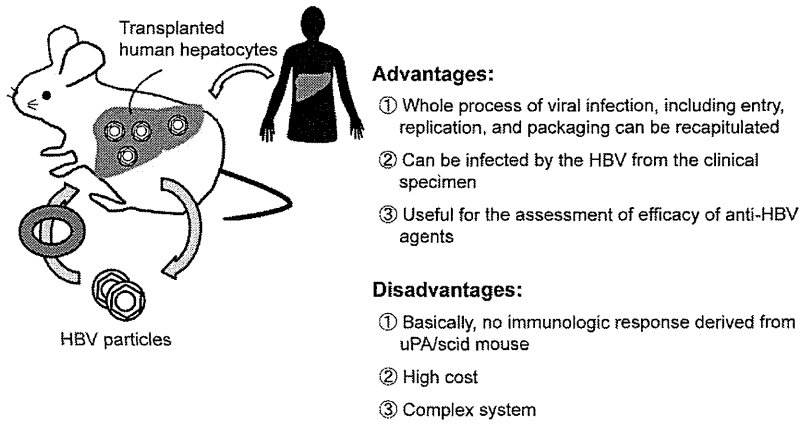
Because the mice are immunocompromised following the injection of 1 million human hepatocytes into the mouse spleen, a proportion of the transplanted cells engraft in the liver after migrating via the splenic and portal veins. A few days post-transplantation, small clusters of human hepatocytes begin to proliferate within the mouse liver, forming larger regenerative nodules that eventually merge together and replace the diseased liver parenchyma. The levels of human chimerism can be estimated by measuring the levels of human albumin circulating in mouse serum.

These two types of human hepatocyte chimeric mice are susceptible to HBV infections (see Figure 3) [53–55]. The establishment of HBV infection is generally first achieved in a small minority of human hepatocytes and several weeks are needed to accomplish viral spreading. After that, nearly all human hepatocytes stain HBcAg-positive and viremia reaches a stable plateau, which directly correlates with the levels of human chimerism [53–56].

Figure 3. Chimeric mouse model.



Because human hepatocyte chimeric mice are immunocompromised, they are not suited for vaccine studies or evaluation of immune responses. These mice, however, are a promising tool for evaluation of anti-HBV agents [54,57,58] and of susceptibility of mutant strains to various drugs [59]. More importantly, the largest advantage of the human hepatocyte chimeric mice is that they are the sole model fully recapitulating the genomic maintenance of nuclear HBV cccDNA. Using this model, Belloni *et al* demonstrated that IFN-α suppresses HBV replication through the mechanism of epigenetic control of cccDNA function and transcription [60]. Since cccDNA can be an important therapeutic target to achieve complete eradication of HBV, chimeric mice experiments aiming at elucidating the molecular mechanism whereby cccDNA activity is controlled would help the development of more effective therapeutics.

2.5. Genetically Humanized Mice

Murine hepatocytes do not support the entry of HBV and hepatitis C virus (HCV), due to the lack of receptor molecules specific for HBV and HCV. Based on the observation that CD81 and occluding (OCLN) comprise the minimal set of human factors required to render mouse cells permissive to HCV entry [61], Dorner *et al.* showed that either transient or stable expression of these two human genes is

sufficient to allow viral uptake and support HCV infection in immunocompetent inbred mice [62,63]. In principle, similar strategy can be applied for the generation of mouse model in which the entire HBV life cycle is recapitulated. However in case of HBV, despite the identification of sodium taurocholate cotransporting polypeptide (NTCP) as a long-sought functional receptor for HBV [34,64–66], recent study demonstrated that in mouse hepatocytes NTCP expression allows HBV entry but is not sufficient to support HBV infection, suggesting the existence of murine restriction factors that limit HBV infection [67]. Thus, future studies for the identification of such factors would be required for the development of immunocompetent genetically humanized mice that support HBV infection.

2.6. Humanized Mice with Human Immune System and Liver Tissues

Due to the absence of a functional immune system, the above-described uPA/scid and $Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}$ mouse models support HBV infection but no liver disease is observed [48,53]. To reproduce human immune response to HCV in a small animal model, Washburn *et al.* developed humanized mice reconstituted with human immune system and liver tissues (AFC8-hu HSC/Hep) [68]. They used Balb/C $Rag2^{-/-}\gamma C$-null mice that were genetically engineered to express a fusion protein of FK506 binding protein (FKBP) and caspase 8 with inducible suicidal activity in hepatocytes under the control of albumin promoter (AFC8). Co-transplantation of human CD34+ hematopoietic stem cells and human hepatocyte progenitors into the transgenic mice treated with FKBP dimerizer allowed for the successful engraftment of immune cells and hepatocytes. AFC8-hu HSC/Hep became infected with HCV in the livers, generated a human immune T cell response against the virus, and developed hepatitis and fibrosis [68]. Thus, HBV infection experiments using these mice are expected to uncover heretofore unsuspected virologic and immunologic aspects of HBV infection.

3. Conclusions

The history of the fight between HBV and humans began in 1965 when Baruch Blumberg *et al.* discovered the Australia antigen later determined to be HBsAg [69], followed by the discovery of the association between the Australia antigen and specific hepatitis viral infection. After that, many basic and clinical studies have shed light on the virology and pathophysiology of HBV and have attempted to establish mouse models for HBV infection. The mechanisms of a wide range of immune responses against HBV and the resultant clinical phenotypes have not yet been determined. To gain further insight into the host-virus interaction during HBV infection, further progress toward establishing suitable animal models for detailed studies of HBV infection and thus the development of a robust animal model are required.

Author Contributions

Tadashi Inuzuka, Ken Takahashi, Tsutomu Chiba and Hiroyuki Marusawa contributed equally to the writing of this review.

Conflicts of Interest

The authors declare no conflict of interest.

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Human Plasmacytoid Dendritic Cells Sense Lymphocytic Choriomeningitis Virus-Infected Cells *In Vitro*

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We previously reported that exosomal transfer of hepatitis C virus (HCV) positive-strand RNA from human Huh-7 hepatoma cells to human plasmacytoid dendritic cells (pDCs) triggers pDC alpha/beta interferon (IFN-α/β) production in a Toll-like receptor 7 (TLR7)-dependent, virus-independent manner. Here we show that human pDCs are also activated by a TLR7-dependent, virus-independent, exosomal RNA transfer mechanism by human and mouse hepatoma and nonhepatoma cells that replicate the negative-strand lymphocytic choriomeningitis virus (LCMV).

Interferons (IFNs) are key mediators of the innate immune response to many viruses, including hepatitis C virus (HCV) (1) and the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) (2). Accordingly, HCV and LCMV have evolved mechanisms to block IFN induction in the infected cell (1, 2). Nevertheless, HCV and LCMV infections strongly induce IFN and IFN-stimulated gene (ISG) expression *in vivo* (3–7). Recently, we reported that Huh-7 cells infected with HCV or containing a subgenomic HCV replicon can trigger alpha/beta IFN (IFN-α/β) production *in vitro* by exosomal transfer of positive-strand HCV RNA to cocultured human peripheral blood-derived plasmacytoid dendritic cells (pDCs) in a Toll-like receptor 7 (TLR7)-dependent manner without infecting them (1, 8, 9). Here, we have extended those observations to a negative-strand RNA virus. The broad host cell range of LCMV allowed us to show that human pDCs can be activated by a wide variety of infected human and mouse cell lineages, a process that required cocultivation of pDCs and infected cells but no infection of pDCs.

