liver disease patients. Studying the HCV-specific cellular immune response may shed light on this issue. At present, what needs to be clarified is whether the prognosis of chronic liver diseases is really good once there has been spontaneous clearance of HCV RNA from the serum of HCV antibody-positive patients.

TABLE I. Clinical, Virological, and Histological Features of Nonviremic HCV-Seropositive Patients Without IFN Therapy*

Patient no.	Date of liver biopsy	Age (yr)	Sex	Previous blood transfusion	History of liver disease	Serum ALT (U/L)[a]	Serum HCV RNA	Antibodies in RIBA-3				HCV core antibody titer (unit)	Liver histology[b]				Liver tissue	
								anti-c22	anti-c33c	anti-c100	anti-NS5		I	II	III	IV	HCV RNA	HBV DNA
1	Jan. 2000	71	F	—	Non-A, non-B CLD	20	—	4+	1+	1+	—	18	0	1	1	0	—	—
2	Jan. 2000	70	M	—	Non-A, non-B CLD	25	—	4+	3+	2+	2+	20	1	1	1	3	+	—
3	May 1998	69	M	—	Non-A, non-B CLD	17	—	1+	1+	—	—	2.3	0	1	1	3	NT	NT
4	Jul. 1999	51	F	—	Acute hepatitis C	11	—	4+	4+	4+	—	24	0	0	1	1	—	—
5	Sep. 1999	63	M	46 yr before	Fatty liver	50	—	4+	3+	1+	—	30	0	4	1	0	—	—
6	Sep. 1999	52	F	—	—	9	—	4+	2+	—	—	7.2	1	1	1	0	—	—
7	Oct. 1999	61	F	—	—	19	—	1+	4+	—	—	≤0.5	0	1	1	0	—	—
8	Nov. 1999	70	M	—	—	16	—	3+	—	—	—	4.2	0	0	0	0	—	—
9	Nov. 1999	62	M	49 yr before	—	50	—	4+	1+	—	—	4.6	0	1	0	0	—	—

*CLD, chronic liver disease; NT, not tested. In patient 1, the liver biopsy specimen was subjected to only virological tests, and the histological data correspond to those obtained at the former liver biopsy in the long nonviremic HCV-seropositive period.
[a]Upper normal limit of ALT = 33 U/L.
[b]Four categories were scored: I, piecemeal necrosis; II, lobular necrosis and inflammation; III, portal inflammation; and IV, fibrosis.
All dashes indicate "negative."

In the current study, patient 2 with occult HCV infection also showed reductions of HCV antibody levels to core and nonstructural proteins. At the end of the follow-up, only the positive HCV RNA strand was found in the liver; no negative-strand RNA-replicative intermediate was found, thus indicating the absence of HCV replication. This patient also showed amelioration of liver histology. The previous laparoscopy had shown established cirrhosis with an apparently nodular liver surface. However, no histological feature of cirrhosis was seen when two liver biopsies were carried out at 16 and 17 years after laparoscopy. Only bridging fibrosis remained. Thus, it seems that occult HCV infection in the liver was accompanied by very low levels of replication and was well compartmentalized to restrict exposure to the host immune system and to exert little virulence. During the long-term follow-up of nonviremic HCV-seropositive states, regression of the liver fibrosis stage and improvement of the necroinflammatory grade were also evident for the other two chronic liver disease patients (patients 1 and 3). Complete loss of bridging liver fibrosis was observed in patient 1,and the histological features of liver cirrhosis had disappeared in patient 3. As for the persistence of occult HCV infection, patient 1 did not have detectable levels of HCV RNA in the liver, and patient 3 could not be tested for liver HCV RNA. Although further studies with more patients are necessary, the data obtained suggest that circulating HCV clearance in patients with chronic liver disease confers favorable liver histological outcomes even if the patients have cirrhosis. Occult HCV infection, if any, may be brought under full control by the host immune system with abrogation of further liver damage.

REFERENCES

Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, Tanaka S, Yoshiba M, Kohara M. 1999. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J Clin Microbiol 37:2899–2903.

Beld M, Penning M, van Putten M, Lukashov V, van den Hoek A, McMorrow M, Goudsmit J. 1999. Quantitative antibody responses to structural (core) and nonstructural (NS3, NS4, and NS5) hepatitis C virus proteins among seroconverting injecting drug users: impact of epitope variation and relationship to detection of HCV RNA in blood. Hepatology 29:1288–1298.

