Ⅲ．研究成果の刊行一覧

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻・号	ページ	出版年
Ifuku H, Kusumoto S, Tanaka Y, Totani H, Ishida T, Okada M, Murakami S, **Mizokami M**, Ueda R, Iida S	Fatal reactivation of hepatitis B virus infection in a patient with adult T-cell leukemia-lymphoma receiving the anti-CC chemokine receptor 4 antibody mogamulizumab.	***Hepatol Res***.			2015
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Ⅳ. 研究成果の刊行物・別刷

Hepatology Research

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Case Report

Fatal reactivation of hepatitis B virus infection in a patient with adult T-cell leukemia–lymphoma receiving the anti-CC chemokine receptor 4 antibody mogamulizumab

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We report an adult T-cell leukemia–lymphoma (ATL) patient suffering from fatal reactivation of hepatitis B virus (HBV) infection after treatment with the anti-CC chemokine receptor 4 (CCR4) monoclonal antibody, mogamulizumab. HBV reactivation occurred without liver damage in this hepatitis B surface antigen (HBsAg) negative patient, who was seropositive for antibodies against the viral core and surface antigens at baseline, after two cycles of CHOP regimen (cyclophosphamide, doxorubicin, vincristine and prednisolone) followed by six cycles of THP-COP regimen (cyclophosphamide, pirarubicin, vincristine and prednisolone). Unexpectedly, mogamulizumab monotherapy for relapsed CCR4 positive ATL induced sudden and fatal liver failure due to HBV reactivation, despite antiviral prophylaxis with entecavir. This clinical course may not only offer important suggestions to prevent critical HBV reactivation in HBsAg positive cancer patients who receive immune-enhancing drugs such as anti-CCR4 antibody, but also provide a clue to understanding the pathogenesis of HBV reactivation following systemic chemotherapy.

Key words: antiviral prophylaxis, CC chemokine receptor type 4, hepatitis B virus, mogamulizumab, reactivation

INTRODUCTION

REACTIVATION OF HEPATITIS B virus (HBV) infection is a potentially critical complication in cancer patients following systemic chemotherapy.[1–4] It can occur not only in patients seropositive for hepatitis B surface antigen (HBsAg),[5,6] but also in those with resolved HBV infection who are seronegative for HBsAg but seropositive for antibodies against hepatitis B core antigen (anti-HBc) and/or antibodies against HBsAg (anti-HBs).[3,4,7–9] Antiviral prophylaxis is recommended by some guidelines to prevent HBV reactivation-related hepatitis and death for HBsAg-positive patients.[10–13]

Adult T-cell leukemia–lymphoma (ATL) is an aggressive peripheral T-cell neoplasm and human T-cell leukemia virus type 1 (HTLV-1) plays a role in its pathogenesis. Recently, the anti-CC chemokine receptor 4 (CCR4) monoclonal antibody, mogamulizumab, was developed and introduced into the management of ATL in Japan.[14–18]

We report here fatal HBV reactivation in an ATL patient after mogamulizumab monotherapy, despite antiviral prophylaxis.

CASE REPORT

A 72-YEAR-OLD JAPANESE man was admitted with the major complaint of bilateral cervical lymphadenopathy. The laboratory findings showed no abnormal lymphocytes in the peripheral blood, but elevated levels of

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lactate dehydrogenase (492 U/L) and soluble interleukin-2 receptor (89 903 U/L). He was seropositive for anti-HTLV-1 antibody. A computed tomographic scan revealed systemic lymphadenopathy, hepatomegaly, a low-density lesion in the spleen, and the presence of pleural effusion and ascites. A diagnosis of lymphoma-type ATL was made on the basis of histopathological examination of a cervical lymph node biopsy and the monoclonal integration of HTLV-1, detected by Southern blotting.

He received two cycles of CHOP regimen (cyclophosphamide, doxorubicin, vincristine and prednisolone), followed by five cycles of THP-COP regimen (cyclophosphamide, pirarubicin, vincristine and prednisolone), and achieved best objective response of partial response to ATL. However, his right cervical lymph node was enlarged just before the sixth cycle of THP-COP regimen and progressive disease of CCR4 positive ATL was judged by the histopathological examination of a second lymph node biopsy (Fig. 1).

He was seronegative for HBsAg before initiating CHOP chemotherapy but neither anti-HBc nor anti-HBs was measured at baseline. When he received a transfusion of red blood cells after the third cycle of THP-COP regimen, his serological HBV markers, which we examined using a preserved sample, were as follows: HBsAg negative (0.02 IU/mL, chemiluminescent immunoassay [CLIA]), anti-HBc positive (9.32 s/co, CLIA) and anti-HBs-positive (68.8 mIU/mL, CLIA). However, the reappearance of HBsAg and seropositivity of hepatitis B e-antigen were confirmed after the sixth cycle of THP-COP regimen, when a high HBV DNA level was detected (>9.1 log copies/mL) by real-time polymerase chain reaction assay before initiating salvage chemotherapy for ATL using mogamulizumab (Fig. 1). Liver damage did not develop, although a very high level of HBV DNA was detected at that point.

He began to receive weekly mogamulizumab monotherapy for relapsed CCR4 positive ATL and received anti-HBV treatment with entecavir (0.5 mg/day) at the same time, according to Japanese guidelines for preventing HBV reactivation-related hepatitis. His cervical lymphadenopathy improved, however, sudden and severe liver damage (alanine aminotransferase, 2410 U/L; total bilirubin, 6.01 mg/dL) developed after the third cycle of mogamulizumab (Figs 1,2). A diagnosis of acute liver failure was made on sustained jaundice with hepatic coma and the decline to less than 40% of prothrombin time, according to Japanese criteria,[19] despite the daily administration of entecavir and Stronger Neo-Minophagen C (40 mg/day). He was transferred to another hospital for intensive care, including plasma exchange, and his HBV DNA levels decreased to 3.6 log copies/mL immediately (Fig. 1), which indicated that entecavir was