LCMV is a noncytolytic enveloped virus with a bisegmented negative-strand RNA genome (1, 2, 10, 11). LCMV causes a long-term chronic infection in its natural host, the mouse. Human infections occur through mucosal exposure to aerosols or by direct contact of abraded skin with infectious material (3–7, 11). LCMV infection of humans can result in severe disease that in some cases can be fatal (12). LCMV infection of mice is associated with an initial burst of type I interferon produced in large part by infected dendritic cells (DCs) (7, 13–15). However, LCMV nucleoprotein (NP) efficiently blocks interferon regulatory factor 3 (IRF3) activation and thus IFN production in LCMV-infected cells (16). This might explain why only a small fraction of LCMV-infected dendritic cells produce IFN in the infected mice (7). Interestingly, however, IFN production also occurs in pDCs in the spleen in the absence of active LCMV replication, suggesting that pDCs can sense LCMV infection independently of virus production (7). Thus, in this study we asked if pDCs can sense LCMV-infected cells by a mechanism similar to that described for sensing of HCV-infected cells (8, 9).

Blood was collected from healthy adult human volunteers after informed consent was obtained according to procedures approved by the Scripps Research Institute Human Research Committee. In a first set of experiments, we infected Huh-7.5.1c2 cells, a subclone of the human hepatoma Huh-7 cell line that is highly permissive for HCV infection (17), with LCMV (Armstrong strain) (multiplicity of infection [MOI] = 0.1) 3 days before coculture with human peripheral blood-derived pDCs as described previously (9). The supernatant harvested after 24 h of coculturing LCMV-infected Huh-7.5.1c2 cells (2×10^5) with human pDCs (2×10^4) contained up to 100 ng/ml of IFN-α (Fig. 1A, lane 5). This was ≥10-fold higher than the amount of IFN-α produced by pDCs that had been cocultured with Huh-7.5.1c2 cells infected by the cell culture-adapted HCV JFH-1 D183 variant (9, 18) (Fig. 1A, lane 4), which correlated with the relative intracellular viral RNA levels in the HCV- and LCMV-infected cells (Table 1). Interestingly, similar amounts of IFN-α were produced in pDC cocultures with cells infected with a single-cycle recombinant LCMV (scrLCMVΔGP/GFP [33]) that cannot produce infectious virus (Fig. 1A, lane 6), suggesting that production of LCMV infectious progeny was not required to trigger IFN-α production by the pDCs. Notably, inoculation of human pDCs with a high dose (MOI = 10) of LCMV for 24 h in the absence of Huh-7 cells did not trigger IFN-α production in the pDCs (Fig. 1A, lane 7). Likewise, pDCs did not produce IFN-α after incubation with the cell culture supernatant (Fig. 1A, lane 8) of the LCMV-infected Huh-7.5.1c2 cells used for the coculture shown in lane 5 of Fig. 1A. These results indicated that production of IFN-α by pDCs did not require that they be infected by LCMV. Human pDCs incubated for 3 days with infectious LCMV were negative for LCMV nucleoprotein (NP) expression by fluorescence-activated cell sorter (FACS) analysis (data not shown), indicating that human pDCs are not likely to be productively infected by LCMV *in vitro*. It is noteworthy that pDC IFN-α production levels were equally robust when infected Huh-7.5.1c2 cells or infected parental Huh-7 cells were used (Fig. 1A, lane 10). Importantly, neither Huh-7 cells nor Huh-7.5.1c2 cells produced IFN-α themselves either before or

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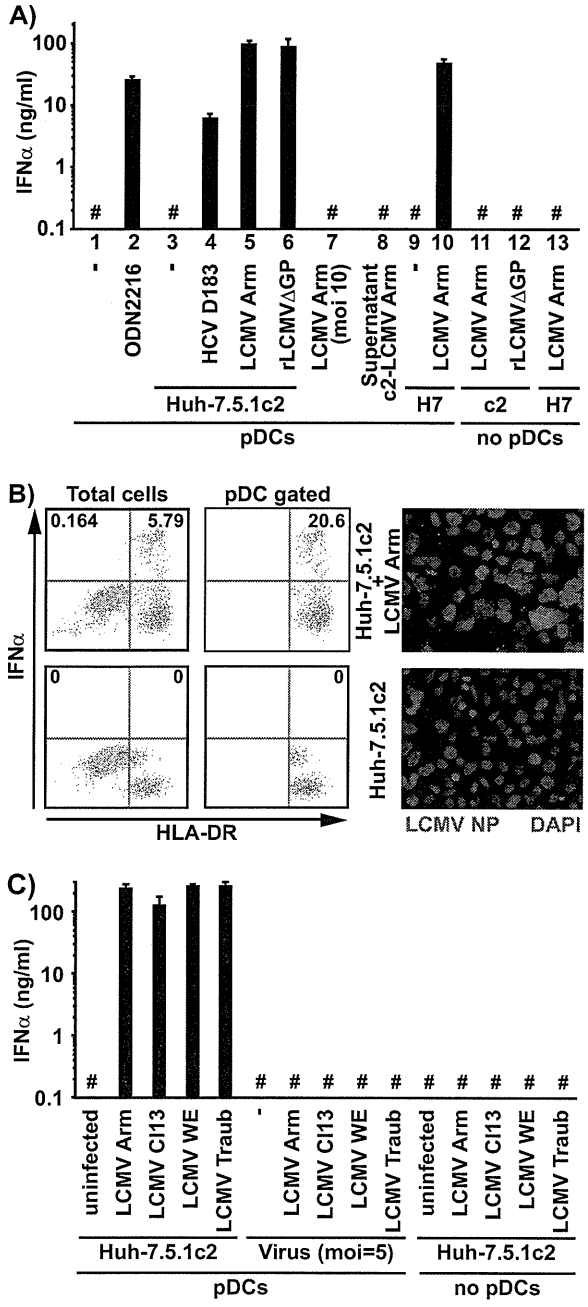

FIG 1 LCMV-infected Huh-7-derived cells trigger IFN-α production in cocultured human peripheral pDCs. (A) Huh-7.5.1c2 or Huh-7 cells (2×10^5) infected with LCMV or HCV D183 (18) at MOI = 0.1 3 days earlier were cocultured with 2×10^4 freshly purified human peripheral pDCs in wells of a 96-well round-bottom plate for 24 h before IFN-α was quantified in the coculture supernatant by enzyme-linked immunosorbent assay (ELISA) as described previously (9). c2, Huh-7.5.1c2 cells; H7, Huh-7 parental cells. (B) Cocultures grown as described for panel A were fixed and analyzed by FACS as described previously (9). All cells are shown in the left panels, and cells gated for HLA-DR and CD123 (pDCs) are shown in the right panels. Huh-7.5.1c2 cells infected with LCMV as described for panel A were analyzed by immunofluorescence as described previously (9) using an anti-NP MAb (1.1.3 [34]) and a secondary Alexa 555-conjugated goat anti-mouse antibody (Invitrogen). (C) IFN-α was quantified in the supernatant of cocultures of Huh-7.5.1c2 cells infected with different LCMV strains (39, 40) with pDCs as described for panel A. #, below the limit of detection of the IFN-α ELISA (36 pg/ml). Error bars represent means ± standard deviations (SD) ($n = 3$).