Diodati G, Bonetti P, Noventa F, Casarin C, Rugge M, Scaccabarozzi S, Tagger A, Pollice L, Tremolada F, Davite C, Realdi G, Ruol A. 1994. Treatment of chronic hepatitis C with recombinant human interferon-α2a: results of a randomized controlled clinical trial. Hepatology 19:1–5.

Dries V, von Both I, Muller M, Gerken G, Schirmacher P, Odenthal M, Bartenschlager R, Drebber U, zum Buschenfelde KHM, Dienes HP. 1999. Detection of hepatitis C virus in paraffin-embedded liver biopsies of patients negative for viral RNA in serum. Hepatology 29:223–229.

Hagiwara H, Hayashi N, Mita E, Takehara T, Kasahara A, Fusamoto H, Kamada T. 1993. Quantitative analysis of hepatitis C virus RNA in serum during interferon alfa therapy. Gastroenterology 104:877–883.

Haydon GH, Jarvis LM, Blair CS, Simmonds P, Harrison DJ, Simpson KJ, Hayes PC. 1998. Clinical significance of intrahepatic hepatitis C virus levels in patients with chronic HCV infection. Gut 42:570–575.

Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, Kiernan TW, Wallman J. 1981. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology 1:431–435.

Kwok S, Higuchi R. 1989. Avoiding false positives with PCR. Nature 339:237–238.

Lau DTY, Kleiner DE, Ghany MG, Park Y, Schmid P, Hoofnagle JH. 1998. 10-year follow-up after interferon-α therapy for chronic hepatitis C. Hepatology 28:1121–1127.

Marcellin P, Boyer N, Gervais A, Martinot M, Pouteau M, Castelnau C, Kilani A, Areias J, Auperin A, Benhamou JP, Degott C, Erlinger S. 1997. Long-term histologic improvement and loss of detectable intrahepatic HCV RNA in patients with chronic hepatitis C and sustained response to interferon-α therapy. Ann Intern Med 127:875–881.

Saracco G, Rosina F, Abate ML, Chiandussi L, Gallo V, Cerutti E, Di Napoli A, Solinas A, Deplano A, Tocco A, Cossu P, Chien D, Kuo G, Polito A, Weiner AJ, Houghton M, Verme G, Bonino F, Rizzetto M. 1993. Long-term follow-up of patients with chronic hepatitis C treated with different doses of interferon-α2b. Hepatology 18:1300–1305.

Takamizawa A, Mori C, Fuke I, Manabe S, Murakami S, Fujita I, Onishi E, Andoh T, Yoshida I, Okayama H. 1991. Structure and organization of the hepatitis C virus genome isolated from human carriers. J Virol 65:1105–1113.

Tomimatsu M, Endou H, Takahashi H, Iga D, Kato Y, Tamai N, Nakajima H, Okano A, Mori H, Hisatomi H. 1997. Negative strand of hepatitis C virus RNA in the liver of patients with chronic hepatitis C after interferon treatment. J Gastroenterol Hepatol 12:629–632.

Vogt M, Lang T, Frosner G, Klingler C, Sendl AF, Zeller A, Wiebecke B, Langer B, Meisner H, Hess J. 1999. Prevalence and clinical outcome of hepatitis C infection in children who underwent cardiac surgery before the implementation of blood-donor screening. N Engl J Med 341:866–870.

Yuki N, Hayashi N, Hagiwara H, Takehara T, Katayama K, Kasahara A, Fusamoto H, Kamada T. 1993a. Changes in antibodies to specific hepatitis C virus antigens with interferon-α therapy: analysis by recombinant immunoblot assay. Am J Gastroenterol 88:914–918.

Yuki N, Hayashi N, Hagiwara H, Takehara T, Katayama K, Kasahara A, Fusamoto H, Kamada T. 1993b. Quantitative analysis of antibodies to hepatitis C virus during interferon-a therapy. Hepatology 17:960–965.