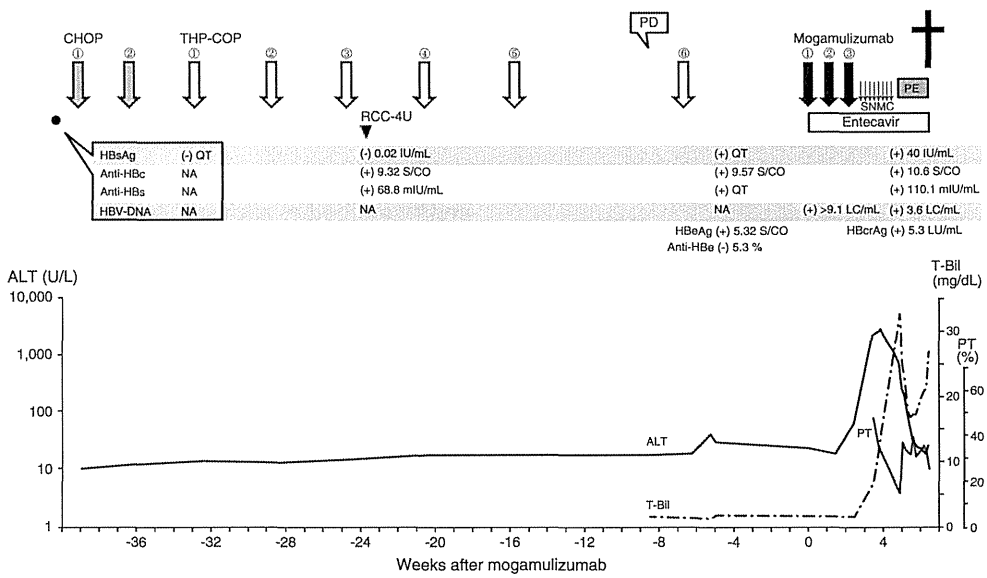

Figure 1 Clinical course of a patient with adult T-cell leukemia–lymphoma before and after treatment with the anti-CC chemokine receptor 4 monoclonal antibody mogamulizumab. ALT, alanine aminotransferase; anti-HBc, antibody against hepatitis core antigen; anti-HBe, antibody against hepatitis B e-antigen; anti-HBs, antibody against HBsAg; CHOP, cyclophosphamide, doxorubicin, vincristine and prednisolone; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; LC/mL, log copies/mL; LU/mL, log U/mL; NA, not available; PD, progressive disease; PE, plasma exchange; PT, prothrombin time; QT, qualitative test; RCC, red cells concentrates; sIL-2R, soluble interleukin-2 receptor; SNMC, Stronger Neo-Minophagen C; T-Bil, total bilirubin; THP-COP, pirarubicin, vincristine and prednisolone.

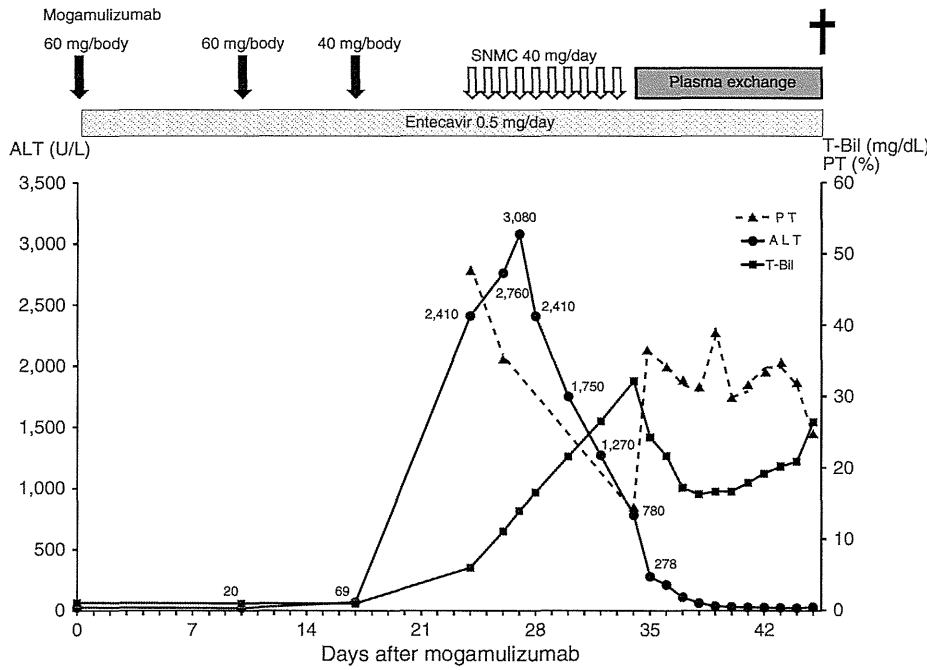

Figure 2 Detailed clinical course of fatal HBV reactivation after mogamulizumab. ALT, alanine aminotransferase; HBV, hepatitis B virus; SNMC, Stronger Neo-Minophagen C; PT, prothrombin time; T-Bil, total bilirubin.

effective in suppressing the replication of HBV DNA. There was no evidence of the emergence of resistance to this antiviral drug at that time. However, acute liver failure progressed rapidly and he died approximately 7 weeks after the first administration of mogamulizumab.

Retrospective analysis using a sample stored 2 weeks after hepatitis onset (at 5 weeks after the first administration of mogamulizumab) showed that he was seropositive for HBsAg (40 IU/mL, CLIA), seropositive for anti-HBc (10.6 s/co, CLIA), seropositive for anti-HBs (110.1 mIU/mL, CLIA) and seropositive for HBc-related antigen (5.3 log U/mL, CLIA). Furthermore, analysis showed that the virus was HBV genotype C, without any mutations in the precore region or basal core promoter, but a HBsAg escape mutation (G145K) was found. This retrospective analysis was approved by the institutional review board of Amagasaki Central Hospital and Nagoya City University.

