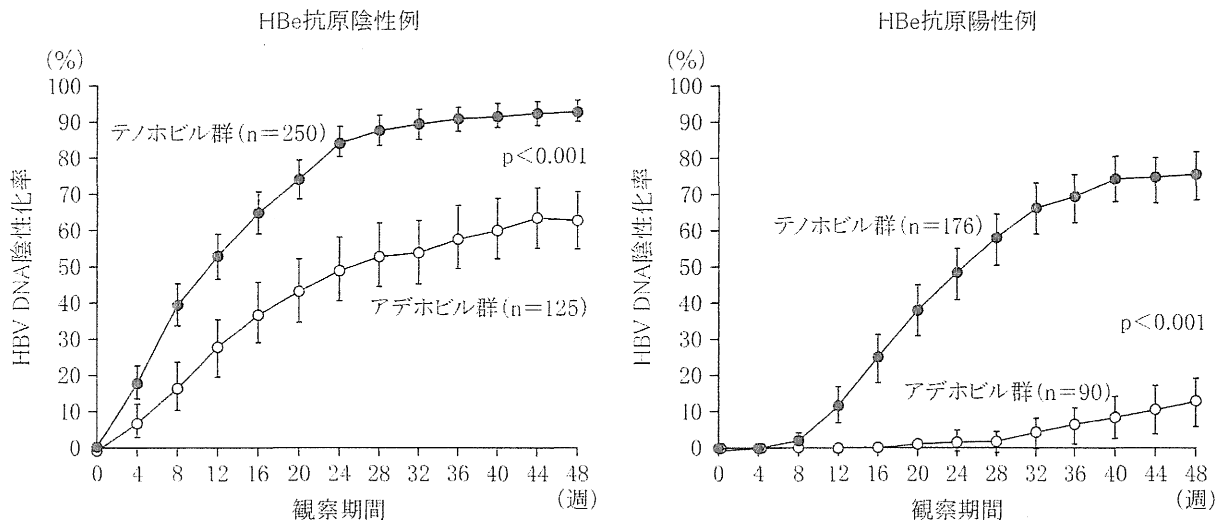

HBe 抗原陰性例・陽性例いずれにおいても，治療 48 週後の HBV DNA 陰性化率は，テノホビル治療群でアデホビル治療群よりも有意に高率だった。

(Marcellin P et al：N Engl J Med 359, 2442-2455, 2008, 一部改変)

図4 HBe 抗原の有無による HBV DNA 陰性化の頻度

て，B型慢性肝疾患に対するテノホビルの良好な抗ウイルス効果が確認されたことから，2014年3月保険承認となった。しかしながら，これらの臨床試験は治療48週までの評価であり，長期的な投与による効果に関しては，今後の臨床成績の集積が待たれる。

III 国外におけるテノホビルの治療成績

前述のように，テノホビルは，既に113の国と地域でB型慢性肝疾患に対する治療薬として保険承認されている。本項では，国外での臨床試験成績ならびに既報を基にテノホビルの治療成績について述べる。

まず，B型慢性肝疾患核酸アナログ未治療例（naïve例）に対して米国，ドイツなど15カ国で実施された2つの臨床試験（GS-US-174-0102およびGS-US-174-0103試験）について示す。HBe抗原陰性例を対象としたGS-US-174-0102試験では，テノホビル48週間投与におけるHBV DNA低下量は，−4.57 Log copies/mLであり，HBV DNA陰性化率は91.2％（228例/250例），ALT正常化も76.3％（180例/236例）と良好な治療成績が示された[8]。一方，HBe抗原陽性例を対象としたGS-US-174-0103試験での治療成績もHBV DNA低下量は，−6.17 Log copies/mLであり，HBV DNA陰性化率は68.8％（121例/176例），ALT正常化も68.0％（115例/169例）と良好であったが，既存の核酸アナログ治療と同様，HBe抗原陽性例においてHBV DNA陰性化率ならびにALT正常化率はやや低い結果となった[8]。また，これら2つの臨床試験は，アデホビル単独治療との48週間の二重盲検試験として実施されており，アデホビル治療との抗ウイルス効果が比較されている。HBe抗原陰性例における治療48週後のHBV DNA陰性化率は，テノホビル治療群91.2％，アデホビル治療群56.0％とテノホビル治療群で有意に高率であり，その傾向は，HBe抗原陽性例に対する試験においても，同様の結果（テノホビル群68.8％ vs アデホビル群8.9％）であり，テノホビルの強力な抗ウイルス効果が示されているといえる（図4）。

Ⅳ テノホビル耐性の出現率

核酸アナログ治療は，治療を中止すると高率に再燃することから，長期的な継続投与が必須であり，その結果，薬剤耐性ウイルスの出現が大きな問題となる。テノホビルに関しては，国外からの報告ではあるが，前述したGS-US-174-0102およびGS-US-174-0103試験症例の後観察において，治療288週（約6年）までテノホビル耐性ウイルスの出現は認められていない[9]。ただし，288週治療まで到達している症例数が限られており，さらに本臨床試験では，治療中にHBV DNA低下が不良である症例ついては，FTC・テノホビル併用療法への切替や治療中止が行われていることから，比較的ハイリスク（薬剤耐性出現の可能性が高い）症例が除外されている可能性があることを考慮に入れて，データを解釈する必要がある。実際，テノホビル耐性ウイルス出現の症例報告も散見される[10)～12)]。これらの報告では，48週の治療期間での耐性ウイルス出現が報告されており，これまでの核酸アナログ製剤と同様，ポリメラーゼRT領域のアミノ酸変異が薬剤耐性に強く関与している可能性がある[11)12)]。したがって，これまでの核酸アナログ治療と同様，テノホビル治療においても，頻度は低いながらも薬剤耐性出現の可能性があることを念頭におき，定期的なHBV DNAのモニタリングを行っていくことが重要と考えられる。

Ⅴ テノホビル治療の副作用

核酸アナログ治療は，副作用が少なく，治療効果が非常に高いことから，現在のB型慢性肝疾患治療の中心となっているが，腎機能障害や低リン血症，Fanconi症候群等の副作用出現例が散見されており，副作用の出現には注意深い経過観察が必要である。テノホビルの国内臨床試験における有害事象の発現頻度は23.1％であり，肝機能検査値異常（AST，ALTおよびγ-GTP増加等）を4.9％に認めたほか，クレアチニン増加2.8％，アミラーゼ・リパーゼ増加2.1％，悪心2.1％，腹痛1.4％であったと報告され，本邦の保険承認時には重篤な副作用は認められていない。しかしながら，海外からの報告をみると，重大な副作用として重度の腎機能障害，乳酸アシドーシスおよび脂肪沈着による重度の肝腫大，膵炎，低リン血症，骨軟化症等が挙げられる[7)13)14)]。特に，腎機能障害，低リン血症，骨軟化症は，本邦でのアデホビル投与例においても散見されており[15)～17)]，今後，テノホビルの長期投与例が増加するに従い，出現する可能性があり，治療経過中のクレアチニン値やリン，カルシウムの推移には十分注意が必要である。

Ⅵ 妊婦，産婦，授乳婦等への投与

テノホビルは，FDA pregnancy categoryにおいてcategory Bに区分され，他の核酸アナログ（いずれもcategory C）に比べ，妊娠に対する安全性が比較的高いと考えられることから，妊娠中もしくは妊娠を希望する症例に対して使用される可能性もある。

しかしながら，本邦において，妊娠中の安全性に関する明確なエビデンスはないこと，動物実験においてテノホビルが胎盤を通過すること，ヒト乳汁中へ移行が確認されていることを踏まえると，妊婦，産婦，授乳婦等に対する使用には，十分な注意が必要と言える。

Ⅶ 今後の展望

テノホビルの保険適応に伴い，B型慢性肝疾患初回治療例に対する治療選択肢が増えるとともに，これまで，ラミブジン・アデホビル併用療法やエンテカビル・アデホビル併用療法を行っても，十分な抗ウイルス効果が得られなかった症例に対しても，ラミブジン・テノホビル併用療法やエンテカビル・テノホビル併用療法といった治療が可能となる。テノホビルは，強力な抗ウイルス効果を持つことから，より良

好なウイルス増殖抑制が可能と考えられるが，一方で，一部のアデホビル耐性変異と交叉耐性を示す可能性も示唆されており，注意が必要といえる。4種類の核酸アナログ製剤を工夫しながら使用することで，B型慢性肝疾患コントロールの向上が期待できる一方，いずれの薬剤にも耐性を示す多剤耐性ウイルスの出現も危惧されることから，専門医と綿密に連携を取った上で，治療していくことが重要と考えられる。

文　献

1) Hosaka T, Suzuki F, Kobayashi M et al : Long-term entecavir treatment reduces hepatocellular carcinoma incidence in patients with hepatitis B virus infection. Hepatology 58 : 98-107, 2013.
2) Liaw YF, Sung JJ, Chow WC et al : Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 351 : 1521-1531, 2004.
3) Matsumoto A, Tanaka E, Rokuhara A et al : Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B : A multicenter retrospective study of 2795 patients. Hepatol Res 32 : 173-184, 2005.
4) Suzuki Y, Kumada H, Ikeda K et al : Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. J Hepatol 30 : 743-748, 1999.
5) Ono A, Suzuki F, Kawamura Y et al : Long-term continuous entecavir therapy in nucleos(t)ide-naive chronic hepatitis B patients. J Hepatol 57 : 508-514, 2012.
6) Tenney DJ, Rose RE, Baldick CJ et al : Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naive patients is rare through 5 years of therapy. Hepatology 49 : 1503-1514, 2009.
7) Karatayli E, Idilman R, Karatayli SC et al : Clonal analysis of the quasispecies of antiviral-resistant HBV genomes in patients with entecavir resistance during rescue treatment and successful treatment of entecavir resistance with tenofovir. Antivir Ther 18 : 77-85, 2013.
8) Marcellin P, Heathcote EJ, Buti M et al : Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. N Engl J Med 359 : 2442-2455, 2008.
9) Kitrinos KM, Corsa A, Liu Y et al : No detectable resistance to tenofovir disoproxil fumarate after 6 years of therapy in patients with chronic hepatitis B. Hepatology 59 : 434-442, 2014.
10) Berg T, Marcellin P, Zoulim F et al : Tenofovir is effective alone or with emtricitabine in adefovir-treated patients with chronic-hepatitis B virus infection. Gastroenterology 139 : 1207-1217, 2010.
11) Liaw YF, Sheen IS, Lee CM et al : Tenofovir disoproxil fumarate(TDF), emtricitabine/TDF, and entecavir in patients with decompensated chronic hepatitis B liver disease. Hepatology 53 : 62-72, 2011.
12) Sheldon J, Camino N, Rodes B et al : Selection of hepatitis B virus polymerase mutations in HIV-coinfected patients treated with tenofovir. Antivir Ther 10 : 727-734, 2005.
13) Heathcote EJ, Marcellin P, Buti M et al : Three-year efficacy and safety of tenofovir disoproxil fumarate treatment for chronic hepatitis B. Gastroenterology 140 : 132-143, 2011.
14) Snow-Lampart A, Chappell B, Curtis M et al : No resistance to tenofovir disoproxil fumarate detected after up to 144 weeks of therapy in patients monoinfected with chronic hepatitis B virus. Hepatology 53 : 763-773, 2011.
15) Shimizu Y, Hiraoka A, Yamago H et al : Hypophosphatemia in patients with hepatitis B virus infection undergoing long-term adefovir dipivoxil therapy. Hepatol Res 2013.
16) Tamori A, Enomoto M, Kobayashi S et al : Add-on combination therapy with adefovir dipivoxil induces renal impairment in patients with lamivudine-refractory hepatitis B virus. J Viral Hepat 17 : 123-129, 2010.
17) Tanaka M, Suzuki F, Seko Y et al : Renal dysfunction and hypophosphatemia during long-term lamivudine plus adefovir dipivoxil therapy in patients with chronic hepatitis B. J Gastroenterol 49 : 470-480, 2014.