after LCMV infection (Fig. 1A, lanes 11 to 13), suggesting that IFN-α production reflects activation of the cocultured human pDCs. This was confirmed by FACS analysis of cocultures of human pDCs and LCMV-infected Huh-7.5.1c2 cells (9) after staining for pDC markers HLA-DR (allophycocyanin [APC]-mouse anti-HLA-DR; eBioscience) and CD123 (phycoerythrin [PE]-Cy7-mouse anti-CD123; Biolegend) and intracellular IFN-α (PE-mouse anti-IFN-α; Miltenyi, Auburn, CA). Approximately 20% of the cocultured HLA-DR-positive (HLA-DR$^+$) pDCs but none of the LCMV-infected HLA-DR$^-$ Huh-7.5.1c2 cells produced IFN-α (Fig. 1B) even though all of the Huh-7.5.1c2 cells were infected, as shown by LCMV NP-specific immunofluorescence (IF) analysis using an anti-NP monoclonal antibody (MAb) (1.1.3 [34]) as described previously (9) (Fig. 1B). Levels of infectious virus-independent pDC activation were similarly robust for all four different strains of LCMV tested (Fig. 1C). Together, these results demonstrate that LCMV-infected human Huh-7-derived hepatoma cells are sensed by human pDCs that respond by producing IFN-α even more strongly than when they are stimulated by HCV JFH1-infected cells (9). The lack of IFN-α production by human pDCs directly exposed to infectious LCMV virions strongly suggested that, in similarity to their response to HCV-infected cells, they likely responded to something other than the virus particles themselves.

Next, we asked whether production of IFN-α by human pDCs in response to coculture with LCMV-infected cells was also related to the exosome-mediated mechanism by which they respond to HCV-infected cells (8, 9). Human pDC activation by LCMV-infected Huh-7.5.1c2 cells was inhibited by the TLR7-specific antagonist IRS661 (Fig. 2A), suggesting that activation of pDCs is mediated by TLR7 as we have previously described for HCV (8, 9) and as has also been observed in the spleen of LCMV-infected mice (15). Likewise, as previously described for HCV (9), human pDC activation by LCMV-infected cells was cell-cell contact dependent since cultivation of LCMV-infected Huh-7.5.1c2 cells and human pDCs in transwell chambers did not result in detectable levels of IFN-α production by the pDCs (Fig. 2B). Next, we performed a series of experiments to determine whether LCMV-infected cell-mediated pDC activation might be exosome dependent. The two structurally unrelated exosome release inhibitors GW4869 (10 μM) and spiroepoxide (20 μM) strongly reduced the ability of LCMV-infected Huh-7.5.1c2 cells to trigger IFN-α production by human pDCs (Fig. 2C) without affecting intracellular LCMV RNA levels in the Huh-7.5.1c2 cells (data not shown). Furthermore, cytochalasin D (0.1 μg/ml), an inhibitor of actin-dependent endocytosis (35) that does not affect LCMV infection (36), completely blocked LCMV-infected cell-mediated IFN-α production by pDCs (Fig. 2C). In control experiments, GW4869, spiroepoxide, and cytochalasin D had little or no effect on TLR7 agonist (resiquimod)-triggered IFN-α production by pDCs (Fig. 2C). These results suggest that exosome release from infected cells and active endocytosis by the pDCs are required for pDC stimulation by LCMV-infected cells. These findings, together with the observation that supernatants of scrLCMVΔGP/GFP-infected cells that do not produce infectious virus nevertheless contain membrane-protected LCMV RNA (data not shown), suggest that LCMV RNA is likely to be transferred to pDCs via exosomes as we have previously described for HCV (8). These findings are consistent with the notion that, in similarity to the situation described for HCV (8, 9), human pDCs sensed LCMV-infected hepatoma

TABLE 1 Cell lines and viruses triggering IFN-α production by pDCs

Cell line	Reference(s)	Species	Cell of origin	Virus	Virus strain/genotype	Infection (log GE/μg RNA)[a]	IFN-α log (ng/ml)[b]
Huh-7.5.1c2	17, 19, 20	Human	Hepatocyte	HCV	JFH1/2a	7	1
				LCMV	Armstrong	8	2
					Cl13	8	2
					WE	8	2
					Traub	8	2
					rLCMVΔGFP	8	2
Huh-7	20, 21	Human	Hepatocyte	HCV	JFH1/2a	7	1
				LCMV	Armstrong	8	2
HepG2	22	Human	Hepatocyte	LCMV	Armstrong	8	2
HepG2.2.15	23	Human	Hepatocyte	HBV	ayw	7	ND[f]
				HBV + Res[c]	ayw/TLR7-agonist	7	1
				HBV + LCMV	ayw/Armstrong	7/8	1–2
Hep3B	22	Human	Hepatocyte	LCMV	Armstrong	8	2
HeLa	24	Human	Cervix	LCMV	Armstrong	8	1
HEK293T	25, 26	Human	Embryonic kidney	LCMV	Armstrong	8	ND
PHH[d]		Human	Liver	LCMV	Armstrong	7	1
AML12	27	Mouse	Hepatocyte	LCMV	Armstrong	8	0
NIH 3T3	28	Mouse	Embryonic fibroblast	LCMV	Armstrong	7	1
CV-1	29	Monkey	Kidney	LCMV	Armstrong	nt[e]	ND
LMH D2	30–32	Chicken	Hepatocyte	DHBV	DHBV3	8	ND
				DHBV + Res[c]	DHBV3/TLR7-agonist	8	1
				DHBV + LCMV	DHBV3/Armstrong	8/8	1

[a] Approximate magnitude of intracellular viral RNA content in log genome equivalents per μg of total cellular RNA (log GE/μg RNA) at the start of the coculture with human pDCs.
[b] Approximate magnitude of IFN-α production [in log(ng/ml)] after 24 h of coculture of the infected cell line with human pDCs.
[c] Cocultures of HepG2.2.15 and LMH D2 cells and human pDCs were simultaneously treated with 50ng/ml of the TLR7 agonist resiquimod (Res).
[d] Freshly isolated primary human hepatocytes (PHH); Life Technologies, Carlsbad, CA.
[e] nt, not tested.
[f] ND, not detected (<36 pg/ml).

cells by a short-range exosome-mediated and TLR7-dependent mechanism.

Unlike HCV, LCMV has a broad host cell range in terms of both type and species, which enabled us to determine if the ability to trigger IFN-α production by human pDCs could be extended to other cell types and species. Coculture of human pDCs with either LCMV-infected human cervical epithelium-derived (HeLa) or human hepatoma-derived (HepG2 and Hep3B) cells triggered strong IFN-α production by the human pDCs (Fig. 3A). In contrast, coculture of human pDCs with LCMV-infected human embryonic kidney 293T cells did not result in production of IFN-α, though 293T cells were infected at levels similar to those of all the other cell lines (Fig. 3B). Importantly, human pDCs did not produce IFN-α when cocultured with the uninfected cell lines or when incubated with supernatants of the LCMV-infected cell lines; neither was it produced by LCMV infection of any of the cell lines examined (Fig. 3A). Next, we determined whether freshly prepared cultures of primary human hepatocytes (PHHs) (Life Technologies, Carlsbad, CA) (1.25×10^5 cells per well in 48-well plates) infected with LCMV would also be capable of triggering IFN-α production by cocultured human blood-derived pDCs. As shown in Fig. 4A, 1.25×10^5 PHHs in a 48-well plate (maintained according to the manufacturer's instructions) infected with LCMV Arm for 3 days (d3) or 4 days (d4) triggered IFN-α production by 1×10^5 cocultured human blood-derived pDCs whereas the uninfected PHHs did not. Importantly, the LCMV-infected PHH cells did not produce IFN-α despite containing high levels of LCMV RNA (Fig. 4B) and despite most of the PHH cells being positive for LCMV NP (Fig. 4C), suggesting that pDCs, rather than PHH cells, were the source of IFN-α in the cocultures (Fig. 4A). These results demonstrated that sensing of virus-infected cells by human pDCs is not restricted to Huh-7-derived cells but also extends to cells of nonhepatic human origin (i.e., HeLa cells), to other human hepatoma-derived cell lines (HepG2 and Hep3B), and, most importantly, to primary human hepatocytes.