DISCUSSION

THIS CASE IS the first report of HBV-related death in an ATL patient who received mogamulizumab, despite antiviral prophylaxis with entecavir before hepatitis onset, according to some guidelines.[10–13] Mogamulizumab is a humanized anti-CCR4 monoclonal antibody, which targets CCR4 molecules expressed not only on tumor cells of ATL, but also normal T-helper type 2 and regulatory T cells.[14,15,20] Mogamulizumab has been demonstrated to be effective for CCR4 positive relapsed/refractory ATL or peripheral T-cell lymphoma,[16,18,21] however, Stevens–Johnson syndrome, a seriously adverse skin event, has been reported to occur, partly because of an enhanced cellular immune response following a remarkable reduction in the number of regulatory T cells.[22]

In this case, the initial systemic chemotherapy (CHOP or THP-COP regimen), possibly in addition to the highly immunocompromised situation caused by ATL itself,[20,23] might have led to the reappearance of HBsAg and an abrupt increase of serum HBV DNA, and HBV reactivation might have occurred before the administration of mogamulizumab. Although HBV reactivation has been reported to be a rare complication following anti-CD20 monoclonal antibody, rituximab-free chemotherapy,[8,9] this clinical course suggested that regular HBV DNA monitoring-guided pre-emptive antiviral therapy is necessary to prevent HBV reactivation in such patients.[3,10] Interestingly, liver damage associated with HBV reactivation did not manifest, even when a very high HBV DNA level was detected. Acute liver failure with hepatic coma developed suddenly after the administration of mogamulizumab and the patient died of hepatitis B in spite of intensive care.

As to why the sudden acute liver failure occurred after mogamulizumab, this anti-CCR4 antibody should rapidly deplete not only CCR4 positive ATL cells, a certain subset of which functions as regulatory T cells, but also CCR4 positive endogenous non-ATL regulatory T cells.[16,18,23] We surmise that this depletion resulted in a rapid provocation of the patient's immune system, especially the cellular immune response to HBV-infected hepatocytes, leading to acute liver failure and HBV-related death. HBV reactivation has been reported to occur most frequently in cancer patients after completion of systemic chemotherapy and recovery of the immune response.[3] Antiviral prophylaxis is recommended by some guidelines to prevent HBV reactivation-related hepatitis and death in HBsAg positive patients before initiating systemic chemotherapy.[10–13] Recently, a randomized controlled trial demonstrated that the prophylactic use of entecavir significantly decreased the risk of HBV reactivation in HBsAg positive patients with low viral loads (serum HBV DNA levels <3 log copies/mL), who received rituximab-containing chemotherapy, compared with the prophylactic use of lamivudine.[24] However, there is limited evidence of prevention of HBV reactivation in HBsAg positive patients, especially those with high viral loads who may have a greater risk of HBV reactivation. This case is also the first report of HBV reactivation-related death during antiviral treatment with a new generation anti-HBV nucleoside analog, entecavir, that has both greater potential to suppress HBV replication and a high barrier to viral resistance mutations.[25,26] Therefore, if a potentially immune-enhancing drug is used for HBsAg positive cancer patients with high HBV DNA levels, it may be necessary to reduce the HBV DNA level as much as possible by antiviral treatment in advance, before initiating systemic chemotherapy.

Why did liver damage not occur, even when a very high HBV DNA level was detected after initial chemotherapy? Again, ATL patients usually have severe cellular immunodeficiency and a diagnosis of ATL is often made with the onset of opportunistic infections, such as pneumocystis pneumonia and cytomegalovirus infection.[27,28] On the other hand, the immune response, especially the HBV-specific T-cell response, is thought to be largely responsible for the onset of hepatitis and viral clearance during HBV infection.[29,30] Therefore, we speculate that his impaired cellular immune response could have allowed replication of the virus but could not attack the HBV-infected hepatocytes and induce hepatitis B just after the THP-COP regimen, suggesting that his immune response against HBV was weak before mogamulizumab treatment. One of the reasons may be limited virus replication due to the absence of precore and/or basal core promoter mutations that are associated with enhanced virus replication in patients with fulminant hepatitis B.[31,32]

What is the clinical significance of the HBsAg escape mutation in HBV reactivation? Retrospective analysis using his preserved sample after HBV reactivation also showed that he had a high titer of anti-HBs (110.1 mIU/mL, Fig. 1) and a common escape mutation (G145K) in the viral S region. Most HBV reactivation occurs after the decrease and disappearance of anti-HBs in patients initially seropositive for anti-HBs.[33] Escape mutations in HBsAg have been reported to be associated with sustained titers of anti-HBs during HBV reactivation,[34] which indicates that regular monitoring of anti-HBs titers may be insufficient to predict adequately HBV reactivation in such patients.

In summary, we first reported the sudden onset of acute liver failure, and death due to HBV reactivation, in an ATL patient who received anti-CCR4 antibody mogamulizumab monotherapy, despite antiviral prophylaxis with entecavir. This clinical course may not only offer important suggestions to prevent critical HBV reactivation in HBsAg positive cancer patients who receive immune-enhancing drugs such as anti-CCR4 antibody, but also provide a clue to understanding the pathogenesis of HBV reactivation following systemic chemotherapy.