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新たな保険外併用の仕組みとして「患者申出療養制度」が創設へ

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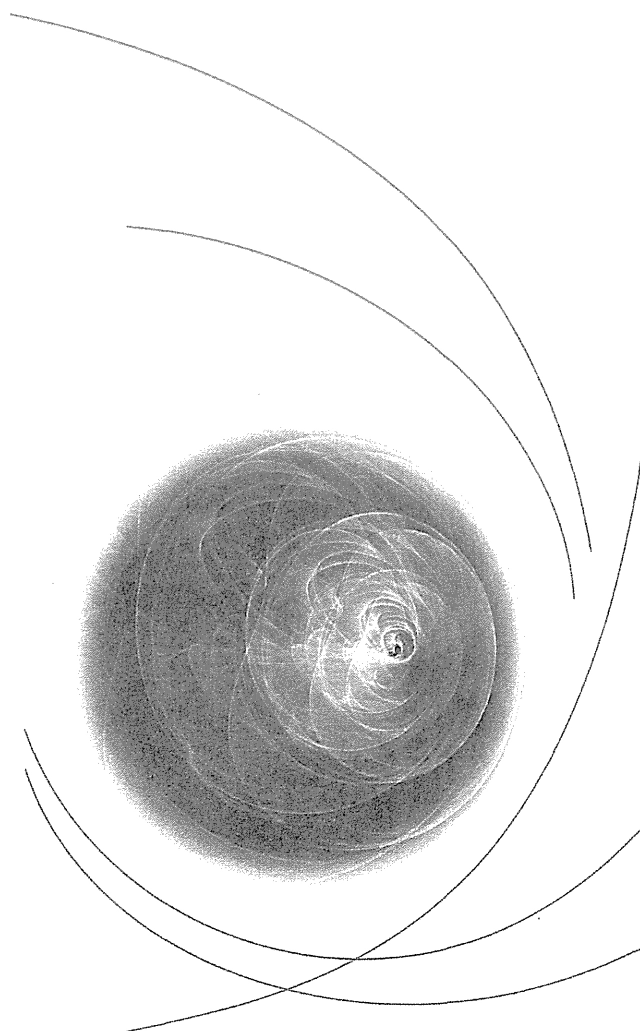

臨床各科

差分解説

◉新知見のエッセンスをピックアップ！
◉知識の更新・アップデートのために！

差分【さぶん・patch】
一旦完成したプログラムの一部を修正すること。また，修正を行うために変更点（差分情報）のみを抜き出して列挙したファイル。「パッチファイル」「差分ファイル」などとも呼ばれる。バグ（不具合）の修正や，小規模なバージョンアップなどを行う際に，ソフトウェア全体を入れ替えるのは効率的でないため，修正点だけを抜き出してパッチ作成し，これを既存のソフトに組み込むことで修正を行う。
（IT用語事典 e-Wordsより引用）

■内科：肝胆膵

B型慢性肝疾患診療におけるHBs抗原測定

従来，B型慢性肝疾患診療においては，HBeセロコンバージョンを誘導し，ALTを正常化することが重要と考えられてきた。しかし，HBe抗原陰性化後もHBV DNAが高く肝炎の持続する症例があること，HBV DNA量と肝発癌に有意な相関が認められることから，2013年9月に改訂された日本肝臓学会の「B型肝炎治療ガイドライン」では，抗ウイルス療法の目標をALT正常化（30IU/L以下），HBeセロコンバージョン，HBV DNA増殖抑制を短期目標とし，HBs抗原消失を最終目標と設定した[1)]。

近年，HBs抗原測定は定量可能となり，測定意義も変化している。ペグインターフェロン治療中のHBs抗原量により，治療終了後のHBeセロコンバージョンやHBV DNA量，HBs抗原の消失率が予測可能であるといった報告[2)]や，核酸アナログ治療を中止する際に，HBs抗原量80IU/mL未満かつHBVコア関連抗原量3.0Log U/mL未満であれば，治療中止後に再燃リスクが低いといった報告[3)]もある。

B型慢性肝疾患診療において，定期的なHBs抗原定量は治療効果や再燃予測のマーカーとして意義が大きくなっている。

【文献】

1）日本肝臓学会肝炎診療ガイドライン作成委員会，編：B型肝炎治療ガイドライン．第1.2版．2013.
2）Piratvisuth T, et al: Hepatol Int. 2011 Jun 24. [Epub ahead of print]
3）Matsumoto A, et al: Hepatol Res. 2012;42(2):139-49.

【解説】

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Postexposure Prophylactic Effect of Hepatitis B Virus (HBV)-Active Antiretroviral Therapy against HBV Infection

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Retrospective study indicates that hepatitis B virus (HBV)-active nucleoside (nucleotide) analogues (NAs) used for antiretroviral therapy reduce the incidence of acute HBV infections in human immunodeficiency virus (HIV)-infected patients. Learning from HIV postexposure prophylaxis (PEP), we explored the possibility of using NAs in PEP following HBV exposure, if preexposure prophylaxis is feasible clinically. Using freshly isolated primary human hepatocytes cultured *in vitro*, we analyzed the effect of HBV-active tenofovir and lamivudine in primary HBV infection and also the effect of treatment with these NAs after HBV infection. HBV-active NAs applied from 24 h before inoculation could not prevent the secretion of hepatitis B surface antigen into the culture medium, and cessation of the NAs after inoculation allowed the cells to establish an apparent HBV infection. In contrast, hepatitis B immune globulin was able to prevent HBV infection completely. NA treatment before infection, however, can control the spread of HBV infection, as detected by immunohistochemistry. Practically, starting NA treatment within 2 days of primary HBV infection inhibited viral spread effectively, as well as preexposure treatment. We demonstrated that preexposure NA treatment was not able to prevent the acquisition of HBV infection but prevented viral spread by suppressing the production of mature progeny HBV virions. The effect of postexposure treatment within 2 days was similar to the effect of preexposure treatment, suggesting the possibility of HBV PEP using HBV-active NAs in HIV- and HBV-susceptible high-risk groups.

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Patients infected with human immunodeficiency virus (HIV) are at high risk of hepatitis B virus (HBV) infection, and an estimated 10% of HIV-infected individuals worldwide have chronic hepatitis B (1). Because of the shared transmission routes of the two viruses, i.e., sexual intercourse and blood contact (2), HIV-infected individuals, particularly men who have sex with men (MSM) and intravenous drug users, are at high risk of acute HBV infection.

Two recent retrospective studies from Japan and the Netherlands indicated that HBV-active nucleoside (nucleotide) analogues (NAs) used for antiretroviral therapy (ART) reduced the incidence of acute HBV infections in HIV-infected patients (3, 4). The prophylactic effects of regimens containing NAs as reverse transcription (RT) inhibitors are well accepted against HIV infection (5, 6), because RT is an initial essential step following HIV entry into susceptible cells. However, considering the differences between the HIV and HBV replication cycles, this notion cannot be applied simply to HBV infection.

Unlike HIV, when HBV enters the hepatocyte, its genomic DNA is transported to the nucleus and converted to covalently closed circular DNA (cccDNA), which serves as the template for transcription. One transcript, the pregenomic RNA, is converted to genomic DNA by RT, which is the target of NAs.

Studies of occult HBV infections strongly suggest that HBV cccDNA remains in the hepatocyte nuclei for a long time after resolution of acute infection (7), often leading to reestablishment of infection (HBV reactivation) following immunosuppressive therapy, i.e., anticancer chemotherapy (8). The phenomenon of HBV reactivation suggests that cccDNA remaining in the hepatocytes can produce infectious virions, leading to very rapid viral spread. Therefore, establishment of cccDNA in the nuclei means HBV infection of susceptible cells.

Nonetheless, human clinical studies have shown that HBV-active ART protects against the occurrence of *de novo* HBV infection, indicating that NA-based strategies inhibit the serological changes of HBV markers (hepatitis B surface antigen [HBsAg], hepatitis B surface antibody [anti-HBs], and hepatitis B core antibody [anti-HBc]) that provide evidence of HBV infection and have a clinical prophylactic effect against incident HBV infection. Learning from HIV postexposure prophylaxis (PEP), we consider the possibility of NA-based PEP against HBV exposure, given that preexposure prophylaxis (PrEP) is feasible clinically. Therefore, using freshly isolated primary human hepatocytes (PHH) cultured *in vitro*, we analyzed the effect of HBV-active NAs on primary HBV infection and showed the possibility of prophylaxis by NAs not only as PrEP but also as PEP against acute HBV infection.

MATERIALS AND METHODS

Freshly isolated PHH. Freshly isolated primary human hepatocytes (PHH) from severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator, whose livers were repopulated with human hepatocytes, were purchased from Phoenix Bio Co., Ltd. (Higashihiroshima, Japan), without cryopreservation. The isolation and culture of PHH were described previously (9).