Long-Term Histologic and Virologic Outcomes of Acute Self-Limited Hepatitis B

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The long-term impact of acute self-limited hepatitis B on the liver is unknown. Fourteen patients were recalled at a median of 4.2 years (range, 1.8-9.5 years) after the onset of acute hepatitis B. All showed clinical and serologic recovery with circulating hepatitis B surface antigen (HBsAg) clearance. Antibody to HBsAg (anti-HBs) had developed in 12 patients. Nine underwent liver biopsies at a median of 7.2 years, and histologic findings were evaluated using Ishak scores. Serum samples and frozen liver tissue were subjected to real-time detection polymerase chain reaction (PCR) to quantify the surface and X regions of the hepatitis B virus (HBV) genome and qualitative PCR to detect the covalently closed circular (ccc) HBV DNA replicative intermediate. Three patients had low levels of circulating HBV DNA up to 8.9 years after the onset, whereas both HBV DNA surface and X regions were found in the liver of all 9 patients examined, including 7 negative for serum HBV DNA. Liver viral loads assessed by the 2 regions showed a significant correlation (r = 0.946; P = .008), and all patients tested positive for ccc HBV DNA. Liver fibrosis and mild inflammation persisted in 8 patients. The fibrosis stage had relation to peak serum HBV DNA in the acute phase (P = .046) but not to liver viral loads in the late convalescent phase. In conclusion, occult HBV infection persists in the liver and is accompanied by abnormal liver histology for a decade after complete clinical recovery from acute self-limited hepatitis B. (HEPATOLOGY 2003;37:1172-1179.)

The clearance of circulating hepatitis B surface antigen (HBsAg) and appearance of antibody to HBsAg (anti-HBs) with normalization of liver function have been generally accepted as evidence of clinical and serologic recovery from acute hepatitis B. However, in chronic HBsAg carriers, there is growing evidence that hepatitis B virus (HBV) DNA sequences persist in the liver for years after seroclearance of HBsAg and seroconversion to anti-HBs.[1-3] Although the clinical and pathologic implications of occult HBV infection in the liver are unknown, viral eradication is unlikely to be achieved once chronic HBV infection has been established. The cytotoxic T-lymphocyte (CTL) response is weak or undetectable in chronic HBV infection. In contrast, a vigorous, polyclonal, and HBV-specific CTL response against multiple HBV epitopes is readily detectable during acute self-limited HBV infection.[4-7] HBV-specific CTLs further persist in the blood for several decades after recovery from acute hepatitis B.[7,8] In the face of an enhanced immune response leading to disease resolution, the virologic outcomes of acute self-limited hepatitis B may differ from those of HBsAg seroclearance and anti-HBs seroconversion in the course of chronic HBV infection.

At present, the long-term histologic and virologic impact of acute self-limited hepatitis B on the liver is unexplored. Studies using polymerase chain reaction (PCR) to detect HBV DNA sequences have shown that low levels of circulating HBV DNA can persist after clinical and serologic recovery from acute hepatitis B but tend to disappear after long-term follow-up.[9,10] Peripheral blood mononuclear cells are known as the site of persistent HBV infection long after recovery from acute hepatitis B[11-13] and may contribute to continuous priming of the HBV-specific CTL response. However, direct evidence is very limited for the possibility of HBV infection persisting in the liver in the late convalescent phase and exerting viru-

Abbreviations: HBsAg, hepatitis B surface antigen; anti-HBs, antibody to hepatitis B surface antigen; HBV, hepatitis B virus; CTL, cytotoxic T lymphocyte; PCR, polymerase chain reaction; anti-HBc, antibody to hepatitis B core antigen; ccc, covalently closed circular; WHV, woodchuck hepatitis virus.

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Received April 18, 2002; accepted January 30, 2003.

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0270-9139/03/3705-0027$30.00/0
doi:10.1053/jhep.2003.50171

lence. To address these issues, liver histologic and virologic outcomes were investigated in patients with a remote history of acute self-limited HBV infection.