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Cyclosporin A and Its Analogs Inhibit Hepatitis B Virus Entry Into Cultured Hepatocytes Through Targeting a Membrane Transporter, Sodium Taurocholate Cotransporting Polypeptide (NTCP)

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Chronic hepatitis B virus (HBV) infection is a major public health problem worldwide. Although nucleos(t)ide analogs inhibiting viral reverse transcriptase are clinically available as anti-HBV agents, emergence of drug-resistant viruses highlights the need for new anti-HBV agents interfering with other targets. Here we report that cyclosporin A (CsA) can inhibit HBV entry into cultured hepatocytes. The anti-HBV effect of CsA was independent of binding to cyclophilin and calcineurin. Rather, blockade of HBV infection correlated with the ability to inhibit the transporter activity of sodium taurocholate cotransporting polypeptide (NTCP). We also found that HBV infection-susceptible cells, differentiated HepaRG cells and primary human hepatocytes expressed NTCP, while nonsusceptible cell lines did not. A series of compounds targeting NTCP could inhibit HBV infection. CsA inhibited the binding between NTCP and large envelope protein *in vitro*. Evaluation of CsA analogs identified a compound with higher anti-HBV potency, having a median inhibitory concentration <0.2 μM. *Conclusion*: This study provides a proof of concept for the novel strategy to identify anti-HBV agents by targeting the candidate HBV receptor, NTCP, using CsA as a structural platform. (HEPATOLOGY 2014;59:1726-1737)

Hepatitis B virus (HBV) infection is a substantial public health problem, affecting ~350 million people worldwide.[1-3] HBV-infected patients have an elevated risk for developing liver cirrhosis and hepatocellular carcinoma. Currently, clinical treatment for HBV infection includes interferon alpha (IFN-α) and nucleos(t)ide analogs. IFN-α therapy yields long-term clinical benefit in only less than 40% of patients and can cause significant side effects. Nucleos(t)ide analog treatment can suppress HBV replication and is accompanied by substantial biochemical and histological improvement; however, it may select for drug-resistant viruses, which limit the efficacy of long-term treatment. To overcome these problems, the development of new anti-HBV agents targeting a different step of the HBV life cycle is urgently needed.

As HBV has only one viral gene encoding an enzymatic activity, the polymerase, there is no apparent strategy to develop a new class of antiviral agents other than polymerase inhibitors. Hence, it is important to define alternative molecular targets for anti-HBV agents as well as to identify potential anti-HBV

Abbreviations: CN, calcineurin; CsA, cyclosporin A; CyPs, cyclophilins; HBs, viral envelope protein; HBV, hepatitis B virus; HCV, hepatitis C virus; HCVpp, HCV pseudoparticles; IFN, interferon; LHBs, large envelope protein; MDR, multidrug resistance; MHBs, middle envelope protein; MRP, MDR-related protein; NTCP, sodium taurocholate cotransporting polypeptide; PHH, primary human hepatocytes; PPIase, peptidyl prolyl cis/trans-isomerase; SHBs, small envelope protein; TCA, taurocholic acid; TUDCA, tauroursodeoxycholic acid.

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compounds.[3,4] Myrcludex-B is a peptide mimicking pre-S1, which is crucial for the virus-cell membrane interaction. Pretreatment with this peptide has been shown to prevent virus entry and spread of virus infection.[5,6] Phenylpropenamide derivatives and heteroarylpyrimidines (HAP) suppressed HBV replication through capsid disassembly.[7-10] Although the development of the former was discontinued because of significant toxicity,[3] HAP exhibited anti-HBV efficacy in the absence of robust toxicity.[8,10] Deoxynojirimycin derivatives are iminosugars that inhibit alpha-glucosidases. Although treatment with these compounds suppressed HBV secretion in both cell culture and mouse models,[11,12] further investigation will be required to assess their anti-HBV efficacy and the specificity to HBV. Thus, it is an attractive strategy to identify a cellular factor that is specifically involved in HBV infection and relevant for the development of anti-HBV agents.

Cyclosporin A (CsA) is an immunosuppressant clinically used for suppression of the immunological failure of xenograft tissues. CsA primarily targets cellular peptidyl prolyl cis/trans-isomerase (PPIase) cyclophilins (CyPs).[13] The resultant CsA/CyP complex subsequently binds to and inhibits calcineurin (CN), a phosphatase that dephosphorylates nuclear factor of activated T cell (NF-AT) to allow nuclear translocation and transactivation of downstream genes. This CN inhibition contributes to the suppression of immune responses. In addition, CsA is known to inhibit the transporter activity of membrane transporters, including the multidrug resistance (MDR) and MDR-related protein (MRP) families.[14] Previously, we demonstrated that CsA and its nonimmunosuppressive derivatives suppress hepatitis C virus (HCV) replication,[15,16] with the anti-HCV activity being mediated by the inhibition of CyPs.[17-19] Currently, a series of drugs classified as CyP inhibitors are in clinical development for treatment of HCV-infected patients.[20,21]

In this study we report that CsA and its analogs inhibited HBV entry through a CyP-independent mechanism. We established a screening system that can identify small molecules inhibiting HBV entry. Screening in this system revealed that CsA blocked HBV entry. The anti-HBV activity of CsA was not correlated with binding to CyPs and CN. CsA inhibited the transporter activity of sodium taurocholate cotransporting polypeptide (NTCP), a recently reported candidate for the HBV entry receptor,[22] and interrupted the binding between NTCP and large envelope protein *in vitro*. Other NTCP inhibitors also blocked HBV infection. Analog testing identified CsA-related compounds with higher anti-HBV potency than CsA. Thus, CsA and NTCP inhibitors can be used as a platform to develop a novel class of anti-HBV agents.

Materials and Methods

Cell Culture. HepaRG (Biopredic), HepAD38 (kindly provided by Dr. Christoph Seeger at Fox Chase Cancer Center), and primary human hepatocytes (PHHs) (Phoenixbio) were cultured as described previously.[23]

HBV Preparation and Infection. The HBV used in this study was mainly derived from the culture supernatant of HepAD38 cells. HBV infection was performed as described previously.[23] More detailed procedures are given in the Supporting Information.

Indirect Immunofluorescence Analysis, Real-Time Polymerase Chain Reaction (PCR), Southern Blot Analysis, 3-(4,5-Dimethylthiazol-2-yl)−2,5-diphenyltetrazolium Bromide (MTT) Assays, and Reporter Assays. Indirect immunofluorescence analysis, real-time PCR, southern blot analysis, MTT assays, and reporter assays were performed essentially as described.[23] More detailed procedures are given in the Supporting Information.

Detection of HBs and HBe Antigens. HBs antigen was quantified by enzyme-linked immunosorbent assay (ELISA) as described previously.[23] HBe antigen was detected by a Chemiluminescent Immuno-Assay (Mitsubishi Chemical Medience).