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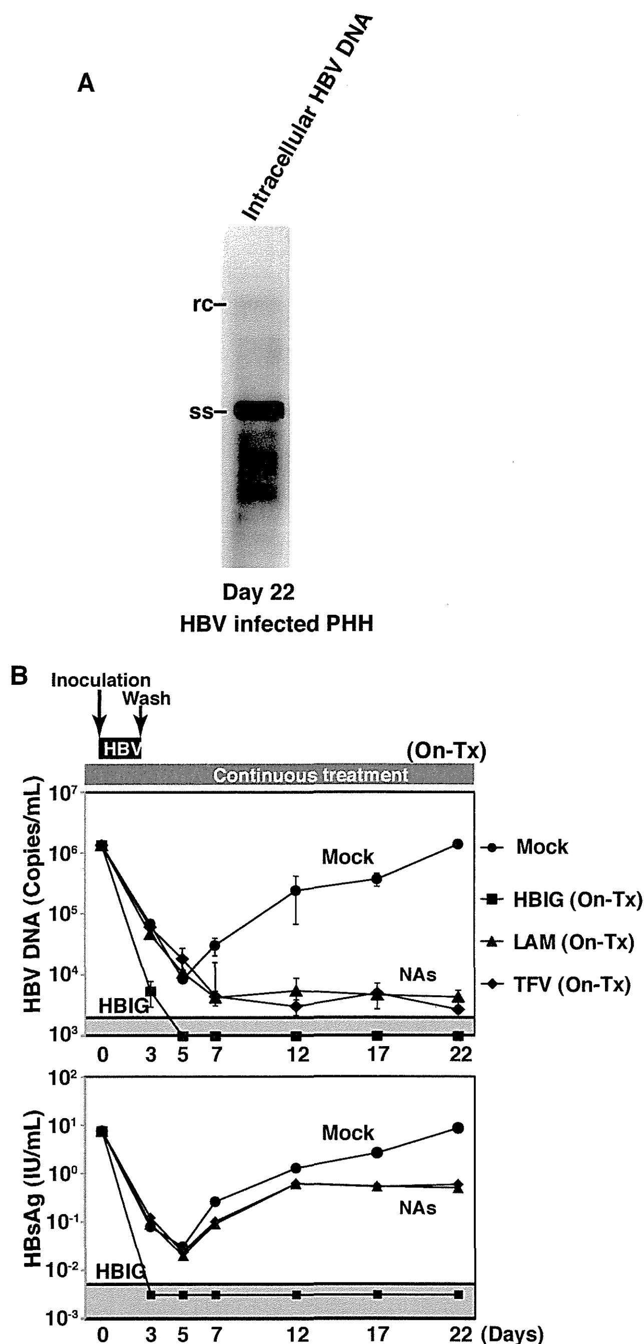

FIG 1 Incomplete prevention of primary HBV infection by NA. (A) Freshly isolated PHH susceptible to HBV infection. Single-stranded HBV DNA (ssDNA), a replicative intermediate in PHH on day 22 postinfection, was visualized by Southern blotting. rcDNA, relaxed-circular, partially double-stranded HBV DNA. ssDNA denotes HBV replication following infection in susceptible cells. (B) The kinetics of HBV DNA (top) and hepatitis B surface antigen (HBsAg; bottom) concentrations in the culture medium of HBV-infected PHH with various treatments. PHH (2.1×10^5 cells/cm^2) were inoculated with HBV (2×10^6 copies/ml) for 48 h at 37°C, and then PHH were washed several times and sampling of the supernatants throughout the course (days 3, 5, 7, 12, 17, and 22 postinfection) was performed. The concentrations of HBV DNA and HBsAg in the culture supernatants, mainly remaining inoculum, decreased until day 5; however, HBV DNA and HBsAg began to increase after around day 5 because of HBV replication in PHH. On-Tx, HBV-infected PHH were treated continuously with lamivudine (LAM), tenofovir (TFV), or hepatitis B immune globulin (HBIG) up to 22 days. Ongoing treatment with LAM or TFV resulted in a continuous decrease in HBV DNA in the supernatant. However, the production of HBsAg increased after day 5. Data are presented as means ± standard deviations (SD) (n = 3 experiments). The NAs were LAM and TFV. Mock, the HBV infection control experiment without treatment.

HBV infection experiments. Inoculation of PHH with 10 HBV genome equivalents per cell was carried out in culture medium without polyethylene glycol for 48 h at 37°C. PHH were washed with medium three times on days 2 and 3 to remove the inoculum. Supernatants were collected, and the culture media were replenished on days 3, 5, 7, 12, 17, and 22 postinfection. To specifically block HBV attachment to the PHH, the HBV inoculum (2×10^6 copies/ml) was preincubated with 1,000 mIU/ml of hepatitis B immunoglobulin (HBIG) for 2 h, and the mixture of HBIG and HBV was added to the PHH. Also, 2.5 mM lamivudine (LAM) or 100 μM tenofovir (TFV) was added to the culture medium, starting 24 h before inoculation. According to previous results, using the HBV-expressing cell lines (Hep2.2.15), the 50% effective concentration (EC_{50}) value of LAM (10) is around 0.12 μM and that of TFV (11) is around 1.1 μM. Thus, the doses of LAM and TFV used in our study are 20,000 times and 100 times higher, respectively, than the EC_{50} for HBV inhibition and thus provide sufficient excess for the desired clinical effect. In addition, the high concentration of LAM and TFV could not cause any cytotoxicity on the cells.

Determination of HBsAg and HBV DNA levels. The levels of HBsAg were determined by chemiluminescent enzyme immunoassay as described previously (12). The detection limit of the HBsAg assay is 0.05 IU/ml. HBV DNA was quantified by quantitative PCR as described previously (13). The detection limit of HBV DNA was set to 2.0×10^3 copies/ml. cccDNA was measured by quantitative PCR with primers cccF2 and cccR4, described previously (14), and a fluorescent probe, cccP2 (5′-FAM-CTGTAGGCATAAATTGGT-MGB-3′ [FAM is 6-carboxyfluorescein]). The detection limit of cccDNA was set to 1.0×10^2 copies/μg DNA.

Southern hybridization. Southern hybridization was performed with full-length probes for HBV as described previously (12, 15).

Immunofluorescence assay. Fluorescence staining of intracellular HBsAg was performed by standard methods using goat anti-HBs (Bioss, Inc., MA, USA) and donkey anti-goat IgG conjugated with Alexa Fluor 488 (Life Technologies, Maryland, USA).

RESULTS

Effects of HBV-active NAs against primary infection. Freshly isolated PHH were confirmed to be susceptible to HBV primary infection by using Southern blot analysis (Fig. 1A). We examined the effect of NAs on the establishment of HBV infection in the hepatocytes. PHH were treated with the HBV-active NAs, LAM and TFV, at extremely high concentrations (2.5 mM and 100 μM, respectively) starting 24 h before HBV inoculation to gain full efficacy of NAs. Figure 1B shows the kinetics of HBV DNA (top) and HBsAg (bottom) concentrations in the culture medium of HBV-infected PHH. After day 5, HBV DNA concentrations increased in the supernatants of nontreated cells, indicating HBV replication, i.e., accumulation of cccDNA in the cell and release of progeny virions into the culture medium. Because NAs inhibit RT activity, treatment with LAM or TFV results in a continuous decrease of HBV DNA in the supernatants. In contrast, HBIG, which inhibits viral entry by blocking receptor binding (16), effectively prevented HBV infection, and HBV DNA could not be detected for 22 days. As shown in the bottom panel of Fig. 1B, LAM and TFV treatment have a small effect on the HBsAg concentration in the

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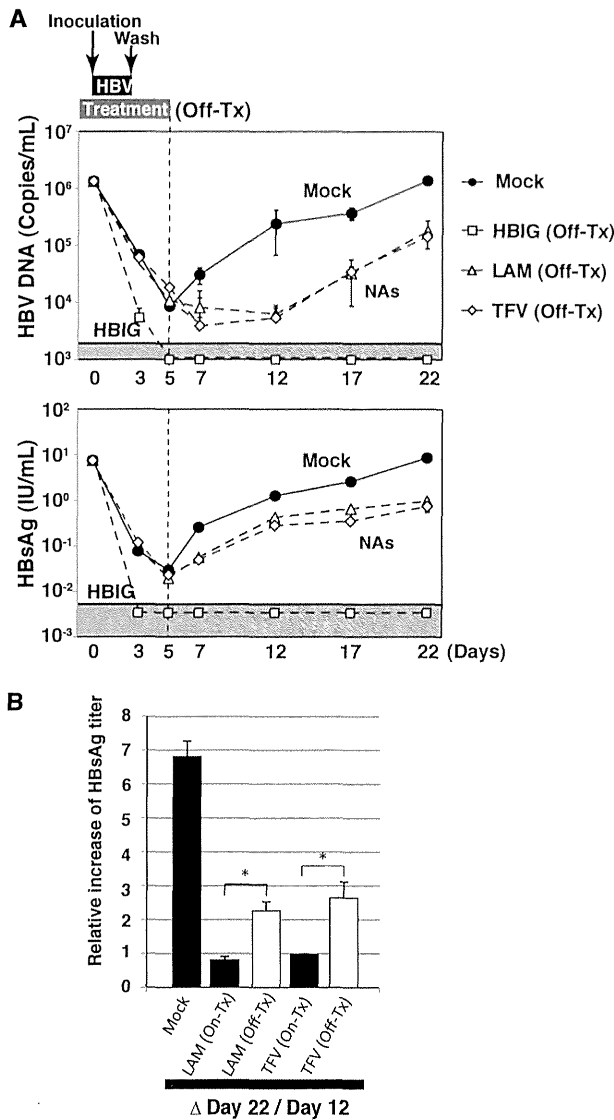

FIG 2 Preexposure NA treatment fails to prevent HBV infection. (A) PHH were inoculated with HBV for 48 h at 37°C and washed several times, and sampling of the supernatants was performed (as described in Fig. 1B). The concentration of HBV DNA increased gradually after day 12 when the LAM or TFV treatment was stopped on day 5, despite the complete cessation of hepatitis B immune globulin (HBIG) treatment. The production of HBsAg in the control experiment increased further after day 12, and HBsAg productions in cases in which NA treatment was stopped on day 5 (Off-Tx) gradually increased in the supernatant. Off-Tx, HBV-infected PHH were treated for only 5 days with lamivudine (LAM), tenofovir (TFV), or HBIG. Data are presented as means ± SD ($n = 3$ experiments). (B) Increasing production of HBsAg in the supernatants during the period from day 12 to day 22. In order to confirm the change of HBsAg production, the HBsAg titer on day 22 was divided by that on day 12. The increase of the rate in the nontreatment experiment was 6.8 from day 12 to day 22; otherwise, the rates of the cells treated continuously with NAs (On-Tx of both LAM and TFV) were maintained at steady levels (the ratio was approximately 1). In the cases with cessation of NA treatment on day 5, the increase of HBsAg production during the period from day 12 to day 22 was observed in the culture medium (Off-Tx of LAM, 2.3; Off-Tx of TFV, 2.6). Data are presented as means ± SD ($n = 3$ experiments). Mock, the HBV infection control experiment without treatment. Statistical analysis of the difference was conducted using the analysis of variance with a nonparametric Mann-Whitney U test. *, $P < 0.05$.