Since mice are the natural host of LCMV, we asked if LCMV-infected mouse cell lines would trigger IFN-α production by human pDCs. Both LCMV-infected murine hepatoma (AML12) and fibroblast (NIH 3T3) cells triggered IFN-α production by cocultured human pDCs, while the corresponding uninfected cells did not (Fig. 5A). Furthermore, the LCMV-infected cells themselves did not produce IFN-α, indicating that the pDCs in the cocultures were the source of the IFN-α (Fig. 5A). Interestingly, we observed substantial differences in the magnitude of IFN-α production by pDCs depending on the LCMV-infected cell type used in the coculture, although the levels of LCMV infection were similar in all cell lines tested (Fig. 5B) as determined by LCMV-NP-specific immunofluorescence and reverse transcription-quantitative PCR (RT-qPCR) using LCMV NP-specific primers (LCMV_NP-up [GTTGCGCATTGAAGAGGTCGG] and LCMV_NP-lo [CCAACCACAGAACGGGCAGT]). This suggested that there may be cell type- and species-specific differences in the efficiency of viral RNA transfer to pDCs.

Table 1 shows a complete list of the IFN-α responses shown by human pDCs during coculture with LCMV-infected cells of dif-

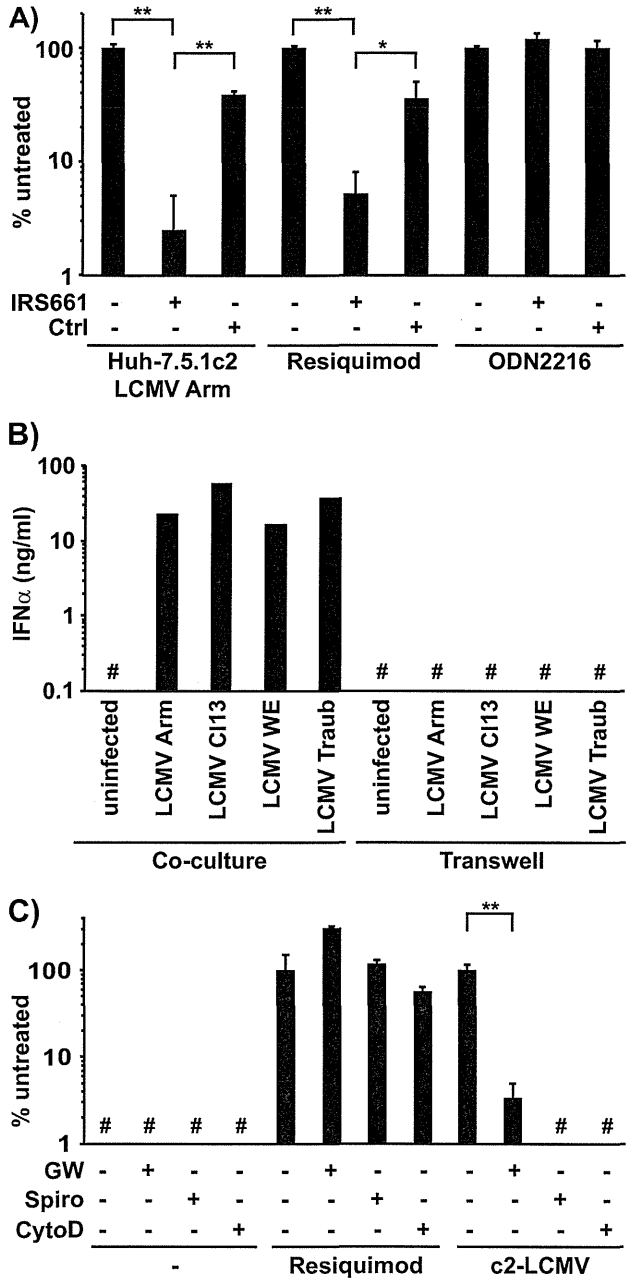

FIG 2 Mechanism of activation of pDCs by LCMV-infected Huh-7.5.1c2 cells. (A) Human peripheral pDCs were cocultured with LCMV Armstrong-infected Huh-7.5.1c2 cells or incubated with a TLR7 (Resiquimod) or TLR9 (ODN2216) ligand and left untreated or treated with a TLR7 antagonist or a control oligonucleotide (Ctrl) exactly as described previously (9). IFN-α production is shown as a percentage of the untreated control level in each group. (B) IFN-α production was quantified by ELISA in the supernatant of cocultures of LCMV-infected Huh-7.5.1c2 cells and pDCs set up exactly as described in the legend to Fig. 1A but either seeded together on top of the membrane of transwell chambers (Coculture) or separated by the membrane (Transwell). Data of individual wells are shown. (C) Human peripheral pDCs (2×10^4) were cocultured with 6.7×10^3 LCMV Armstrong-infected Huh-7.5.1c2 cells or incubated with a TLR7 agonist (Resiquimod) and left untreated or treated with the exosome release inhibitors GW4869 (GW, 10 μM) and spiroepoxide (Spiro, 20 μM) or the endocytosis inhibitor cytochalasin D (CytoD, 0.1 μg/ml) as described previously (8). IFN-α production is shown as a percentage of the untreated control group level. #, below the limit of detection of the IFN-α ELISA (150 pg/ml). Error bars represent means ± SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$ (paired Student's t test).

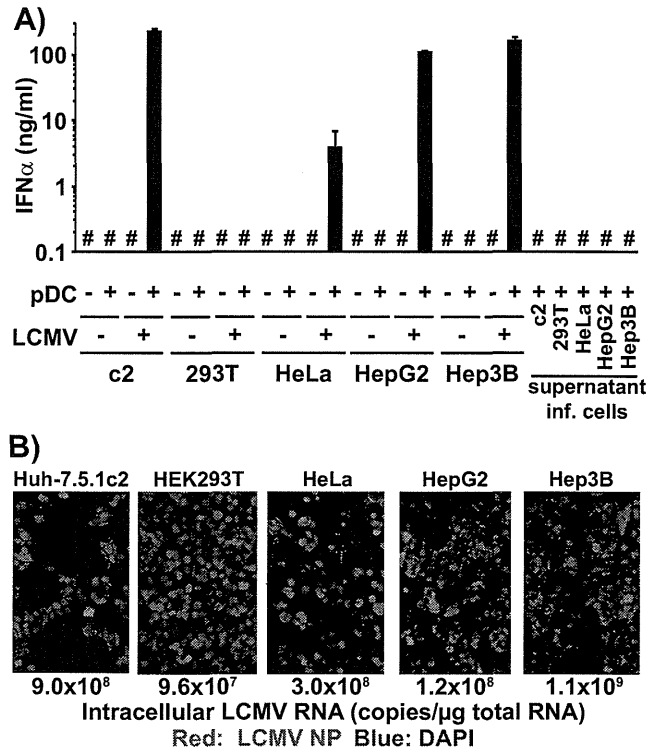

FIG 3 LCMV-infected human cell lines other than Huh-7 trigger IFN-α production in cocultured pDCs. (A) Quantification of IFN-α production in cell culture supernatants of uninfected or LCMV Armstrong-infected cells (LCMV +/−) cocultured or not with human peripheral pDCs (pDC +/−) set up as described for Fig. 1A. Alternatively, 2×10^4 pDCs were incubated with the supernatant of LCMV-infected cells collected 3 days after LCMV inoculation. #, below the limit of detection of the IFN-α ELISA (36 pg/ml); inf, infected. Error bars represent means ± SD ($n = 3$). (B) Analysis of LCMV infection 3 days after LCMV Armstrong inoculation (MOI = 0.1). LCMV-infected cells were visualized by LCMV NP-specific immunofluorescence. Intracellular LCMV RNA levels were determined by LCMV-specific RT-qPCR and normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA levels as described previously (9).