HCV Pseudoparticle Assay. The HCV pseudoparticles (HCVpp), which reproduce HCV envelope-mediated entry, were generated by transfecting the

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Additional Supporting Information may be found in the online version of this article.

expression plasmids for MLV Gag-Pol, HCV E1E2, and a luciferase that can be packaged into the virion (kindly provided by Dr. Francois-Loic Cosset at the University of Lyon) into 293T cells. HCVpp recovered from the culture supernatant of transfected cells were used in a HCV entry assay as described previously.[24]

Transporter Assay. The transporter activity of NTCP was assayed essentially as described[25] using 293 (Sekisui Medical) and HepG2 cells permanently overexpressing human NTCP. Briefly, the cells were preincubated with compounds at 37°C for 15 minutes and then incubated with radiolabeled substrate, [^{3}H]taurocholic acid (TCA), at 37°C for 5 minutes to allow substrate uptake into the cells. The cells were then washed and lysed to measure the accumulated radioactivity. In this assay, we did not observe cytotoxic effects of compounds at any of the concentrations tested. More detailed procedures are given in the Supporting Information.

AlphaScreen Assay. Recombinant NTCP and HBs proteins, which were tagged with 6xHis and biotin, respectively, were synthesized using a wheat cell-free protein system as described previously.[26] Protein-protein interactions were detected using the AlphaScreen IgG (ProteinA) detection kit (PerkinElmer) according to the manufacturer's instruction. Briefly, the recombinant tagged proteins were incubated with streptavidin-coated donor beads and anti-6xHis antibody-conjugated acceptor beads that generate a luminescence signal when brought into proximity by binding to interacting proteins. Luminescence was analyzed with the AlphaScreen detection program of an Envision spectrophotometer (PerkinElmer). More detailed procedures for the AlphaScreen assay are described in the Supporting Information.

Additional experimental procedures are included in the Supporting Information.

Results

Cyclosporin A Blocked HBV Infection. We focused on HBV entry and established a cell culture system to evaluate this step in HBV infection. To identify small molecules inhibiting HBV entry, we pretreated HepaRG cells[27] with compounds for 2 hours, then added a HBV inoculum and continued incubation with compounds for 16 hours (Fig. 1A). After washing out free HBV and compounds, the cells were cultured for an additional 12 days in the absence of compounds (Fig. 1A). For robust chemical screening, HBV infection was monitored by the viral envelope protein (HBs) level secreted from the infected cells at 12 days postinfection by ELISA. This assay could identify heparin, an HBV attachment inhibitor,[28,29] and bafilomycin A1, a v-type H^+ ATPase inhibitor that blocks acidification of vesicles and HBV entry,[30] but not lamivudine, a reverse transcriptase inhibitor,[31] as compounds reducing HBs protein level in the medium (Fig. 1B). In addition, use of an anti-HBs antibody to neutralize viral entry, but not use of an anti-FLAG antibody, reduced viral protein secreted from the HBV-infected cells (Fig. 1B). Thus, this system is likely to evaluate the effect of compounds on the early phase of the HBV life cycle, including attachment and entry, but not effects on HBV replication. A chemical screen with this system revealed that CsA reduced HBs secretion from HBV-infected cells (Fig. 1B). Treatment with CsA significantly decreased HBc protein expression (Fig. 1C) and HBV DNA as well as cccDNA (Fig. 1D) in the cells and HBe in the medium (Fig. 1E), without causing cytotoxicity (Supporting Fig. S1A). This effect of CsA was not limited to infection of HepaRG cells, as we observed a similar anti-HBV effect of CsA for PHHs (Fig. 1F). The anti-HBV effect of CsA was also observed on HBV infection of PHHs in the absence of PEG8000 (Fig. S1B), indicating that the effect of CsA did not depend on PEG8000, which was normally included in the HBV infection experiments. These data suggest that CsA blocked HBV infection.

Effect of Cyclosporin A on HBV Entry. CsA decreased HBs and HBe secreted from the infected cells in a dose-dependent manner (Fig. 2A). We next investigated which step in the HBV life cycle was blocked by CsA. The HBV life cycle can be divided into two phases: the early phase of infection including attachment, entry, nuclear import, and cccDNA formation, and the following late phase representing HBV replication that includes transcription, assembly, reverse transcription, and viral release.[32] Lamivudine drastically decreased HBV DNAs in HepAD38 cells,[33] which reproduce HBV replication but not the early phase of infection (Fig. 2B). In addition, continuous treatment with lamivudine as well as entecavir and interferon-α for 4 days after HBV infection could decrease HBV DNA levels in HBV-infected HepaRG cells, which suggests an inhibition of HBV replication (Fig. 2C). Nevertheless, lamivudine did not show an anti-HBV effect when applied only prior to and during HBV infection (Fig. 1A,B), suggesting that the anti-HBV compounds identified in Fig. 1A interrupted the early phase of the HBV life cycle.

We then examined whether CsA inhibited attachment or entry. For evaluating HBV attachment,[34] cell surface HBV DNA was extracted and quantified from HepaRG cells exposed to HBV at 4°C for 3 hours and