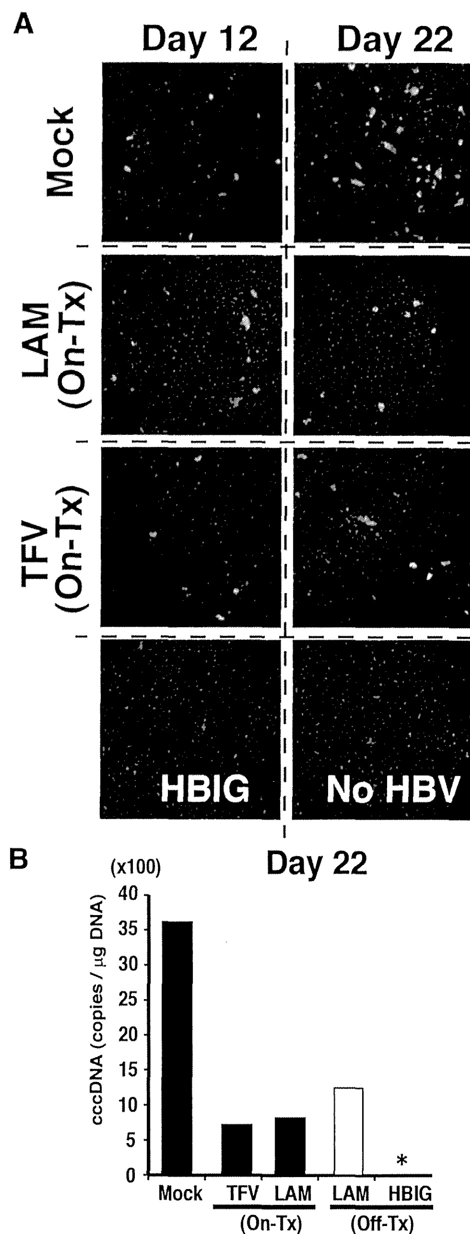

FIG 3 HBV-active NAs can inhibit viral spread after acquisition of HBV infection in freshly isolated PHH. (A) Immunohistochemical staining of HBV-infected PHH with various treatments. Viral spread occurred over 10 days from day 12 to day 22 in the nontreatment experiment (Mock); however, the numbers of cells staining for HBsAg did not change from day 12 to day 22 with continuous treatment by either LAM or TFV. HBIG, treatment with HBIG for 12 days; no HBV, PHH at day 22 without HBV inoculation; green, staining of HBsAg; blue, staining of nuclear DNA. (B) The level of cccDNA in HBV-infected PHH at day 22. Compared to the control experiment (Mock), the levels of cccDNA treated continuously with NAs (On-Tx of both LAM and TFV) were suppressed and that with LAM treatment for only 5 days (LAM Off-Tx) exhibited an increase following the cessation of LAM treatment. The asterisk indicates a value below the detection limit.

supernatant up to day 12; presumably, these NAs could not protect against the formation albeit at high concentration but do inhibit the accumulation of cccDNA as the template for HBsAg. Through the experiments, we did not see evidence of cytotoxicity by 3-(4,5-di-

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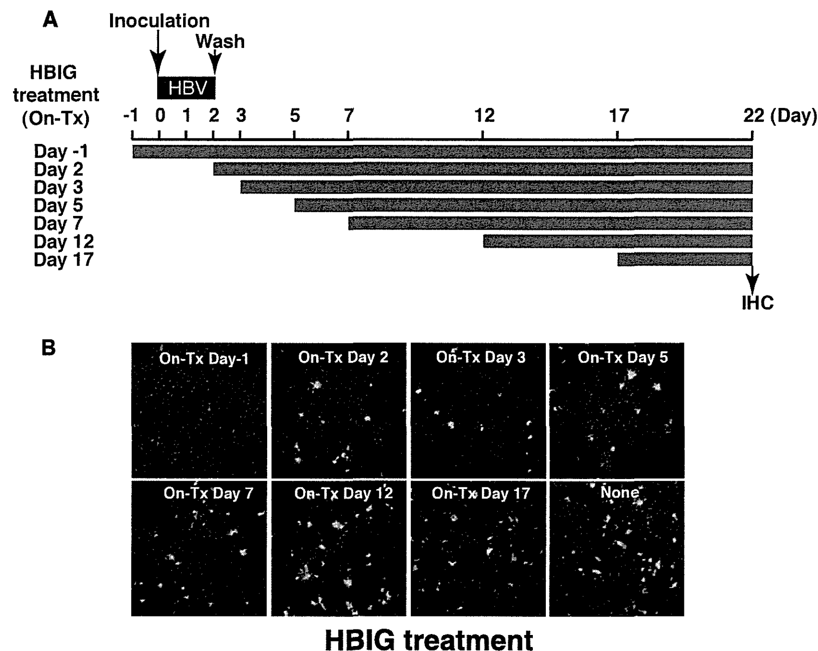

FIG 4 HBIG treatment in freshly isolated PHH post-HBV inoculation inhibits viral spread, while delaying HBIG addition allows viral spread. (A) Schematic representation of the schedule for continuous treatment (On-Tx) with hepatitis B immune globulin (HBIG) after HBV inoculation. IHC, immunohistochemical staining for intracellular HBsAg. (B) IHC of PHH on day 22 with HBIG treatment started at 2, 3, 5, 7, 12, and 17 days after HBV inoculation, as indicated in panel A. The addition of HBIG after inoculation did not protect against primary HBV infection (On-Tx Day 2), and delaying the addition of HBIG allowed gradual viral spread (On-Tx Day 2 to Day 17), detected by fluorescence IHC at day 22 postinoculation.

methyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays or by microscopic examination of cell morphology after treatment with these high concentrations (data not shown).

Next, to confirm the prophylactic effect of NAs, treatment was stopped at day 5 (Fig. 2A). As expected, HBIG continued to prevent HBV infection. When LAM or TFV treatment was stopped on day 5, however, the concentration of HBV DNA increased gradually after day 12, suggesting that these agents did not protect against the establishment of HBV infection (Fig. 2A). The production of HBsAg in the control experiment also increased after day 12, and HBsAg production in cases in which NA treatment had ceased on day 5 (Off-Tx) also slowly increased. Previously, it was thought that the rapid differentiation of PHH leads to a loss of susceptibility to HBV infection within a few days. However, the PHH used in this study were isolated from living chimeric mice without cryopreservation. We therefore confirmed the susceptibility to HBV infection over 10 days using the increase in HBsAg production as a surrogate marker for the accumulation of cccDNA and thus the increasing number of HBV-infected PHH. Although HBsAg production increased markedly in the nontreatment experiment (the relative increase ratio was 6.8) during the period from day 12 to day 22 (Fig. 2B), there was no increase in cells continuously treated with NA (the ratios of LAM and TFV On-Tx were approximately 1). However, in the cases with cessation of NA treatment on day 5, HBsAg production increased in the culture medium from day 12 to day 22 (the ratios of LAM and TFV Off-Tx were 2.3 and 2.6, respectively). These results indicated that freshly isolated PHH, without cryopreservation, have prolonged susceptibility to HBV infection. Although we did not compare fresh and cryopreserved PHH, our observations indicate that freshly isolated PHH remain susceptible to HBV infection at least for 1 month after preparation (data not shown).

To further demonstrate the susceptibility of freshly isolated PHH to HBV infection, we investigated HBsAg expression and measured PHH cccDNA concentration. Detection of HBV-infected PHH by fluorescence immunohistochemistry (IHC) revealed that the number of infected cells on day 22 was approximately 6 times greater than that on day 12, indicating viral spread during this period (Fig. 3A, Mock). In contrast, no increase in the number of infected cells was observed following continuous LAM or TFV treatment. Although the level of cccDNA in cells treated continuously with NAs (LAM and TFV) was suppressed on day 22, compared to the control experiment, those treated with LAM for only 5 days (LAM Off-Tx) exhibited an increase following cessation of LAM treatment (Fig. 3B). Because NAs inhibit the production of infectious virions at the stage of conversion of pregenomic RNA to genomic DNA, the release of progeny virions is inhibited by NAs. This allows us to suggest that secondary infection of neighboring cells by newly produced HBV virions, viral spread, occurred in the *in vitro* system. From these results and basic mechanisms regarding the HBV replication cycle, we conclude that NAs do not prevent the establishment of cccDNA and acquisition of HBV infection but prevent viral spread by suppressing the production of progeny HBV virions.

Feasibility of NA-based HBV postexposure prophylaxis. Considering that HBV-active NAs act only on viral spread, we explored the possibility that the addition of LAM to the culture medium shortly after HBV inoculation might have an effect similar to that of preexposure LAM treatment, as shown in Fig. 1 and 3. Again, to confirm the susceptibility to HBV infection during the

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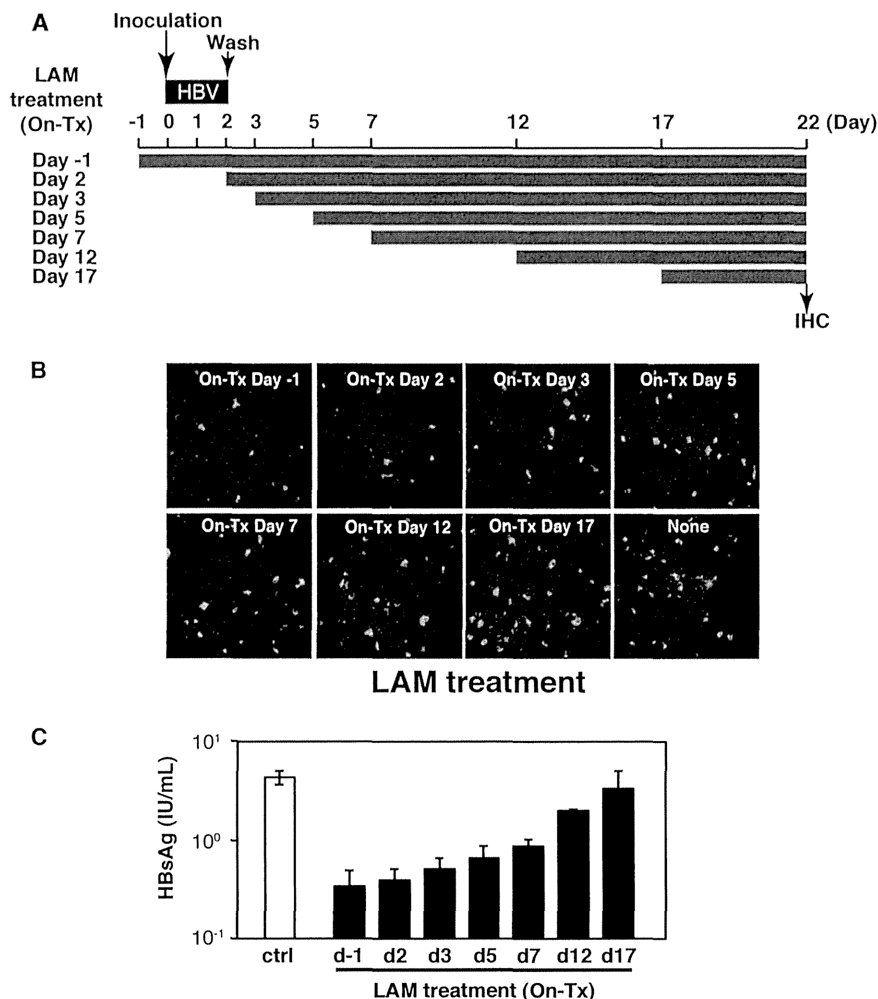