ferent origins and species. Interestingly, human hepatoma cells (HepG2.2.15 [37]) that replicate hepatitis B virus (HBV) and secrete infectious virions did not trigger IFN-α production by pDCs, and the same was true for chicken hepatocyte-derived cells (LMH D2 [38]) that replicate the duck hepatitis B virus (DHBV) (Table 1). For both systems, however, the failure to trigger pDC activation seemed to be virus specific, since superinfection of the same cells with LCMV resulted in strong IFN-α production by human pDCs. Furthermore, pDCs cocultured with HBV- and DHBV-producing HepG2.2.15 and LMH D2 cells were fully able to produce IFN-α in response to the TLR7 agonist resiquimod (Table 1) compared to resiquimod stimulation of human pDCs only (data not shown), suggesting that neither HBV- nor DHBV-infected cells impaired the ability of pDCs to produce IFN-α in response to TLR7 ligation.

While we were unable to detect IFN-α production in *in vitro* cocultures of murine splenic pDCs and LCMV-infected mouse or human cell lines (data not shown), it is well documented that LCMV infection triggers IFN-α production by pDCs *in vivo* in mice (7, 15). Interestingly, it has recently been shown *in vivo* that the majority of IFN-α-producing pDCs in the mouse spleen early

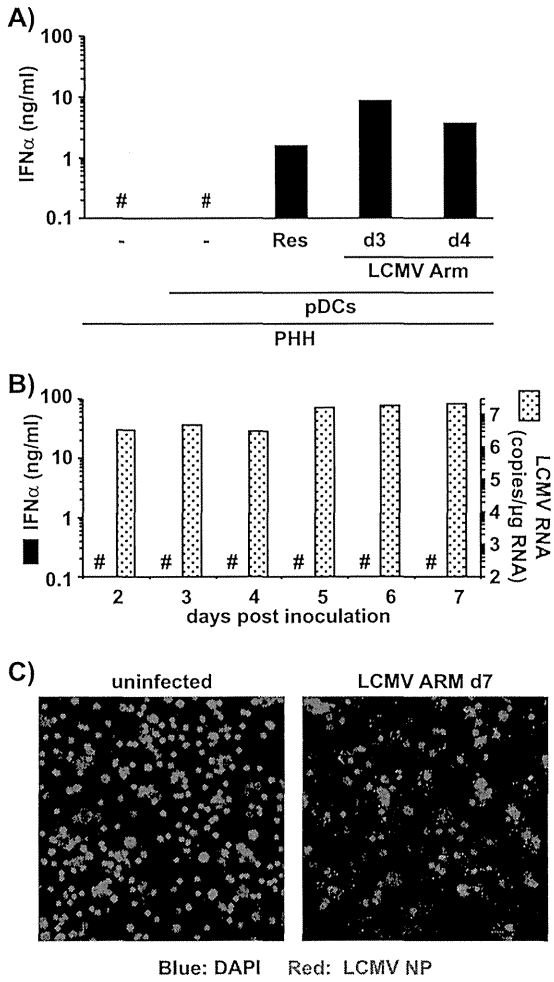

FIG 4 LCMV-infected primary human hepatocytes (PHHs) trigger IFN-α production in cocultured pDCs. (A) Quantification of IFN-α production in cell culture supernatants of uninfected PHHs cocultured (20 h) or not with human peripheral pDCs and cocultures of human pDCs with PHHs infected with LCMV (MOI = 0.1) for 3 days (d3) or 4 days (d4). #, below the limit of detection of the IFN-α ELISA (36 pg/ml); Res, resiquimod. Single wells were analyzed. (B) Analysis of LCMV infection and IFN-α production of PHHs at different time points after LCMV Armstrong inoculation (MOI = 0.1). Intracellular LCMV RNA levels were determined by LCMV-specific RT-qPCR and normalized to GAPDH mRNA levels as described previously (9). #, below the limit of detection of the IFN-α ELISA (36 pg/ml). (C) LCMV-infected PHHs were visualized by LCMV NP-specific immunofluorescence 7 days postinoculation.

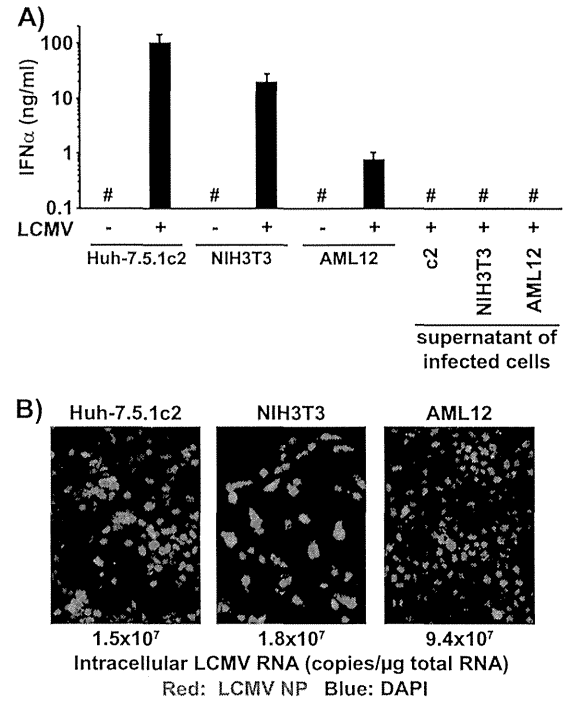

FIG 5 LCMV-infected murine cell lines trigger IFN-α production in cocultured human peripheral pDCs. (A) IFN-α production in cell culture supernatants of uninfected or LCMV Armstrong-infected cells (LCMV +/−) cocultured with human peripheral pDCs (pDC +/−) set up exactly as described for Fig. 1A. Alternatively, pDCs were incubated with the supernatants of LCMV-infected cells collected 3 days after LCMV inoculation and set up exactly as described for Fig. 1A. #, below the limit of detection of the IFN-α ELISA (36 pg/ml). Error bars represent means ± SD (n = 3). (B) Analysis of LCMV infection 3 days after LCMV Armstrong inoculation (MOI = 0.1). LCMV-infected cells were visualized by LCMV NP-specific immunofluorescence. Intracellular LCMV RNA levels were determined by LCMV-specific RT-qPCR and normalized to GAPDH mRNA levels as described previously (9).

after LCMV Cl13 infection are not productively infected and it was suggested that those pDCs might sense infected cells by a mechanism that is independent of intrinsic virus replication in pDCs, e.g., by the sensing of LCMV-infected cells (7). The results reported here might explain how those pDCs sense LCMV infection *in vivo* in the mouse spleen. Together with our previous studies employing analysis of the responsiveness of human pDCs to activation by HCV-infected cells (8, 9), the results presented here support the concept that the ability of noninfected pDCs to direct a strong IFN-α response upon sensing infected cells might be a general mechanism by which the host can circumvent the ability of viruses to block innate signaling in productively infected cells and thus mount efficient innate immune responses that have the potential to control viral infection.