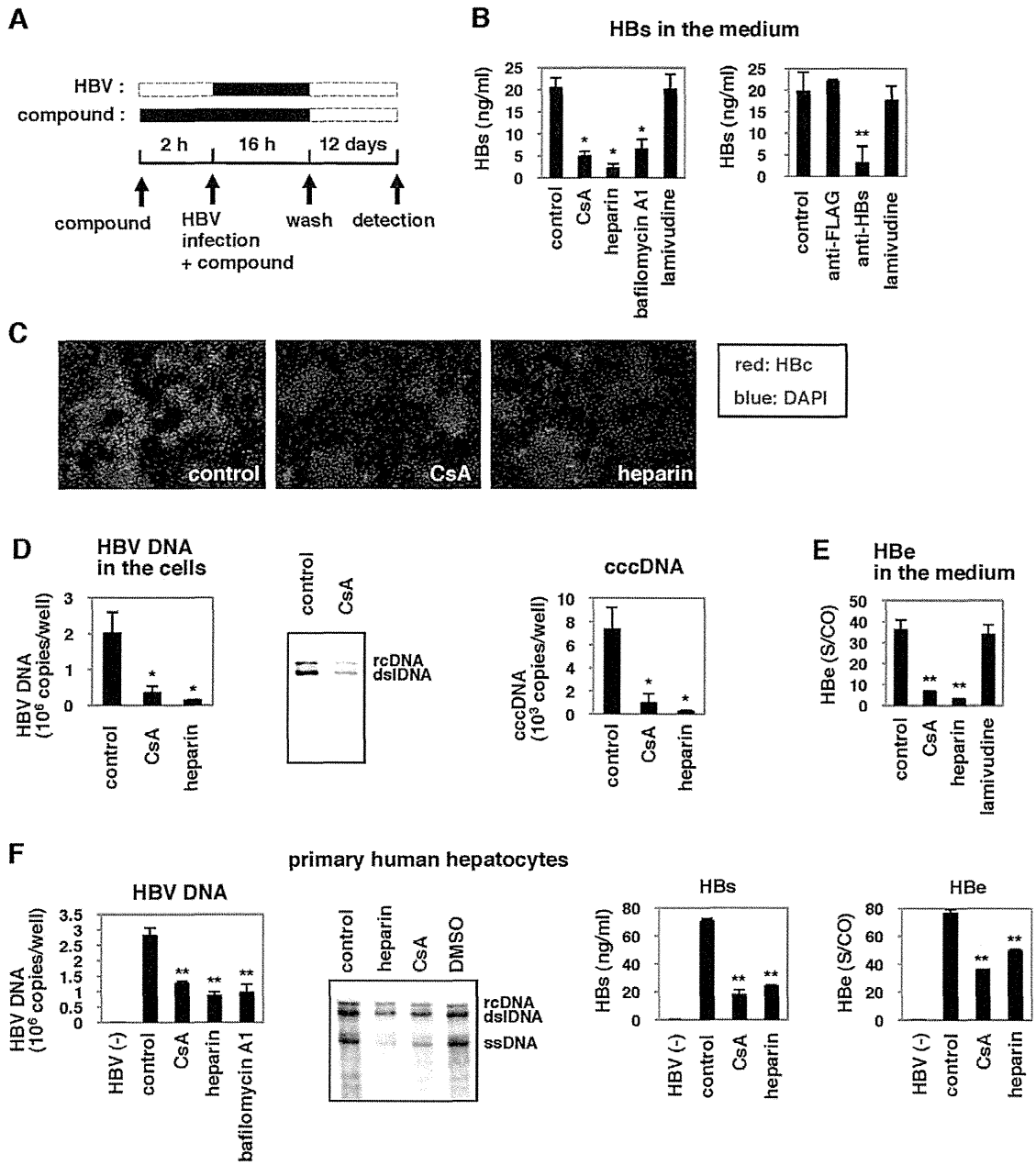


Fig. 1. Cyclosporin A (CsA) blocked HBV infection. (A) Schematic representation of the schedule for exposing HepaRG cells to compounds and HBV. HepaRG cells were pretreated with compounds for 2 hours and then inoculated with HBV for 16 hours. After washing out the free HBV and compounds, the cells were cultured with the medium in the absence of compounds for an additional 12 days to quantify HBs protein secreted from the infected cells into the medium. Black and dotted bars indicate the interval for treatment and without treatment, respectively. (B) CsA 4 μM, heparin 25 U/mL, bafilomycin A1 200 nM, lamivudine 1 μM, anti-FLAG 10 μg/mL, and anti-HBs antibody 10 μg/mL, were tested for effect on HBV infection according to the protocol shown in (A). (C-E) HBc protein (C), HBV DNAs, and cccDNA (D) in the cells as well as HBe antigen in the medium (E) at 12 days postinfection according to the protocol shown in (A) were detected by immunofluorescence, real-time PCR analysis, southern blot, and ELISA. Red and blue in (C) show the detection of HBc protein and nuclear staining, respectively. (F) PHHs were treated with the indicated compounds and infected with HBV using the protocol shown in (A). The levels of HBV DNAs in the cells, as well as of HBs and HBe antigens in the medium, were determined. Statistical significance was determined using the Student t test ($^*P < 0.05$, $^{**}P < 0.01$).

then washed (Fig. 2D-a). For the internalization assay,[34] the above cells, after washing, were further cultured at 37°C for 16 hours to allow HBV to internalize into the cells, and then trypsinized to digest HBV remaining on the cell surface to allow quantification of internalized HBV DNA (Fig. 2D-b). CsA slightly reduced the amount of attached HBV DNA, although the effect was not statistically significant