FIG 5 Effects of LAM on post-HBV inoculation *in vitro*. (A) Schematic representation of the schedule for continuous treatment (On-Tx) with lamivudine (LAM) after HBV inoculation. IHC, immunohistochemical staining for intracellular HBsAg. (B) IHC of PHH on day 22 with LAM treatment started at 2, 3, 5, 7, 12, and 17 days after HBV inoculation, as indicated in panel A. The addition of LAM before and after inoculation did not protect against primary HBV infection (On-Tx Day −1 and Day 2), and the delay in the addition of LAM resulted in viral spread at day 22 postinoculation. (C) The HBsAg concentrations in the culture medium collected on day 22 from the HBV-infected PHH with LAM treatment as shown in panel A. Data are presented as means ± SD ($n = 3$ experiments). ctrl, HBV-infected PHH without treatment.

follow-up periods, the addition of HBIG after inoculation was explored for 22 days (Fig. 4A), because HBIG treatment protects completely against new HBV infection (Fig. 1B, 2A, and 3B). The addition of HBIG after inoculation was not able to protect against primary HBV infection (Fig. 4B, On-Tx Day −1 compared to Day 2), and delaying adding HBIG allowed gradual viral spread, monitored by fluorescence IHC at day 22 postinoculation (Fig. 4B, On-Tx Day 2 to Day 17 compared to None). These results strongly indicate that delaying starting HBIG treatment after HBV inoculation allowed viral spread into the freshly isolated PHH system. Next, the effect of the addition of LAM after inoculation was monitored and quantified, based on the HBsAg titer in the supernatant (Fig. 5). Delaying the addition of LAM (Fig. 5A) allowed gradual viral spread, as observed in the HBIG treatment. However, the addition of LAM on day 2 after inoculation effectively inhibited the viral spread observed on day 22 (Fig. 5B, On-Tx Day 2). Furthermore, the HBsAg levels in the culture medium on day 22 indicate that starting LAM treatment within 2 days of inoculation has a similar effect on viral spread to preexposure treatment (Fig. 5C).

DISCUSSION

Coinfection with HBV and HIV increases the morbidity and mortality beyond those caused by either virus alone, and the burden of coinfection is greatest in developing countries, particularly in Southeast Asia and sub-Saharan Africa (1). Therefore, the assessment of HBV status is warranted for all HIV-positive persons, and hepatitis B vaccination should be offered to all susceptible individuals. Although immunization remains the mainstay of disease prevention, HIV-positive individuals mount poorer antibody responses to hepatitis B vaccine than healthy donors (17, 18). Moreover, as the course of hepatitis B immunization involves injections at 0, 1, and 6 months, a coordinated effort and period of time are required.

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Because NA-based PEP has been used as an HIV prevention strategy for nearly 20 years, we explored the possibility of HBV PEP using NAs, with reference to clinical evidence that PrEP may be feasible for *de novo* HBV infection. In Japan, Gatanaga et al. explored NA-based PrEP using sequential serum samples from HIV-infected MSM who had no detectable HBsAg, anti-HBs, and anti-HBc at baseline (3). Evidence of HBV infection was detected in follow-up samples from 43 of the 354 men (12.1%) after a median time period of 1.6 years. The rate of incident infection was approximately 90% lower for patients taking LAM or TFV than for those receiving no ART or a non-LAM/TFV-containing regimen. Moreover, no new HBV infections occurred in patients taking TDF; however, the 7 men who developed HBV infection despite taking LAM were more likely to be infected with LAM-resistant strains (50.0%) than the 36 individuals not receiving HBV-active ART (7.1%). Another serological follow-up study from the Netherlands reported that LAM and TFV protected against the occurrence of *de novo* HBV infection among HIV-infected MSM over a median of 6.8 years and also demonstrated a superior protective effect on HBV seroconversion in the group taking TFV (4). These clinical data indicated that HBV-active NAs can prevent acute HBV infection, defined by the absence of the serological markers HBsAg, anti-HBs, and anti-HBc, and TFV-based PrEP has a significant effect on HBV primary infection. In the case of HIV infection, the prophylactic effects of NAs as RT inhibitors are well accepted, because these block the initial step following virus entry. However, during HBV infection, RT inhibitors act after cccDNA formation and do not prevent HBV infection completely, confirmed by our original HBV-infected PHH system. Our results clearly indicate that the effect of NAs on HBV primary infection was simply prevention or inhibition of viral spread.

It remains largely unknown how NAs act as prophylaxis. As clinical data showed, treatment with NAs protects against the acquisition of HBV without an antibody response. We speculated that acquired immunity, especially humoral immunity, could not be responsible for the clinical outcome of prophylaxis by NAs. Recent reports show that natural killer (NK) cells contribute to protection against acute HCV infection (19–21), and NK cells may play important roles in the eradication of HBV from hepatocytes (22, 23). Continuous treatment with NAs could prevent viral spread and, consequently, the cccDNA would be confined to the cells infected initially. If this is the case, the small number of cccDNA-containing cells could readily be eradicated by the major innate immune cells, i.e., NK cells, without an antibody response, and/or the cccDNA could be diluted during hepatocyte turnover with subsequent diminishment of hepatocytes harboring cccDNA in the absence of immune responses (24).

Although the extent to which the data from *in vitro* studies can be extrapolated to clinical human studies is largely unknown, we postulate that treatment with NAs should be used for longer periods of HBV prophylaxis than of HIV prophylaxis, typically for 4 weeks in HIV PEP (5, 25), because of the evidence from our *in vitro* data regarding acquisition of HBV infection. Correspondingly, the use of NAs for preexposure and postexposure prophylaxis to prevent HBV transmission must also be studied in the context of HIV prevention in areas where there may be problems in obtaining and/or storing HBIG. Despite the fact that our results with higher concentrations of NAs than those in the clinical setting indicate the possibility of NA-based HBV PEP, as well as PrEP shown by retrospective human studies, further research and human clinical trials are needed to evaluate brief screening, drug selection, as well as dose, timing, and duration of NA-based PEP against *de novo* HBV infection in susceptible high-risk groups.

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We have no conflicts of interest to declare.

REFERENCES

1. **Kourtis AP, Bulterys M, Hu DJ, Jamieson DJ.** 2012. HIV-HBV coinfection—a global challenge. N Engl J Med **366:**1749–1752. http://dx.doi.org/10.1056/NEJMp1201796.
2. **Thio CL.** 2003. Hepatitis B in the human immunodeficiency virus-infected patient: epidemiology, natural history, and treatment. Semin Liver Dis **23:**125–136. http://dx.doi.org/10.1055/s-2003-39951.
3. **Gatanaga H, Hayashida T, Tanuma J, Oka S.** 2013. Prophylactic effect of antiretroviral therapy on hepatitis B virus infection. Clin Infect Dis **56:**1812–1819. http://dx.doi.org/10.1093/cid/cit145.
4. **Heuft MM, Houba SM, van den Berk GE, Smissaert van de Haere T, van Dam AP, Dijksman LM, Regez RM, Brinkman K.** 2014. Protective effect of hepatitis B virus-active antiretroviral therapy against primary hepatitis B virus infection. AIDS **28:**999–1005. http://dx.doi.org/10.1097/QAD.0000000000000180.
5. **Cardo DM, Culver DH, Ciesielski CA, Srivastava PU, Marcus R, Abiteboul D, Heptonstall J, Ippolito G, Lot F, McKibben PS, Bell DM.** 1997. A case-control study of HIV seroconversion in health care workers after percutaneous exposure. Centers for Disease Control and Prevention Needlestick Surveillance Group. N Engl J Med **337:**1485–1490.
6. **Kuhar DT, Henderson DK, Struble KA, Heneine W, Thomas V, Cheever LW, Gomaa A, Panlilio AL, U.S. Public Health Service Working Group.** 2013. Updated US Public Health Service guidelines for the management of occupational exposures to human immunodeficiency virus and recommendations for postexposure prophylaxis. Infect Control Hosp Epidemiol **34:**875–892. http://dx.doi.org/10.1086/672271.
7. **Rehermann B, Ferrari C, Pasquinelli C, Chisari FV.** 1996. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. Nat Med **2:**1104–1108. http://dx.doi.org/10.1038/nm1096-1104.
8. **Torres HA, Davila M.** 2012. Reactivation of hepatitis B virus and hepatitis C virus in patients with cancer. Nat Rev Clin Oncol **9:**156–166. http://dx.doi.org/10.1038/nrclinonc.2012.1.
9. **Yamasaki C, Kataoka M, Kato Y, Kakuni M, Usuda S, Ohzone Y, Matsuda S, Adachi Y, Ninomiya S, Itamoto T, Asahara T, Yoshizato K, Tateno C.** 2010. *In vitro* evaluation of cytochrome P450 and glucuronidation activities in hepatocytes isolated from liver-humanized mice. Drug Metab Pharmacokinet **25:**539–550. http://dx.doi.org/10.2133/dmpk.DMPK-10-RG-047.
10. **Innaimo SF, Seifer M, Bisacchi GS, Standring DN, Zahler R, Colonno RJ.** 1997. Identification of BMS-200475 as a potent and selective inhibitor of hepatitis B virus. Antimicrob Agents Chemother **41:**1444–1448.
11. **Delaney WE, IV, Ray AS, Yang H, Qi X, Xiong S, Zhu Y, Miller MD.** 2006. Intracellular metabolism and *in vitro* activity of tenofovir against hepatitis B virus. Antimicrob Agents Chemother **50:**2471–2477. http://dx.doi.org/10.1128/AAC.00138-06.
12. **Sakamoto T, Tanaka Y, Watanabe T, Iijima S, Kani S, Sugiyama M, Murakami S, Matsuura K, Kusakabe A, Shinkai N, Sugauchi F, Mizokami M.** 2013. Mechanism of the dependence of hepatitis B virus genotype G on co-infection with other genotypes for viral replication. J Viral Hepat **20:**e27–e36. http://dx.doi.org/10.1111/jvh.12022.
13. **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.
14. **Sa-Nguanmoo P, Tanaka Y, Ratanakorn P, Sugiyama M, Murakami S, Payungporn S, Sommanustweechai A, Mizokami M, Poovorawan Y.** 2011. Cross-species transmission of gibbon and orangutan hepatitis B virus to uPA/SCID mice with human hepatocytes. Virus Res **158:**209–215. http://dx.doi.org/10.1016/j.virusres.2011.04.002.