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CASE REPORT

Acute hepatitis B of genotype H resulting in persistent infection

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Abstract

A 47-year-old man presented with general fatigue and dark urine. The laboratory data showed increased levels of hepatic transaminases. The patient was positive for hepatitis B virus (HBV) markers and negative for anti-human immunodeficiency virus. The HBV-DNA titer was set to 7.7 log copies/mL. The patient was diagnosed with acute hepatitis B. The HBV infection route was obscure. The serum levels of hepatic transaminases decreased to normal ranges without any treatment, but the HBV-DNA status was maintained for at least 26 mo, indicating the presence of persistent infection. We isolated HBV from the acute-phase serum and determined the genome sequence. A phylogenetic analysis revealed that the isolated HBV was genotype H. In this patient, the elevated peak level of HBV-DNA and the risk alleles at human genome single nucleotide polymorphisms s3077 and rs9277535 in the human leukocyte antigen-DP locus were considered to be risk factors for chronic infection. This case suggests that there is a risk of persistent infection by HBV genotype H following acute hepatitis; further cases of HBV genotype H infection must be identified and characterized. Thus, the complete determination of the HBV genotype may be essential during routine clinical care of acute hepatitis B outpatients.

Key words: Acute hepatitis; Chronic hepatitis; Genotyping; Hepatitis B virus; Single nucleotide polymorphisms

Core tip: Hepatitis B virus (HBV) genotype H infection is rare in Asia, particularly in Japan. Here, we report a case of acute hepatitis B caused by a genotype H strain with persistent infection, although most adult cases of acute hepatitis B are self-limiting in Japan. This case suggests that the HBV genotype H infection can be a risk factor for persistent infection. Therefore, it is necessary to investigate the characteristics of genotype H infection in an accumulation of cases. Thus, the

complete determination of the HBV genotype may be essential in the routine clinical care of acute hepatitis B patients.

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INTRODUCTION

Hepatitis B is a potentially life-threatening liver infection caused by the hepatitis B virus (HBV); it represents a major global health problem. HBV can cause chronic liver diseases and increases the risk of death from cirrhosis and liver cancer. Worldwide, an estimated two billion people have been infected with HBV and more than 240 million have chronic infections[1]. The HBV genome consists of approximately 3200-nucleotides of DNA; the virus replicates using a reverse transcriptase enzyme that lacks proofreading ability. Therefore, HBV possesses diverse genetic variability, and the viral population is classified into at least eight genotypes that are designated A -H[2-6]. In Japan, genotypes B and C are prevalent among patients with chronic infections. However, in the last decades, the prevalent genotype in acute HBV infections has shifted from genotype C to A[7-9]. There are some differences in the clinical features and outcomes among the genotypes[10-13]. It has been reported that the persistent infection from acute hepatitis is prevalent in adults that are infected with genotype A HBV. Thus, determining the HBV genotype is of increasing importance even in routine clinical practice, although a reliable kit for determination of all HBV genotypes is still uncommon and is not yet covered by insurance. The host factors associated with persistent infection by HBV have also been reported, such as single nucleotide polymorphisms (SNPs) or genotypes in the human leukocyte antigen-DP locus. It may also be useful for identifying the patients who are prone to develop chronic hepatitis.

In this report, we describe a case of acute hepatitis B resulting from infection by a genotype H strain of HBV. Although the laboratory data and symptoms were not distinguishable from acute hepatitis B with other genotypes, this patient developed persistent infection.

CASE REPORT

A 47-year-old man living in Kawasaki, Japan, presented at our hospital with general fatigue and dark urine. Approximately 1 wk before visiting the hospital, the patient developed nausea, loss of appetite, and a feeling of fullness in the abdomen. Four days later, he noted darkening of his skin and urine. Upon admission, the patient's laboratory data revealed elevated serum aspartate aminotransferase, alanine aminotransferase (ALT), lactate dehydrogenase, alkaline phosphatase, γ-glutamylt ranspeptidase, and total bilirubin (T-Bil) levels (Table 1). The prothrombin activity was within the normal range (95%). Test for hepatitis B surface antigen (HBsAg; HISCL-2000i, Sysmex, Kobe, Hyogo, Japan), hepatitis B e-antigen (HBeAg; ARCHITECT® CLIA, Abbott Japan, Tokyo, Japan) and anti-hepatitis B core antigen (anti-HBc) IgM (ARCHITECT® CLIA) were positive. A test for HBV-DNA was also positive, exhibiting a titer of 7.7 log copies/mL (COBAS TaqMan HBV Test v2.0, Roche Diagnostics, Tokyo, Japan). HBsAg had not been detected 2 years previously when the patient had been admitted to another hospital for treatment of acute enterocolitis. Other hepatitis virus markers were negative. Therefore, the patient was diagnosed with acute hepatitis B. The genotype of the infecting HBV, as assessed by the Immunis HBV Genotype Immunis® HBV Genotype EIA Kit (Institute of Immunology, Tokyo, Japan), was determined as genotype C. The patient had not been abroad in the past 12 mo; he had no history of receiving blood or blood-related products, transfusions, or drug injections, and he reported no personal or family history of liver disease. The man was unmarried and declared that he was heterosexual, with no history of sexual contact with commercial sex workers or strangers. Anti-human immunodeficiency virus (HIV) was not detected. In the absence of medication, the patient's condition and elevated ALT level improved within a month. Anti-HBe became detectable, and HBeAg disappeared 2 mo after onset of the symptoms. HBsAg became undetectable at 5 mo, but the patient still tested positive for HBV-DNA, a status that persisted for at least 26 mo following his presentation at our hospital (Figure 1). We are now preparing to administer anti-viral medication.

For further analysis of the HBV infecting this patient, HBV-DNA was extracted from the acute-phase serum using a QIAamp DNA Blood Mini kit (QIAGEN, Valencia, CA). The entire HBV genome sequence was determined after polymerase chain reaction (PCR) amplification using the following primers [the number of nucleotides (nt) added to the primers were deduced from the prototype HBV/C clone, with accession no. AB246344]. For the amplification of half of the HBV genome, the outer primers were 5'-ATTCCACCAAGCTCTGCTAGATCCCAGAGT-3' (nt 10-39) and 5'-GGTGCTGGTGAACAGACCAATTTATGCCTA-3' (nt 1813-1784), and the inner primers were 5'-CCTATATTTTCCTGCTGGTGGCTCCAGTTC-3' (nt 46-75) and 5'-TAGCCTAATCTCCTCCC CCAACTCCTCCCA-3' (nt 1760-1731). For the other half of the HBV genome, the outer primers were 5'- ACGTCGCATGGAGACCACCGTGAACGCCCA-3' (nt 1601-1630) and 5'-AAGTCCACCACGAGTCTAGACTCTGTGGTA-3' (nt 266-237), and the inner primers were 5'-CCAGGTCTTGCCCAAGGTCTTACATAAGAG-3' (nt 1631-1660) and 5'-CCCGCCTGTAACACGAGCAGGGGTCCTAGG-3' (nt 207-178). The PCR was performed in a thermal cycler for 30 cycles