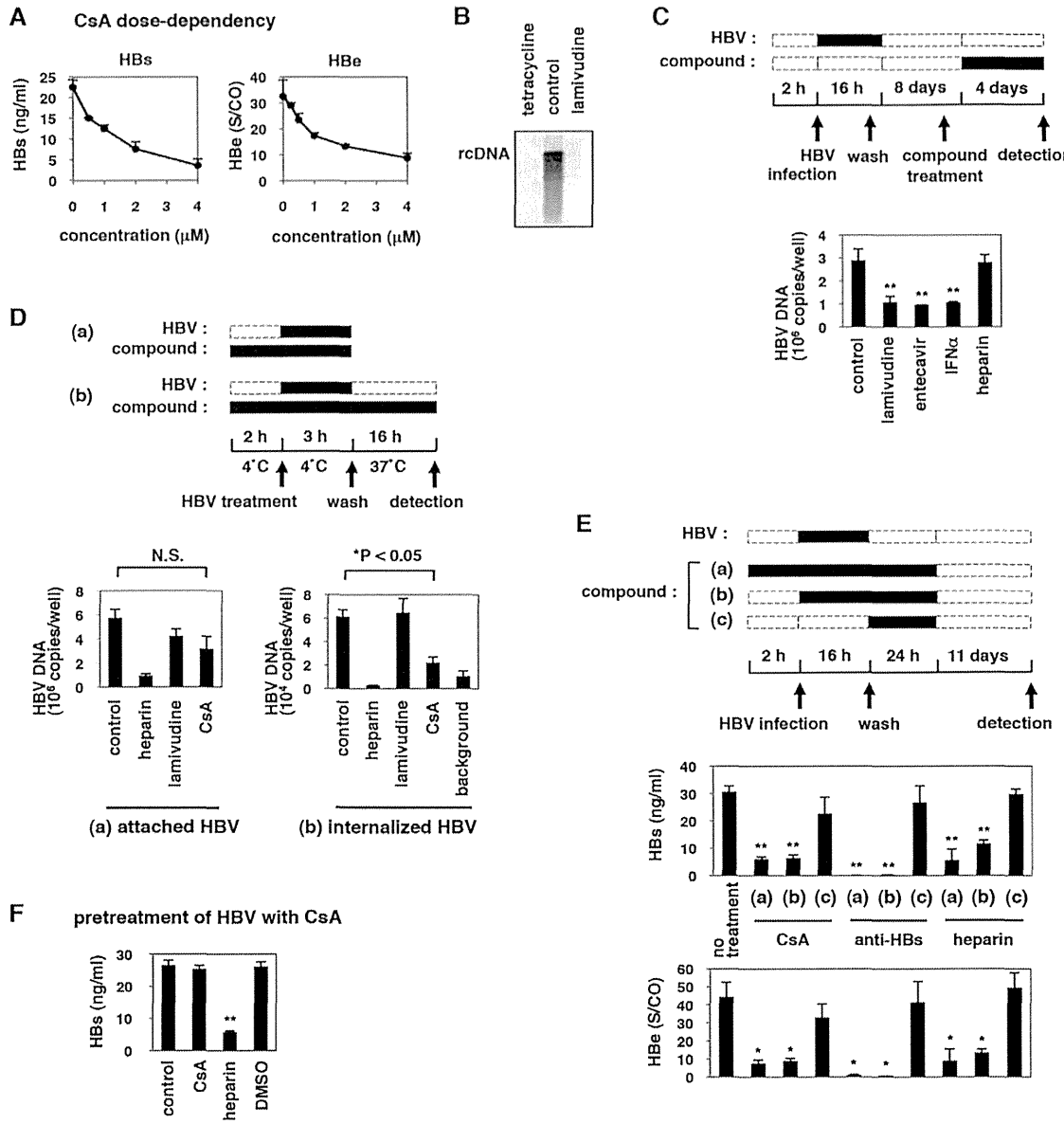


Fig. 2. CsA reduced internalized HBV. (A) HepaRG cells were treated with or without various concentrations of CsA (0.5, 1, 2, and 4 μM) as shown in Fig. 1A. HBV infection was monitored by HBs and HBe secretion. (B) HBV DNA in core particles was detected by southern blot analysis of DNA extracts from HepAD38 cells treated for 6 days with or without tetracycline 0.5 μg/mL and lamivudine 1 μM. (C) Upper scheme indicates the treatment schedule of HepaRG cells with compounds and HBV. HepaRG cells were infected with HBV for 16 hours. After washing out the input virus, cells were cultured in the absence of compounds for 8 days. The cells were then cultured with compounds (lamivudine 1 μM, entecavir 1 μM, IFN-α 100 IU/mL, or heparin 25 U/mL) for 4 days and recovered for detection of HBV DNA. Black and dotted boxes indicate the periods with and without treatment, respectively. Lower graph shows the quantified relative HBV DNA level in cells treated according to the above scheme. (D) Upper scheme shows the experimental procedure for examining the attached and internalized HBV. (a) The cells were pretreated with compounds (heparin 25 U/mL, lamivudine 1 μM, or CsA 4 μM) at 4°C for 2 hours and then treated together with HBV at 4°C for 3 hours to allow HBV attachment to the cells. After washing out the free virus, cell surface HBV DNA was extracted and quantified by real-time PCR. (b) After attachment of HBV at 4°C for 3 hours and the following wash, the cells were cultured in the presence or absence of compounds at 37°C for 16 hours to allow the cells to internalize bound HBV. The cells were then trypsinized and extensively washed prior to quantifying the cellular HBV DNA. The lower graphs show the level of HBV DNA attached to the cells (a) and internalized inside the cells (b). "Background" in (b) indicates the signal from cells incubated at 4°C, instead of 37°C, for 16 hours after washing out the virus in (b), which shows the background signal level of the assay. (E) The upper scheme shows the procedure for the time of addition experiment. Compounds (CsA 4 μM, anti-HBs antibody 10 μg/mL, or heparin 25 U/mL) were applied beginning 2 hours prior to HBV infection (a), beginning during HBV infection (b), or beginning immediately after HBV infection (c) until 24 hours postinfection. HBs and HBe protein secretion were measured at 12 days postinfection. Middle and lower graphs indicate HBs and HBe secretion, respectively, from the cells treated according to the above scheme. (F) Preincubation of HBV with compounds. HBV was preincubated with the indicated compounds for 30 minutes at 37°C. Compounds were then removed by ultrafiltration. The recovered compound-treated HBV was used to infect HepaRG cells (16 hours incubation), and HBV infection was monitored with HBs antigen secreted into the medium at 12 days postinfection. $*P < 0.05$, $**P < 0.01$, N.S., not significant.