Patients and Methods

Patients. Between December 1990 and August 1998, 19 patients with acute hepatitis B were admitted to our institution. A diagnosis of acute hepatitis B was based on elevated serum alanine aminotransferase activity, detection of HBsAg and immunoglobulin M antibody to hepatitis B core antigen (anti-HBc) in the serum, and the recent onset of jaundice and other typical symptoms. Coinfection with hepatitis A, C, D, or E; Epstein-Barr virus; or cytomegalovirus was ruled out because of the negative results of serologic tests. Confounding etiology of liver disease, human immunodeficiency virus infection, and other immunodeficient diseases were not found in any patient. In 2000, this complete series of patients with acute hepatitis B was contacted for follow-up. Fourteen patients (74%) revisited our institution and were reevaluated for clinical, serologic, histologic, and virologic recovery from the disease. The group comprised 11 men and 3 women ranging in age from 26 to 65 years (median, 43 years). All patients were negative for hepatitis C virus antibody by an enzyme-linked immunosorbent assay (Ortho Diagnostic Systems Co., Ltd., Tokyo, Japan) and serum hepatitis C virus RNA by PCR.[14] They had no history of administration of hepatotoxic drugs and showed no evidence of autoimmune liver disease. Their daily alcohol intake was reevaluated based on a detailed questionnaire and interview. Of the 14 patients, 5 were nondrinkers. Four patients did not have a habit of daily drinking, and their alcohol intake was less than 5 g/d. Alcohol consumption exceeded 5 g/d in the remaining 5 patients, but the levels were relatively low (10-15 g/d in 4 patients and 40-45 g/d in 1 patient). After a median of 4.2 years (range, 1.8-9.5 years) from the onset of the disease, the liver function of the 14 patients was reevaluated using blood chemistry and ultrasonography. Serum samples were obtained from all patients and subjected to serologic and virologic tests for HBV. Ultrasound-guided liver biopsies were performed on 9 patients at 1.8 to 9.5 years (median, 7.2 years) after resolution, and paired serum and liver samples were obtained. The study was approved by the local research ethics committee in accordance with the 1975 Declaration of Helsinki, and all patients provided written informed consent.

Serologic and Virologic Assays. Serum samples were tested for HBsAg, anti-HBs, hepatitis B e antigen, and antibody to hepatitis B e antigen with enzyme immunoassays (Abbott Laboratories, North Chicago, IL). Anti-HBc and immunoglobulin M class anti-HBc were measured with radioimmunoassays (Abbott Laboratories). Serum HBV DNA was quantitatively detected by real-time detection PCR based on TaqMan chemistry as previously reported.[15] Serial serum samples collected at 1- to 4-week intervals in the acute phase of infection and the late convalescent sera had been stored at −80°C without thawing and were subjected to HBV DNA PCR. In brief, total DNA was extracted from 100 μL of serum. Purified DNA was resuspended in 20 μL of distilled water, and a 10-μL aliquot of DNA solution (50-μL serum equivalent) was used for real-time detection PCR. Amplification was performed using primers corresponding to conserved sequences of the surface and X regions. A portion of the HBV surface region was amplified using set 2 primers: antisense primer HBSF2 (5′CTTCATCCTGCTGCTATGCCT3′, nucleotide positions 406-426) and sense primer HBSR2 (5′AAAGCCCAGGATGATGGGAT3′, nucleotide positions 608-627). Another portion of the HBV X region was further amplified using set 3 primers: antisense primer HBXF1 (5′ACGTCCTTTGTTTACGTCCCGT3′, nucleotide positions 1414-1435) and sense primer HBXR1 (5′CCCAACTCCTCCCAGTCCTTAA3′, nucleotide positions 1723-1744). Using serum samples obtained at the onset of the disease, HBV genotypes were further determined by restriction fragment length polymorphism analysis of the surface gene region as described previously.[16]

Percutaneous needle liver biopsies were performed using 14-gauge Tru-Cut needles (Hakko Medical Co., Ltd., Nagano, Japan), and biopsy specimens sufficient for histologic and virologic evaluation were obtained. Liver specimens for PCR testing were immediately frozen and then stored at −80°C until PCR testing. For the detection of HBV DNA, total DNA was extracted from liver tissue using a commercially available kit (SMI test EX R and D; Sumitomo Metal Industries, Tokyo, Japan). Purified total hepatic DNA was resuspended in 500 μL of distilled water. A 25-μL aliquot of DNA solution was subjected to quantitative real-time detection PCR using set 2 and set 3 primers, and the relative amounts of hepatic HBV DNA loads were obtained. In preliminary experiments, the efficacy of real-time detection PCR with 2 sets of primers (sets 2 and 3) was evaluated by quantitatively measuring sequential levels of synthetic standard HBV DNA. The detection limit of this system was as few as 10 DNA copies/reaction for each set of primers, and a linear standard curve was obtained between 10^1 and 10^8 DNA copies/reaction.[15] Without gel electrophoresis, the specificity of the assay was confirmed via hybridization with a target-specific fluorogenic probe. To further confirm the sizes of the amplified products, the assay was repeated