Table 1 Laboratory findings at first visit to our hospital

Hematology		Blood chemistry		Viral markers		Immunology		Coagulation	
WBC	7400/μL	TP	7.4 g/dL	Anti-HA IgM	(-)	IgA	183 mg/dL	PT%	95%
Neutrophil	72.0%	Albumin	4.5 g/dL	Anti-HCV	(-)	IgG	1168 mg/dL	APTT	36.4 s
Eosinophil	1.0%	T-Bil	11.1 mg/dL	HBsAg	(+) 197333	IgM	220 mg/dL		
Basophil	0.0%	D-Bil	8.0 mg/dL	Anti-HBc IgM	(+) 25.5 C.O.I	ANA	× 40, homogeneous		
Monocyte	10.0%	AST	1942 IU/L	HBeAg	(+) 253 C.O.I				
Lymphocyte	17.0%	ALT	2963 IU/L	Anti-HBe	(-) 0.0 %				
RBC	457/μL	ALP	612 IU/L	HBV-DNA	7.7 log copies/mL				
Hemoglobin	16.0 g/dL	γGTP	756 IU/L	Anti-HIV	(-)				
Hematocrit	46.4%	LDH	739 IU/L	RPR	(-)				
Platelet	36.6×10^4/μL	BUN	8.2 mg/dL	TPHA	(+)				
		Creatinine	0.64 mg/dL	Anti-CMV IgG	(+)				
		T-Chol	225 mg/dL	Anti-CMV IgM	(-)				
				Anti-EBV EBNA	(+)				
				Anti-EBV EA IgG	(-)				
				Anti-EBV VCA IgG	(+)				
				Anti-EBV VCA IgM	(-)				

WBC: White blood cells; RBC: Red blood cells; ANA: Antinuclear antibody; TP: Total protein; T-Bil: Total bilirubin; D-Bil: Direct bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; γGTP: γ-glutamyltranspeptidase; LDH: Lactase dehydrogenase; BUN: Blood urea nitrogen; T-Chol: Total cholesterol; PT: Prothrombin activity; APTT: Activated partial thromboplastin time; C.O.I: Cutoff index; HA: Hepatitis A; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen; HBc: Hepatitis B core; HBeAg: Hepatitis B e-antigen; HBV: Hepatitis B virus; HIV: Human immunodeficiency virus; RPR: Rapid plasma regain; TPHA: Treponema pallidum hemagglutination assay; CMV: Cytomegalovirus; EBV: Epstein-Barr virus; EBNA: Epstein-Barr virus nuclear antigen; EA: Early antigen; VCA: Viral capsid antigen.

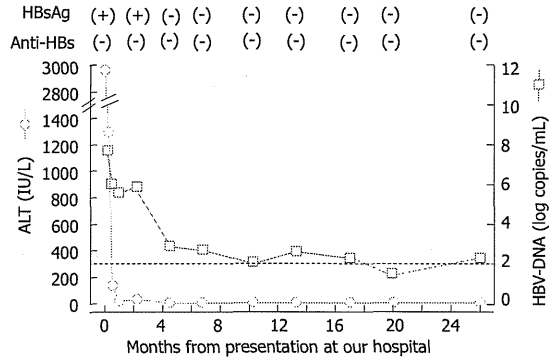

Figure 1 Clinical course of the patient infected with the genotype H strain. The dotted line indicates the detection limit of HBV-DNA (2.1 log copy/mL); the titer of the HBV-DNA was below the lower limit at 18 mo. HBsAg: Hepatitis B surface antigen; Anti-HBs: Antibody to hepatitis B surface antigen; ALT: Alanine aminotransferase; HBV: Hepatitis B virus.

(94 ℃, 30 s; 60 ℃, 30 s; 72 ℃, 30 s) with TAKARA LA Taq® DNA polymerase (TAKARA, Shiga, Japan). The amplified fragments were sequenced directly with an automated DNA sequencer (3500 Genetic Analyzer, Applied Biosystems, Foster City, CA, United States).

The genome of the infecting HBV (designated as B-MHJ9014) was 3215 bases in size. A phylogenetic analysis was performed with this strain and several database reference strains. B-MHJ9014 sorted with the genotype-H branch of the tree and clustered with the genotype-H strains previously isolated from Japanese patients (Figure 2). The substitutions at nt 1762 and nt 1764 (the basal core promoter region) and at nt 1896 (the precore region) were not observed. The length of the deduced amino acid sequences of the S, X, Core, and P proteins were identical to those encoded by other genotype H strains in the databases. The α determinant region of the S protein of B-MHJ9014 harbored an amino acid polymorphism (phenylalanine to leucine) at residue 134. The predicted B-MHJ9014 reverse transcriptase did not include any of the amino acid substitutions known to be associated with nucleotide analog resistance. To assess the complexity of the infecting virus, S region sequences from 51 clones in acute phase serum were determined. The detected sequences were genotype H and were closely related to the consensus sequence determine by direct sequencing with 1-3 amino acids polymorphisms (data not shown).

To assess the presence of human genome SNPs in the HLA-DP locus that are associated with persistent infection by HBV[14,15], a blood specimen was obtained from the patient (who had previously provided informed consent). Genomic DNA was extracted from buffy coat samples with the QIAamp DNA Mini kit (QIAGEN); DNA for SNPs rs3077 and rs9277535 were amplified with the appropriate primers and TAKARA LA Taq® DNA polymerase and were sequenced directly. The patient was homozygous (G/G) at both of these SNPs; these alleles are considered to be risk alleles for persistent infection.

DISCUSSION

HBV genotype H was first reported in 2002[5]. Infections by this genotype have been found mainly in Nicaragua, Mexico, and California; this genotype is considered to be rare in Asia, particularly in Japan[5,16-18]. However, since the first recognition of genotype H in Japan in 2005, eight strains have been isolated from Japanese patients (Table 2)[18-25]. All reported genotype H strains were isolated from male patients aged 35 to 65 years old, and the major route of infection was sexual transmission (5/8, 62.5%). Four cases (50%) represent transmissions that