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15. **Fujiwara K, Tanaka Y, Paulon E, Orito E, Sugiyama M, Ito K, Ueda R, Mizokami M, Naoumov NV.** 2005. Novel type of hepatitis B virus mutation: replacement mutation involving a hepatocyte nuclear factor 1 binding site tandem repeat in chronic hepatitis B virus genotype E. J Virol **79:**14404–14410. http://dx.doi.org/10.1128/JVI.79.22.14404-14410.2005.
16. **Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, Fu L, Song M, Chen P, Gao W, Ren B, Sun Y, Cai T, Feng X, Sui J, Li W.** 2012. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. eLife **1:**e00049. http://dx.doi.org/10.7554/eLife.00049.
17. **Rivas P, Herrero MD, Puente S, Ramirez-Olivencia G, Soriano V.** 2007. Immunizations in HIV-infected adults. AIDS Rev **9:**173–187.
18. **Launay O, van der Vliet D, Rosenberg AR, Michel ML, Piroth L, Rey D, Colin de Verdiere N, Slama L, Martin K, Lortholary O, Carrat F, ANRS HB03 VIHVAC-B Trial.** 2011. Safety and immunogenicity of 4 intramuscular double doses and 4 intradermal low doses vs standard hepatitis B vaccine regimen in adults with HIV-1: a randomized controlled trial. JAMA **305:**1432–1440. http://dx.doi.org/10.1001/jama.2011.351.
19. **Sugden PB, Cameron B, Mina M, Lloyd AR, HITS Investigators.** 2014. Protection against hepatitis C infection via NK cells in highly-exposed uninfected injecting drug users. J Hepatol **61:**738–745. http://dx.doi.org/10.1016/j.jhep.2014.05.013.
20. **Werner JM, Heller T, Gordon AM, Sheets A, Sherker AH, Kessler E, Bean KS, Stevens M, Schmitt J, Rehermann B.** 2013. Innate immune responses in hepatitis C virus-exposed healthcare workers who do not develop acute infection. Hepatology **58:**1621–1631. http://dx.doi.org/10.1002/hep.26353.
21. **Knapp S, Warshow U, Hegazy D, Brackenbury L, Guha IN, Fowell A, Little AM, Alexander GJ, Rosenberg WM, Cramp ME, Khakoo SI.** 2010. Consistent beneficial effects of killer cell immunoglobulin-like receptor 2DL3 and group 1 human leukocyte antigen-C following exposure to hepatitis C virus. Hepatology **51:**1168–1175. http://dx.doi.org/10.1002/hep.23477.
22. **Maini MK, Peppa D.** 2013. NK cells: a double-edged sword in chronic hepatitis B virus infection. Front Immunol **4:**57. http://dx.doi.org/10.3389/fimmu.2013.00057.
23. **Rehermann B.** 2013. Pathogenesis of chronic viral hepatitis: differential roles of T cells and NK cells. Nat Med **19:**859–868. http://dx.doi.org/10.1038/nm.3251.
24. **Summers J, Jilbert AR, Yang W, Aldrich CE, Saputelli J, Litwin S, Toll E, Mason WS.** 2003. Hepatocyte turnover during resolution of a transient hepadnaviral infection. Proc Natl Acad Sci U S A **100:**11652–11659. http://dx.doi.org/10.1073/pnas.1635109100.
25. **U.S. Public Health Service.** 2001. Updated U.S. Public Health Service guidelines for the management of occupational exposures to HBV, HCV, and HIV and recommendations for postexposure prophylaxis. MMWR Recomm Rep **50:**1–52.

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B型急性肝炎の遷延化（Genotype Aを中心に）

渡 邊 綱 正* 田 中 靖 人*

索引用語：HBV遺伝子型A，急性肝炎遷延化，慢性化，HLA，ウイルス増殖効率

1 はじめに

B型肝炎ウイルス（HBV）感染者数は全世界で4億2千万人，さらに死亡者数は年間約50万人以上といわれ，HIV，HCVと並び世界的に重要な感染症の一つである．本邦での感染率は約0.7％と推定され，感染者数は80万人程度と考えられている．

分子系統樹解析の進歩に伴い，HBVは現在A型からJ型までの9つの遺伝子型（遺伝子型IはCの亜型）に分類され[1]，その臨床的差異が数多く報告されている[2]．HBV遺伝子型A，Dはヨーロッパ諸国および地中海沿岸に広く分布する一方，遺伝子型B，Cは日本を含む東アジアを中心に広く分布している．これまでの国内HBV感染者は，そのほとんどが母児感染による遺伝子型Cであったが，母子感染防止事業により新規の垂直感染例は稀となった．しかし，その一方では若年者の性行為感染によるB型急性肝炎，特に遺伝子型AのHBV（HBV/A）感染が増加しており，今後わが国におけるHBV感染症に大きな影響を及ぼすことが危惧される．

本稿では，HBV感染症について最近報告のあったいくつかの論文を中心に遺伝子型Aの特徴，特に肝炎遷延化についてこれまでわかっていることについて概説する．

2 わが国における遺伝子型AによるB型急性肝炎の推移

1991～2009年に国立病院を中心とした全国28カ所のB型急性肝炎患者（547例）における遺伝子型分布の調査が，Tamadaらにより英文雑誌GUTに報告された[3]．B型急性肝炎例のうちHBV遺伝子型がAである割合は，1991～1996年が9例（6.0％），1997～2002年は19例（15.4％），2003～2008年では89例（39.4％），さらに2009年のみでは20例（41.7％）であり，全国的にHBV/AによるB型急性肝炎の増加が確認された（図1）．さらに，2006年から2008年の首都圏におけるB型急性肝炎の実態を調査した山田らの報告[4]

Tsunamasa WATANABE et al : Mechanism of progression to chronicity by acute hepatitis B virus genotype A infection

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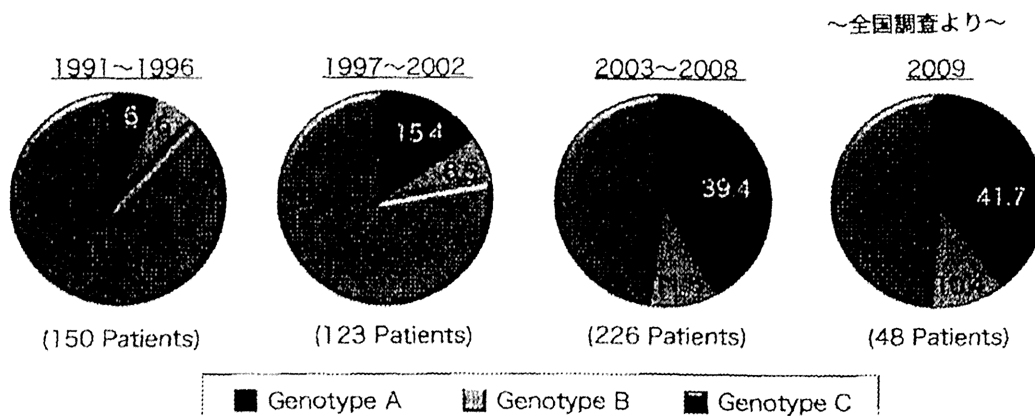

図1　B型急性肝炎におけるHBV Genotypesの継時推移(文献3より改変)

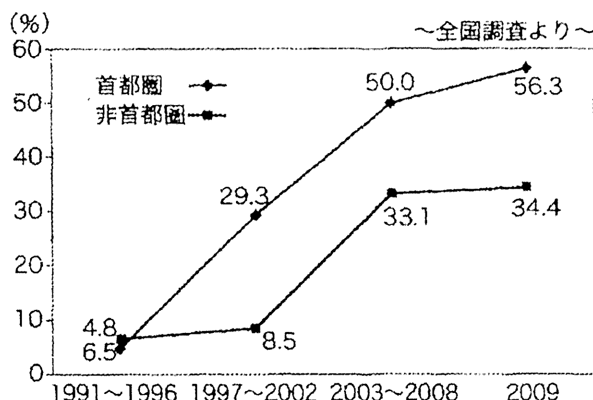

図2　HBV Genotypes Aによる急性肝炎の頻度(文献3より改変)

と同様に，この全国調査の中でも1997年から2002年に首都圏でHBV/Aが急激に増化し(1991年～1996年が4.8%，1997年～2002年は29.3%，$p<0.05$)，その後の2003年から2008年に非首都圏でHBV/Aが増加したこと(1997年～2002年が8.5%，2003年～2008年は33.1%，$p<0.0001$)が報告されている(図2).

3　B型急性肝炎の遷延化機序

近年，本邦において急性肝炎でありながら慢性の経過をたどるB型肝炎例の報告が散見され，その多くがHBV/Aであることが報告されている[5-7]．これまで，B型急性肝炎はHBVの初感染によって起こるself-limitingな疾患であり，多くは2～3カ月の経過をもってウイルスは排除され臨床的治癒に至ると考えられていた．一般に，急性肝炎とは症状発現後6カ月以内に臨床的治癒に至るとされるが，HBV/Aによる急性肝炎ではHBs抗原の消失が遷延し，従来の急性肝炎とは臨床経過が異なる可能性が示唆される．これは本邦のみにおける特有な経過ではなく，以前から欧米の報告でも急性肝炎の約10%が慢性化するとされており，おそらく地域による感染HBV遺伝子型の違いによることが予測される．

HBV遺伝子型による臨床的差異が生じる要因については現在も不明であるが，近年，B型慢性肝炎成立機序に関して，示唆に富む研究がいくつか報告されている．

2009年のチンパンジーを用いた感染実験の結果から[8]，高力価(10^{10}～10^4 copy/mL)ないし低力価(1 copy/mL)の感染ウイルス接種では免疫応答による肝炎発症の後に30週以内にウイルス排除をきたす一方，1頭あたり10 copiesのウイルスを接種したチンパンジーにおいてのみ慢性肝炎(42週以上の持続HBV感染)が成立し，その要因がCD4 T細胞のプライミングであることを，抗体を用いたCD4 T細胞のdepletionにより明らかとした．

表1　HBVに関するこれまでのGWAS報告

Ethnicity	表現型	候補遺伝子	SNP ID	オッズ比（95％CI）またはβ（SE）	Year［ref］
日本人，タイ人	CHB	HLA-DPA1	rs3077	0.57（0.49～0.66）	2009［10］
		HLA-DPB1	rs9277535	0.59（0.51～0.61）	
日本人	CHB	HLA-DQB1	rs2856718	1.56（1.45～1.67）	2011［11］
		HAL-DQB2	rs7453920	1.81（1.62～2.01）	
		HLA-DPA1	rs3077	1.87（1.73～2.01）	
		HLA-DPB1	rs9277535	1.77（1.65～1.91）	
中国人	CHB vs ASC	GRIN2A	rs11866328	1.68（1.40～2.02）	2011［13］
インドネシア人	HB vaccine response	HLA-DR	rs3135363	1.53（1.35～1.74）	2011［14］
		HLA	rs9267665	2.05（1.64～2.57）	
		HLA-DPB1	rs9277535	1.39（1.23～1.59）	
日本人，韓国人	CHB vs control	HLA-DPA1	rs3077	0.42（0.30～0.58）	2012［12］
		HLA-DPB1	rs9277542	0.42（0.31～0.58）	
	CHB vs resolver	HLA-DPA1	rs3077	0.44（0.32～0.61）	
		HLA-DPB1	rs9277542	0.51（0.37～0.70）	