Table 1. Long-Term Virologic and Histologic Outcomes of Acute Self-Limited Hepatitis B

Patient No.	Sex	Age (y)	Peak ALT (ULN)	Peak Bilirubin (ULN)	Peak HBV DNA		HBV Genotype	Follow-up (y)	Alcohol Intake (g/d)	HBsAg/Anti-HBs	Serum HBV DNA		Liver HBV DNA			Histologic Outcome	
					S	X					S	X	S	X	cccDNA	Activity Grade	Fibrosis
1	M	53	23.0	26.4	8.0×10^3	4.7×10^3	C	9.5	<5	−/+	<200	<200	4.5×10^3	2.3×10^3	+	0.0.0.1	1
2	M	65	129.3	10.2	8.9×10^7	2.5×10^7	A	8.9	40-45	−/+	<200	<200	1.4×10^4	2.6×10^3	+	0.0.0.0	3
3	M	47	78.7	6.8	1.2×10^8	3.3×10^7	C	8.9	10-15	−/+	1.3×10^3	<200	1.6×10^4	3.3×10^3	+	0.0.0.1	3
4	F	43	27.5	1.0	5.3×10^7	1.6×10^7	C	7.8	0	−/+	2.4×10^4	7.7×10^3	4.4×10^4	1.4×10^4	+	0.0.0.1	3
5	M	60	37.1	2.2	3.0×10^8	1.1×10^8	A	7.3	10-15	−/+	<200	<200	NA	NA	NA	NA	NA
6	M	34	70.0	12.2	NA	NA	NA	7.2	<5	−/+	<200	<200	3.0×10^3	4.6×10^2	+	0.0.0.0	0
7	M	42	46.1	6.6	1.1×10^5	4.2×10^4	C	4.6	0	−/−	7.7×10^2	<200	NA	NA	NA	NA	NA
8	M	26	65.2	13.7	5.0×10^5	8.2×10^4	C	3.7	<5	−/+	<200	<200	NA	NA	NA	NA	NA
9	M	64	63.5	35.1	1.5×10^5	2.2×10^4	C	3.6	0	−/+	<200	<200	5.7×10^3	1.4×10^3	+	0.0.1.1	1
10	F	27	114.5	6.0	5.0×10^6	7.8×10^5	C	3.5	0	−/+	<200	<200	NA	NA	NA	NA	NA
11	M	29	48.3	18.6	2.0×10^6	2.1×10^5	C	2.9	10-15	−/+	<200	<200	1.6×10^3	1.9×10^2	+	0.0.0.1	3
12	M	31	88.5	9.3	3.6×10^7	8.0×10^6	C	2.6	<5	−/−	<200	<200	8.6×10^3	1.4×10^3	+	0.0.0.1	3
13	F	33	65.4	4.9	5.0×10^4	1.8×10^4	C	1.9	0	−/+	<200	<200	NA	NA	NA	NA	NA
14	M	51	80.7	12.2	1.4×10^6	3.6×10^5	C	1.8	10-15	−/+	<200	<200	5.1×10^4	1.8×10^4	+	0.0.0.1	3

NOTE. HBV DNA was detected by real-time detection PCR with 2 sets of primers derived from the surface (S) and X regions, and results are expressed in genome copies per milliliter serum or milligram liver. Ishak score is given to liver histology in the following order: piecemeal necrosis, 0–4; confluent necrosis, 0–6; focal necrosis, 0–4; portal inflammation, 0–4; and fibrosis, 0–6.

Abbreviations: ALT, alanine aminotransferase; ULN, upper limit of normal; NA, not available.

without the fluorogenic probe and the PCR products were run on 3% agarose gels with ethidium bromide and visualized under UV light.