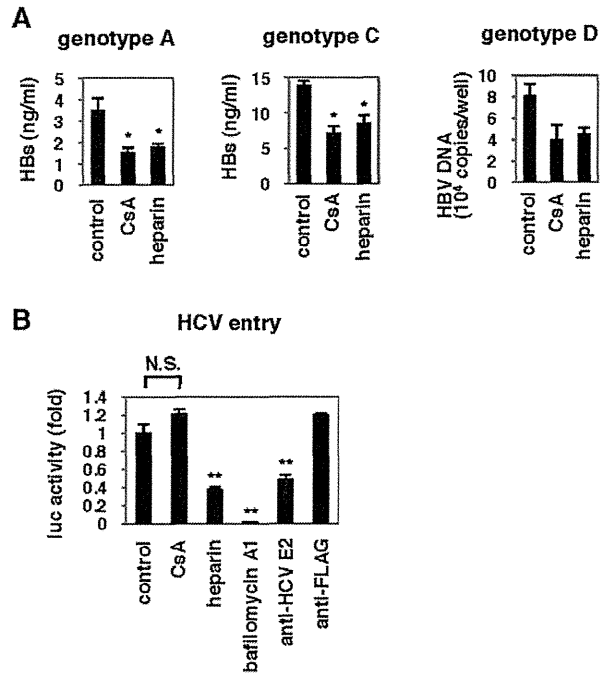
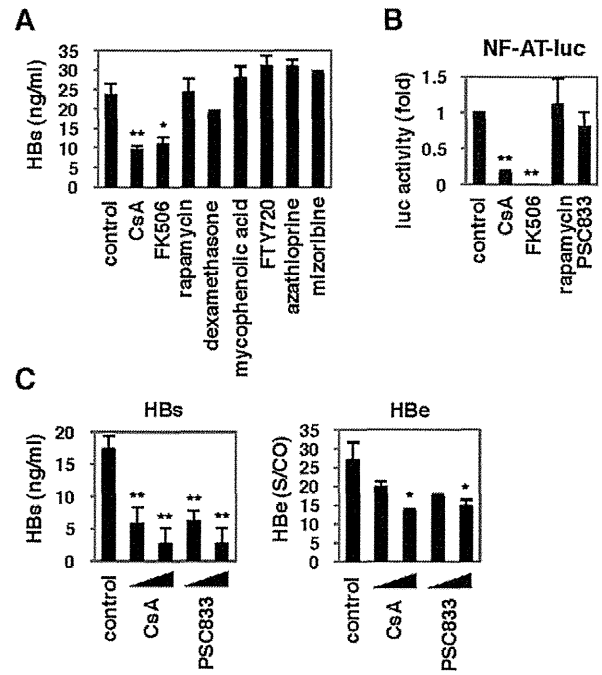


Fig. 3. CsA showed a pan-genotypic anti-HBV effect. (A) PHHs were treated with compounds (CsA 4 μM or heparin 25 U/mL) according to the scheme in Fig. 1A with different genotypes of HBV inoculum, and either HBs protein in the medium or HBV DNA in the cells at 12 days postinfection was quantified. (B) CsA did not affect the entry of HCV. Huh-7.5.1 cells were pretreated with the indicated compounds for 1 hour and then infected with HCVpp for 4 hours. At 72 hours postinfection, intracellular luciferase activity was measured. $*P < 0.05$, $**P < 0.01$, N.S., not significant.

(Fig. 2D-a). In contrast, CsA caused a significant reduction of HBV DNA in the internalization assay (Fig. 2D-b). In the time of addition assay as shown in Fig. 2E, treatment with CsA during HBV infection decreased HBs and HBe production (Fig. 2E-b), while CsA did not have an anti-HBV effect when delivered after HBV infection (Fig. 2E-c). Thus, CsA appears to primarily block the entry step including internalization. To examine whether CsA targeted HBV particles or host cells, we preincubated HBV with CsA and then purified the CsA from the HBV inoculum, followed by measurement of the HBV infectivity using HepaRG cells (Fig. 2F). Preincubation with CsA did not affect HBV infectivity, in contrast to the antagonizing effect of heparin to HBV particles (Fig. 2F), suggesting that CsA did not affect HBV particles but rather targeted host cells.

Cyclosporin A Showed a Pan-Genotypic Anti-HBV Effect. We examined the anti-HBV effect of CsA on the infection of different genotypes of HBV into PHHs. As shown in Fig. 3A, CsA reduced the infection of HBV genotype A, C, or D, which differ in sequences from the virus strain used in all of the other figures. However, CsA did not affect the entry of HCV, in contrast to the inhibition of HCV entry by heparin, bafilomycin A1, or an anti-HCV E2 antibody (Fig. 3B).

Effect of Immunosuppressants on HBV Infection. CsA is used clinically as an immunosuppressant, such as in patients following liver transplantation.[13] We therefore investigated the activity of other immunosuppressants on HBV infection. Among the additional immunosuppressive drugs examined, only FK506 was able to suppress HBV infection (Fig. 4A). CsA is known to have three major cellular targets: cellular cyclophilins (CyPs), calcineurin (CN), and transporters including MDRs and MRPs.[18] Although both CsA and FK506 can inhibit CN (Fig. 4B), this activity



D

IC_{50} for CyP binding

compound	IC_{50} (μM)		
	CyPA	CyPB	CyPD
CsA	0.36 ± 0.01	0.73 ± 0.02	1.25 ± 0.03
FK506	> 10	> 10	> 10
PSC833	> 10	> 10	> 10

Fig. 4. Effect of immunosuppressants on HBV infection. (A,C) HepaRG cells were treated with or without the indicated compounds at 2 μM (FK506 4 μM) in (A), and CsA (2 and 4 μM) and PSC833 (2 and 4 μM) in (C), according to the scheme in Fig. 1A. HBs (A,C) and HBe (C) secretion was determined. (B) Effect of compounds on the activity of the calcineurin/NF-AT pathway. Jurkat cells transfected with pNF-AT-luc and pRL-TK were stimulated with PMA and ionomycin in the presence or absence of CsA, FK506, and PSC833 for 24 hours to measure the luciferase activity. (D) Cyclophilin binding activity of CsA, FK506, and PSC833 was determined in a competitive binding assay as described in the Materials and Methods using a CsA-derived fluorescent probe. IC_{50}s (μM) for the inhibition of probe binding to CyPA, CyPB, and CyPD are shown. $*P < 0.05$, $**P < 0.01$.