（文献22より改変）

従来から，HBV排除にはCD8T細胞が重要であることが報告されていたが[9]，慢性化成立機序にはCD4T細胞応答が重要であることを示唆する報告であった．

このことは，臨床サンプルを用いたヒト要因に関する検討からも類推される．2009年にKamataniらのグループが日本人B型慢性肝炎患者群179検体と対照群934検体を対象としたGWASを実施した結果，HLA-DPA1/DPB1遺伝子領域がB型肝炎慢性化に有意な関連を示すことを報告し[10]，さらに検体数を増やしたGWASからHLA-DQ遺伝子領域がHLA-DPと独立したB型慢性肝炎発症の関連因子であることが報告された[11]．また，独立した日本人集団と韓国人集団を対象としたGWAS研究により，HLA-DP遺伝子がB型肝炎慢性化のみならず，HBVの排除にも寄与することが報告された[12]．その後，相次いで同様の報告がアジア人種を中心としたGWASにより追試された[10-14]（表1）．一方，2012年にドイツのグループが同様の検討を行ったが[15]，アジア人種で報告されたSNPとヨーロッパにおけるHBV感染の臨床経過に関連性は見いだせなかった．すなわち，アジアで報告されたSNPが必須というわけではなく，HLAタイピングによりHBV感染応答が異なることを示唆した．

HLA-DPA1，*DPB1*はMHC class II 分子であるHLA-DPのα，βサブユニットをコードし，ウイルスや細菌などの外来抗原をCD4陽性T細胞に抗原提示する際に重要な役割を担う．このHLA-DP分子の抗原結合部位の多型がCD4T細胞を介した免疫応答に影響し，HBV持続感染に寄与するものと推測される．

また，HBV感染ハイリスク群を主な対象とするHBVワクチン接種において，ある一

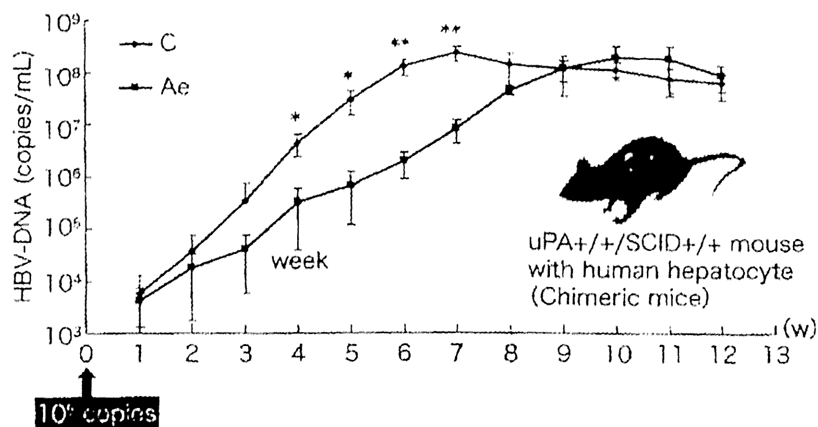

図3　キメラマウス感染実験における初期ウイルス動態(文献19より改変)

定の割合で高い抗体価(HBs抗体価)が得られないことが知られていたが，この要因にもHLA領域(HLA-DR，HLA-DP，HLA class Ⅲ領域)が関連することが，インドネシア人集団約3,600人を対象としたGWASにより明らかとなった[14]．以上より，HBV感染症に関連する遺伝的要因として，HLA領域の重要性が再認識された．上記知見は，HBVの慢性化機序をすべて説明するものではないが，少なくともホスト側の要因であるヒト免疫応答により，その後のHBV感染経過に大きな影響を与えることが示唆される．

一方，ウイルス側の要因であるHBV遺伝子型による病態の違いも注目されている．われわれはこれまでに各HBV遺伝子型の特徴についてキメラマウスを利用した検討を行ってきた．キメラマウスとは，ヒト肝細胞の移植により，ヒト肝臓とマウス肝臓をキメラ状に持つマウスであり，小型哺乳動物におけるHBVやHCVの感染実験を可能とした有用なモデルである[16,17]．キメラマウスでの*in vivo*感染実験において，特に感染初期におけるウイルス複製効率の違いが顕著であることを見いだした(図3)[18,19]．すなわち，遺伝子型によりウイルス増殖効率が異なり，特にHBV/Aでは他の遺伝子型に較べてウイルス増殖効率が低いことを明らかとした．また，チンパンジーを用いたHBV複製効率を概算する実験においても，HBV/Aのdoubling timeは3.4 days [95% confidential interval (CI) 2.6～4.9]であるのに対し，HBV/Cは1.9 days (95% CI 1.6～2.3)であることも報告されている[20]．

先に述べたチンパンジー感染実験におけるウイルス接種量と慢性化成立の関連性と合わせて，感染初期のウイルス量ないしウイルス増殖効率の差が慢性化成立の一端を担う可能性が考えられる．

感染初期のウイルス増殖効率とホストであるヒト免疫応答が相重なり，HBVの遷延化ないし慢性化を形成する機序については，HBV/Aの急性肝炎における臨床経過からも示唆される．Itoらによる2014年英文雑誌Hepatologyの報告[21]によると，2005年～2010年に38施設から212例のB型急性肝炎例をHBs抗原の消失時期別に検討した結果，6カ月以内にHBs抗原が消失するいわゆる急性肝炎の経過を示す症例は，ALT値が高値でHBV/Aの割合が少なく，6カ月以降までHBs抗原陽性で推移する急性肝炎例はALT値が低くHBV/Aの割合が高くさらにHBV-DNA量が高いことを報告した．また，12カ月以上HBs抗原陽性を示した212例中9例は，よ

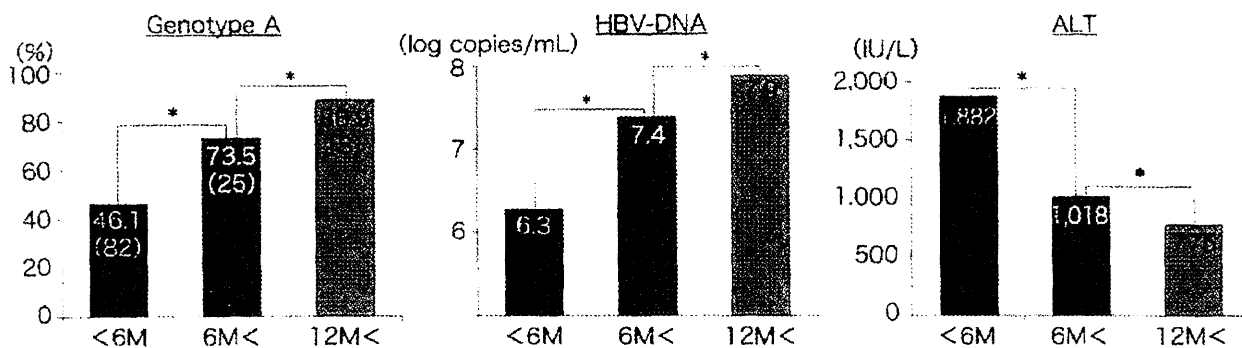

図4　急性肝炎後のHBs抗原消失時期別ALT値，HBV-DNA量，Genotype A割合（文献21より改変）

りALT値が低くHBV-DNAも高値を示し，HBV/Aが9例中8例(88.9％)であった(図4)．すなわち，ALTの低値は肝炎発症時のmildな免疫応答を示唆し，さらにもう一つの臨床的特徴である肝炎発症時の血中ウイルス量高値は遅発性のウイルス増殖を示唆し，上記のヒト免疫応答とHBV/Aのウイルス増殖効率に合致する臨床的特徴と解釈できる．

4　おわりに

ここまで述べたように，わが国におけるB型肝炎の現状は大きく変わってきている．われわれはHIV重複感染例のHBV/Aによる発癌例も経験しており，今後はHBV/A感染例に対する早期核酸アナログ治療介入が肝炎遷延化ないし慢性化を阻止するかどうかの検討が必要と考えられ，さらに治療介入による長期予後の改善も期待される．

文　献

1) Tatematsu K, Tanaka Y, Kurbanov F et al : A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. J Virol 83 : 10538–10547, 2009
2) Miyakawa Y, Mizokami M : Classifying hepatitis B virus genotypes. Intervirology 46 : 329–338, 2003
3) Tamada Y, Yatsuhashi H, Masaki N et al : Hepatitis B virus strains of subgenotype A2 with an identical sequence spreading rapidly from the capital region to all over Japan in patients with acute hepatitis B. Gut 61 : 765–773, 2012
4) 山田　典，四柳　宏，小板橋優，他：首都圏におけるB型急性肝炎の実態と変遷：―Genotype Aに焦点をあてて―．肝臓49：553–559，2008
5) Kobayashi M, Arase Y, Ikeda K et al : Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. J Med Virol 68 : 522–528, 2002
6) 菊池健太郎，宮川　浩，阿部和裕，他：HBV genotype A初感染後キャリア化した夫より夫婦間感染した妻の急性B型肝炎の1例．肝臓39：533–538，1998
7) 新妻宏文，石井元康，小島敏明，他：HBV genotype Aの急性感染からキャリア化した成人例の検討．肝臓40：346–349，1999
8) Asabe S, Wieland SF, Chattopadhyay PK et al : The size of the viral inoculum contributes to the outcome of hepatitis B virus infection. J Virol 83 : 9652–9662, 2009
9) Thimme R, Wieland S, Steiger C et al : CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. J Virol 77 : 68–76, 2003
10) Kamatani Y, Wattanapokayakit S, Ochi H et al : A genome-wide association study identifies variants in the HLA-DP locus associated with chronic hepatitis B in Asians. Nat Genet 41 : 591–595, 2009
11) Mbarek H, Ochi H, Urabe Y et al : A genome-wide association study of chronic hepatitis B identified novel risk locus in a Japanese population. Hum Mol Genet 20 : 3884–3892, 2011
12) Nishida N, Sawai H, Matsuura K et al : Genome-wide association study confirming association of HLA-DP with protection against chronic hepatitis B and viral clearance in Japanese and Korean. PloS one 7 : e39175, 2012

13) Liu L, Li J, Yao J et al : A genome-wide association study with DNA pooling identifies the variant rs11866328 in the GRIN2A gene that affects disease progression of chronic HBV infection. Viral Immunol 24 : 397–402, 2011

14) Png E, Thalamuthu A, Ong RT et al : A genome-wide association study of hepatitis B vaccine response in an Indonesian population reveals multiple independent risk variants in the HLA region. Hum Mol Genet 20 : 3893–3898, 2011

15) Vermehren J, Lotsch J, Susser S et al : A common HLA-DPA1 variant is associated with hepatitis B virus infection but fails to distinguish active from inactive Caucasian carriers. PloS one 7 : e32605, 2012

16) Mercer DF, Schiller DE, Elliott JF et al : Hepatitis C virus replication in mice with chimeric human livers. Nat Med 7 : 927–933, 2001

17) Tateno C, Yoshizane Y, Saito N et al : Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 165 : 901–912, 2004

18) Sugiyama M, Tanaka Y, Kato T et al : Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. Hepatology 44 : 915–924, 2006

19) Sugiyama M, Tanaka Y, Kurbanov F et al : Direct cytopathic effects of particular hepatitis B virus genotypes in severe combined immunodeficiency transgenic with urokinase-type plasminogen activator mouse with human hepatocytes. Gastroenterology 136 : 652–662, 2009