Using PCR primers flanking the direct repeat region, the covalently closed circular (ccc) HBV DNA replicative intermediate in the liver was further detected by PCR. The methodology used was similar to that reported previously except that the unconserved 3′ nucleotide was deleted from the DRR3 primer.[17] In brief, the PCR was performed on a 5-μL aliquot of hepatic DNA solution using primers DRF1 (5′GTCTGTGCCTTCTCATCTGC3′, nucleotide positions 1553-1572) and modified DRR3 (5′AGTATGGTGAGGTGAGCAATG3′, nucleotide positions 2040-2060) for 30 cycles at 94°C for 1 minute, 53°C for 1.5 minutes, and 72°C for 3 minutes. Seminested PCR was then performed using primers DRF1 and DRR2 (5′ACAAGAGATGATTAGGCAGAGG3′, nucleotide positions 1830-1851) for 30 cycles at 94°C for 1 minute, 49°C for 1.5 minutes, and 72°C for 3 minutes. The products were analyzed on 3% agarose gels. In preliminary experiments, PCR products were observed for the liver from HBsAg-positive control patients but not from individuals without HBV infection. The incomplete Dane particle HBV DNA in sera was not amplified with the primers used, showing specificity for double-stranded DNA in the direct repeat region. To rule out the effect of DNA repair activity in liver nuclei on the specificity, DNA extraction was performed on normal liver tissue from 2 HBV-uninfected individuals in the presence of 10^2 to 10^7 copies of the partially double-stranded virion HBV DNA and purified DNA was subjected to the PCR procedure. All samples remained negative, thus confirming the specificity. Direct sequencing of the PCR amplicons was also performed bidirectionally by the dideoxy method with the DNA sequencing kit/ABI Prism Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Tokyo, Japan), and specific amplification was confirmed. The threshold levels of hepatic HBV DNA, which give positive results for ccc HBV DNA, were further determined based on serial 2-fold dilution of hepatic DNA solution containing ccc HBV DNA.

The real-time detection PCR method used is a simple and specific assay. The chance of contamination is reduced via monitoring and calculation of fluorescent signals in a single sample tube with a closed optical cap. To further avoid contamination in all PCR assays, the contamination avoidance measures of Kwok and Higuchi[18] were strictly applied throughout, and positive and negative controls were used.

Histologic Evaluation. Liver biopsy specimens for histologic evaluation were fixed in formalin and embedded in paraffin for routine staining with hematoxylin-eosin. All specimens were examined by the same experienced pathologist, who was unaware of the biochemical, serologic, and virologic data. Biopsy specimens were semiquantitatively evaluated by the modified histologic activity index described by Ishak et al.[19]

Statistical Analysis. Statistical analysis for group comparisons was performed using the Wilcoxon nonparametric test. Correlations between the variables were calculated using Spearman rank order correlations. A P value less than .05 (2-tailed) was considered to indicate significance.

Results

Table 1 shows the characteristics of the 14 patients with a remote history of acute self-limited hepatitis B.

Clinical and Virologic Profiles at the Onset of Acute Hepatitis B. The peak alanine aminotransferase activity in the acute phase ranged from 23.0 to 129.3 times the upper limit of normal (median, 65.3), and the peak bilirubin levels ranged from 1.0 to 35.1 times the upper limit of normal (median, 9.8). HBV genotypes and the peak serum HBV DNA levels were determined for 13 patients. Eleven patients were infected with genotype C, whereas the remaining 2 had genotype A. Real-time detection PCR to quantify the HBV genome was performed on serial serum samples in the acute phase. In each case, the peak point determined by the surface primers coincided with that determined by the X primers. A significant correlation was observed between the peak levels of the HBV DNA surface region (median, 2.0×10^6; range, 8.0×10^3 to 3.0×10^8 copies/mL) and those of the X region (median, 3.6×10^5; range, 4.7×10^3 to 1.1×10^8 copies/mL) ($r = 0.989$; $P = .001$). Liver function was normalized within 3 months, and HBsAg was cleared from the serum within 5 months.