20) Komiya Y, Katayama K, Yugi H et al : Minimum infectious dose of hepatitis B virus in chimpanzees and difference in the dynamics of viremia between genotype A and genotype C. Transfusion 48 : 286–294, 2008

21) Ito K, Yotsuyanagi H, Yatsuhashi H et al : Risk factors for long-term persistence of serum hepatitis B surface antigen following acute hepatitis B virus infection in Japanese adults. Hepatology 59 : 89–97, 2014

22) Chayama K, Ochi H, Miki D : [Recent progress in the study of genetic variants associated with liver diseases]. Nihon Shokakibyo Gakkai zasshi 110 : 1577–1590, 2013

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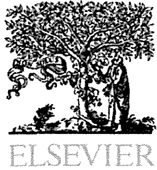

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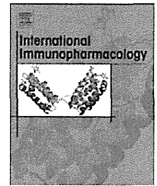

Efficient generation of highly immunocompetent dendritic cells from peripheral blood of patients with hepatitis C virus-related hepatocellular carcinoma☆,☆☆

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ABSTRACT

Background & aims: Immunotherapy using dendritic cells (DCs) is a promising cancer therapy. The success of this therapy depends on the function of induced DCs. However, there has been no consensus on optimal conditions for DC preparation in vitro for immunotherapy of hepatocellular carcinoma (HCC) patients. To address relevant issues, we evaluated the procedures to induce DCs that efficiently function in hepatitis C virus (HCV)-related HCC.
Methods: We studied immunological data from 14 HCC patients. The DC preparation and the surface markers were assessed by flow cytometric analysis. Four different additional activation stimuli (Method I, medium alone; Method II, with OK-432; Method III, with IL-1β + IL-6 + TNF-α; Method IV, with IL-1β + IL-6 + TNF-α + PGE2) were tested and the functions of DCs were confirmed by examination of the ability of phagocytosis, cytokine production and allogeneic mixed lymphocyte reaction (MLR).
Results: The numbers of DCs induced and their cytokine production ability were not different between healthy controls and HCC patients. T-cell stimulatory activity of DCs in MLR was significantly lower in HCC patients than in healthy controls. The maturation of DCs with OK-432 boosted production of cytokines and chemokines, such as IL-2, IL-12p70, IFN-γ, TNF-α, IL-13 and MIP1α, and restored T-cell stimulatory activity of DCs in MLR.
Conclusions: The clinically approved compound OK-432 is a candidate for highly immunocompetent DC preparation and may be considered as a key drug for immunotherapy of HCV-related HCC patients.
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1. Introduction

Hepatocellular carcinoma (HCC) is the seventh most common cancer worldwide and the fourth leading cause of cancer-related deaths in Japan [1]. Hepatitis C virus (HCV) infection is a common cause of chronic liver disease and leads to liver cirrhosis, contributing to the incidence of HCC [2]. Although some therapies for HCC exist, tumor recurrence rates are extremely high in these patients after curative treatments, including hepatic resection and radiofrequency ablation (RFA) [3]. In terms of the reason of this, HCV-related chronic hepatitis and cirrhosis are known to have carcinogenic potential for the development of HCC [4]. For the secondary chemoprevention of HCC patients with HCV-related cirrhosis, recent studies showed the efficacy of long-term and low-dose interferon-α therapy [5]. Among many strategies for HCC recurrence, immunotherapy is considered to be an attractive strategy to eradicate tumor cells completely [6].

Until now, different immunotherapeutic approaches have been tested for patients with HCC [7]. However, tumors have evolved numerous immune escape mechanisms, including the generation of cells with immune suppressor functions, such as Tregs and myeloid-derived suppressor cells [8]. Dendritic cells (DCs) are the most potent professional antigen-presenting cells with the unique ability to initiate and maintain adaptive immunity, and are considered

Abbreviations: DC, dendritic cell; HCV, hepatitis C virus; IFN, interferon; PBMC, peripheral blood mononuclear cell.

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☆☆ Author contributions: Masaaki Kitahara was responsible for analysis of the data and drafting of the article; Eishiro Mizukoshi was responsible for conception and design of the article; Yasunari Nakamoto, Naofumi Mukaida and Kouji Matsushima were responsible for critical revision of the article for important intellectual content; Shuichi Kaneko was responsible for final approval of the article.

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to overcome the immune-suppressive environment produced by tumors [9,10]. DCs are also known to enhance antitumor immunity by activating the functions of endocytosis, trafficking, maturation and cytokine production. Numerous studies have shown that DCs from peripheral blood of HCC patients are reduced in number, have an immature phenotype and an impaired function [11]. Moreover, to date, there is no consensus on optimal conditions for DC preparation in vitro for immunotherapy of HCC patients.

Recently, we have developed the combined immunotherapy of transcatheter hepatic arterial embolization (TAE) with infusion of immature and mature monocyte-derived DCs (MoDCs) for HCC [12, 13]. In this study, we evaluated the procedures to induce MoDCs that efficiently function in the immune-mediated treatments for HCC.

2. Materials and methods

2.1. Patients and healthy controls

Fourteen patients (four women and ten men) attending Kanazawa University Hospital (Ishikawa, Japan) between September 2007 and December 2008 were enrolled in this study. All patients were serologically positive for HCV. HCC was radiologically diagnosed by computed tomography (CT), magnetic resonance imaging (MRI) and CT angiography. Blood samples were taken from 14 patients with HCC and 14 healthy controls having no hepatitis history and serologically negative for both hepatitis B and C. The clinical profiles of the patients and controls analyzed in the present study are shown in Table 1. All patients gave written informed consent to participate in the study in accordance with the Helsinki Declaration and this study was approved by the regional ethics committee (Medical Ethics Committee of Kanazawa University, No. 829).

2.2. Preparation of immature DCs

Immature DCs were separated from peripheral blood mononuclear cells (PBMCs) of patients and healthy controls. PBMCs were isolated by centrifugation using Lymphoprep™ Tubes (Nycomed, Roskilde, Denmark). The cells were resuspended in serum-free medium (GMP CellGro DC Medium; CellGro, Manassas, VA) and allowed to adhere to 6-well tissue culture dishes (Costar, Cambridge, MA) at 1.4×10^7 cells in 2 mL per well. After 2 h at 37 °C, non-adherent cells were removed and adherent cells were cultured in the medium with 50 ng/mL recombinant human IL-4 (GMP grade; CellGro) and 100 ng/mL recombinant human GM-CSF (GMP grade; CellGro) for 5 days to generate immature DCs.

Table 1
Characteristics of healthy controls and patients.

	Controls	HCC patients	P
No. of patients	14	14	
Age (years)	42.6 ± 14.9	68.8 ± 7.6	<.05
Gender (M/F)	7/7	10/4	n.s.
WBC ($X10^2/\mu L$)	ND	43.5 ± 15.4	n.s.
PLT ($X10^4/\mu L$)	ND	13.1 ± 6.0	n.s.
PT (%)	ND	85.2 ± 13.3	n.s.
ALT (IU/L)	ND	59.7 ± 46.8	n.s.
Alb (g/dL)	ND	3.3 ± 0.6	n.s.
T-Bil (mg/dL)	ND	0.8 ± 0.4	n.s.
Histology of non-tumor liver			
Chronic hepatitis	ND	8	n.s.
Cirrhosis (Child-Pugh A/B/C)	ND	6 (5/1/0)	n.s.
TNM stage (I/II/IIIA/IIIB/IIIC/IV)	ND	0/11/1/2/0/0	

Data are expressed as the mean ± SD.
ND: not determined, n.s.: not significant.

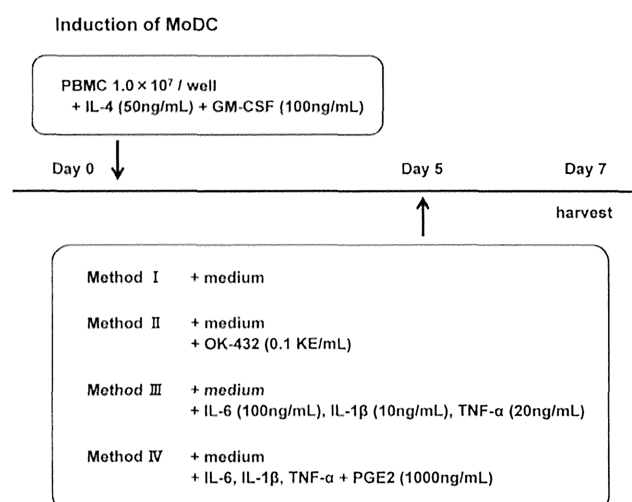

Fig. 1. Protocols for preparation of DCs. DCs, which were derived from PBMCs in the presence of IL-4 and GM-CSF for 5 days, were cultured for 2 additional days with the serum-free medium only (Method I), OK-432 (Method II), and cytokine cocktails consisting of IL-6, IL-1β and TNF-α without (Method III) or with (Method IV) PGE2. On day 7, these cells were harvested and evaluated.

2.3. Activation of immature DCs

Several activation stimuli were tested (Fig. 1). After 5 days of culture, the immature DCs induced by the above method were cultured for 2 additional days in the serum-free medium (Method I) or stimulated with 0.1 KE/mL OK-432 (Chugai Pharmaceuticals, Tokyo, Japan) (Method II), 10 ng/mL IL-1β (GMP grade; CellGro), 100 ng/mL IL-6 (GMP grade; CellGro) and 20 ng/mL TNF-α (GMP grade; CellGro) (Method III), and IL-1β, IL-6, TNF-α and PGE2 (Kaken Pharmaceuticals, Tokyo, Japan) (Method IV). On day 7, the cells were harvested.

2.4. Antibodies

The following anti-human monoclonal antibodies (mAbs) were used for flow cytometry: anti-lin1 (lineage cocktail 1; CD3, CD14, CD16, CD19, CD20 and CD56)-FITC, anti-HLA-DR-PerCP and -FITC (L243), anti-CD11c-APC (S-HCL-3), anti-CD123-PE (9F5), anti-CCR7-PE (3D12), anti-CD14-APC (MφP9) (BD Biosciences Pharmingen, San Jose, CA), anti-CD80-PE (MAB104), anti-CD83-PE (HB15a) and anti-CD86-PE (HA5.2B7) (Beckman Coulter, Fullerton, CA).

2.5. Flow cytometric analysis

Surface markers on DCs were evaluated using flow cytometric analysis. Cells were analyzed on a FACSCalibur™ for four-color flow cytometry. Data analysis was performed using CELLQuest™ software (Becton Dickinson, San Jose, CA).

2.6. Endocytosis assay

The endocytic capacity of DCs was assessed by measurement of FITC dextran (Sigma-Aldrich, St. Louis, MO) uptake. DCs were incubated for 30 min at 37 °C in the presence of 1 mg/mL FITC dextran, washed three times and analyzed using a FACSCalibur™ cytometer.

2.7. Cytokine production assay

The concentrations of cytokines and chemokines in the supernatants of culture medium were measured using enzyme-linked immunosorbent assay (ELISA) kit for IL-12p40 and IFNγ (Biosource International,