Clinical and Serologic Recovery After Follow-up. After 1.8 to 9.5 years (median, 4.2 years) from the onset of acute hepatitis B, the 14 patients were examined for clinical and serologic recovery. None of the patients presented symptoms of liver disease, and all were found to have normal livers on ultrasonography. Liver function data, including serum alanine aminotransferase activity, were normal for all patients. They were all clear of HBsAg, and anti-HBs had developed in 12 patients. All had seroconverted to antibody to hepatitis B e antigen and tested positive for anti-HBc. Using sera diluted 200-fold, anti-HBc was detected in only 1 patient. Overall, all patients showed complete clinical and serologic recovery from acute hepatitis B. When sera collected in the late convalescent phase were subjected to HBV DNA PCR, 3 patients (21%) were found to have low levels of circulating HBV DNA up to 8.9 years after the onset of the disease. The HBV DNA surface region of 7.7×10^2 to 2.4×10^4 copies/mL was detected in the 3 patients, whereas the X region of 7.7×10^3 copies/mL was found in only 1 patient. Neither the surface region nor the X region was amplified in the remaining 11 patients.

Liver Virologic and Histologic Outcomes. Virologic and histologic studies were further performed on liver biopsy specimens obtained from 9 patients at 1.8 to 9.5 years (median, 7.2 years) after the onset of the disease. Although only 2 patients remained positive for circulating HBV DNA, both regions of the HBV genome were found in frozen biopsy specimens from all patients by real-time detection PCR using target-specific fluorogenic probes. The sizes of the PCR amplicons were also confirmed by gel electrophoresis (Fig. 1). The relative amounts of liver HBV DNA measured using the surface primers were significantly correlated to those measured using the X primers ($r = 0.946$; $P = .008$). This figure was similar to that for circulating HBV DNA levels in the acute phase (Fig. 2). Using qualitative PCR to amplify the direct repeat region, PCR products were also observed in all biopsy specimens, thus showing the presence of an intact direct repeat region indicative of the ccc HBV DNA replicative intermediate (Fig. 3). To confirm specific amplification, the PCR amplicons of patients 2 and 3 were subjected to direct sequencing. The amplified sequence showed 94% to 98% homology to the reference sequence of an HBV isolate (GenBank accession no. AY123041) (Fig. 4). None of the serum samples collected in the acute phase or in the late convalescent phase tested positive by the PCR methodology. Based on a serial end-point dilution method, the threshold levels of total hepatic HBV DNA to detect ccc HBV DNA were 10 to 90 copies (median, 35) when assessed by the surface primers and 2 to 20 copies (median, 5) when assessed by the X primers. Thus, the ratio of ccc HBV DNA to total HBV DNA was shown to range within 1 $\log_{10}$.

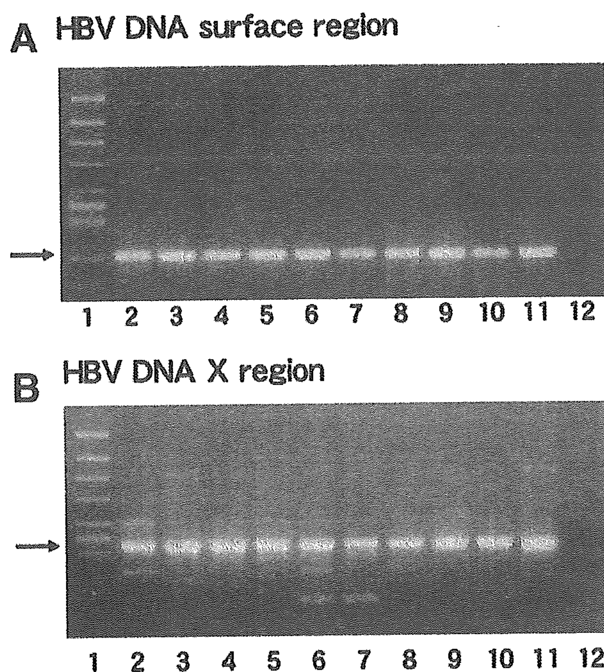

Fig. 1. Detection of the (A) HBV DNA surface and (B) X sequences (222 and 331 base pairs, respectively) in liver tissue from patients with a remote history of acute self-limited hepatitis B. The real-time detection PCR products were run on agarose gels. Lane 1, molecular weight marker (px174 DNA/Hae III [Toyobo]); lanes 2, 3, 4, 5, 6, 7, 8, 9, and 10, liver tissue from patients 1, 2, 3, 4, 9, 11, 12, 14, and 6, respectively; lane 11, positive control (liver tissue from a chronic HBsAg carrier); lane 12, negative control (water).