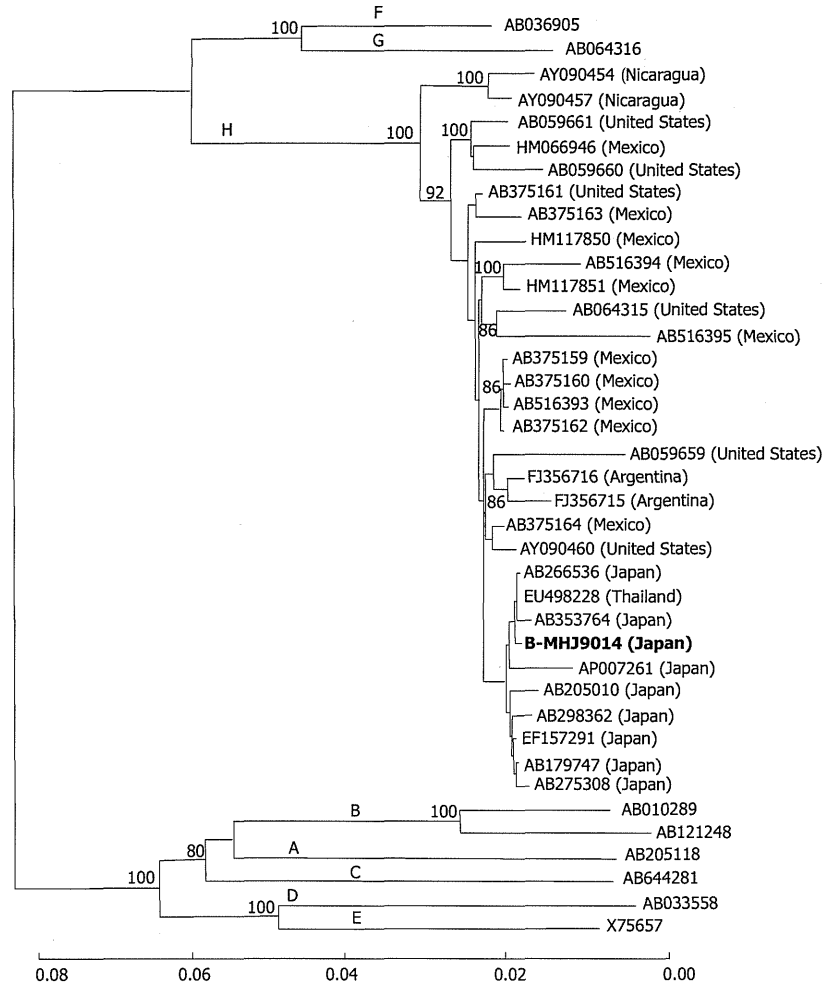

Figure 2 A phylogenetic trees constructed using the neighbor-joining method with the full hepatitis B virus genome sequence of the isolated and reference strains. The strain isolated in this case (B-MHJ9014) is shown in bold. The horizontal bar indicates the number of nucleotide substitutions per site. The reference sequences are shown with the DDBJ/EMBL/GenBank accession numbers. The HBV genotypes are indicated on each branch. The bootstrap values (> 80%) are indicated at the nodes as a percentage of the data obtained from 1000 resamplings. HBV: Hepatitis B virus.

Table 2 Genotype H strains reported in Japan

No.	Patient		Hypothesized source of infection		HIV infection[1]	Clinical feature	Accession number (Ref.)
	Age	Gender	Route	Place			
1	52	Male	Unknown	Japan	NA	Unknown blood donor	AB179747, [18]
2	61	Male	Sexual contact (heterosexual)	Thailand	NA	Chronic	AB205010, [19]
3	46	Male	Sexual contact (bisexual)	South America	(+)	Chronic	AP007261, [20]
4	38	Male	Sexual contact (homosexual)	Unknown	NA	Chronic	AB298362, [21]
5	65	Male	Unknown	Japan	NA	Acute	EF157291, [22]
6	35	Male	Unknown	Japan	NA	Acute	AB266536, [23]
7	60	Male	Sexual contact (homosexual)	Japan	(-)	Acute	AB275308, [24]
8	60	Male	Sexual contact (heterosexual)	Unknown	(+)	Chronic	AB353764, [25]
9	47	Male	Unknown	Japan	(-)	Acute to chronic	AB846650, this paper

[1]NA: Not available; HIV: Human immunodeficiency virus.

occurred in Japan. Co-infection with HIV was not common (2/8, 25%). These characteristics were similar to the case described here. All isolated strains from Japanese patients clustered together as a branch on the phylogenetic tree; therefore, it is possible that a specific strain of genotype H has emerged and spread in Japan. Presumably, the infrequent use of a reliable and convenient detection kit for genotype H infection has hampered the

correct diagnosis of genotype H infection; some cases may be misdiagnosed and considered to be infections by other genotypes. In fact, in the current case, our HBV isolate was originally identified as genotype C by the commercial kit that is covered by insurance in Japan. This kit was developed before the discovery of genotype H; thus, such a misidentification is a potential risk, as noted in the kit's instruction manual. The clinical features of genotype H infection remain obscure. There is a growing need for an accumulation of genotype H infection cases. To this end, the use of a reliable HBV genotyping kit that can correctly distinguish all genotypes is essential for routine clinical practice.

In Japan, most cases of acute hepatitis B are self-limiting, but some cases have been reported to have progressed to persistent infections[9,26-29]. Among the reported cases of genotype H infection, 4 strains were isolated from chronic hepatitis patients; in all cases, the infection was ascribed to sexual contact (Table 2)[19-21,25]. In our case, the HBV-DNA persisted for at least 26 mo. To our knowledge, this report represents the only case of genotype H infection in which chronic hepatitis was observed following acute infection. HBsAg was no longer detected at 4 mo from onset by HISCL-2000i. This disappearance was also confirmed by ARCHITECT® HBsAg (CMIA, Abbott Japan, Tokyo, Japan). In the S protein analysis, we found an amino acid polymorphism in the α determinant region. This polymorphism may affect the sensitivity for detecting HBsAg. HIV infection, a well-known risk factor for prolonged HBV infection[30], was not detected in our patient. Recently, the risk factors for HBV persistent infection have been reported in an analysis of a cohort that excluded patients co-infected with HIV[29]. In that report, infection with genotype A, elevated peak levels of HBV-DNA, and attenuated peak levels of ALT were suggested as risk factors for chronic infection. In the case described here, the peak level of HBV-DNA was 7.7 log copy/mL, which was consistent with increased risk for chronic infection. However, our patient exhibited a peak level of ALT of 2963 IU/L, which is a value that would classify this individual in the self-limiting group. Therefore, the clinical features of this case did not completely fit the risk factors associated with the establishment of chronic infection in the previous analysis[29]. Another reported risk factor for chronic HBV infection is the presence of certain SNP alleles. Specifically, selected SNPs around the HLA-DP locus have been reported to be associated with chronic hepatitis B in Asians[14,15]. With the informed consent of our patient, we determined the sequences for these SNPs (rs3077 and rs9277535) and found that this patient harbored risk alleles at both polymorphisms. This factor may have contributed to the establishment of chronic infection in this case.

In conclusion, we report a case of acute hepatitis B caused by a genotype H strain of HBV. This patient exhibited persistent infection. Our finding suggests that the infection of HBV genotype H can be a risk factor for persistent infection. We believe that it is necessary to use kits that are capable of accurate genotyping to permit an accumulation of cases and to investigate the clinical features of genotype H infection in routine clinical practice.

COMMENTS

Case characteristics

The main symptoms were nausea, loss of appetite, and a feeling of fullness in the abdomen.

Clinical diagnosis

The patient was a case of acute hepatitis B caused by a genotype H strain with persistent infection.

Differential diagnosis

The hepatitis B virus (HBV) genotype was considered to be important to predict the outcome and clinical features.

Laboratory diagnosis

To diagnose this patient, the detection of HBV markers and the complete determination of the HBV genotype were essential.

Treatment

The anti-viral treatment was not administered because we expected this case was self-limiting. Authors are now preparing medication.

Experiences and lessons

The infection of HBV genotype H can be a risk factor for persistent infection and the complete determination of HBV genotype is important.

Peer review

To conclude the association between HBV genotype H and chronic infection, the accumulation of cases of genotype H infection is essential.

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Retroviral vectors for homologous recombination provide efficient cloning and expression in mammalian cells

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ABSTRACT

Homologous recombination technologies enable high-throughput cloning and the seamless insertion of any DNA fragment into expression vectors. Additionally, retroviral vectors offer a fast and efficient method for transducing and expressing genes in mammalian cells, including lymphocytes. However, homologous recombination cannot be used to insert DNA fragments into retroviral vectors; retroviral vectors contain two homologous regions, the 5′- and 3′-long terminal repeats, between which homologous recombination occurs preferentially. In this study, we have modified a retroviral vector to enable the cloning of DNA fragments through homologous recombination. To this end, we inserted a bacterial selection marker in a region adjacent to the gene insertion site. We used the modified retroviral vector and homologous recombination to clone T-cell receptors (TCRs) from single Epstein Barr virus-specific human T cells in a high-throughput and comprehensive manner and to efficiently evaluate their function by transducing the TCRs into a murine T-cell line through retroviral infection. In conclusion, the modified retroviral vectors, in combination with the homologous recombination method, are powerful tools for the high-throughput cloning of cDNAs and their efficient functional analysis.

1. Introduction

A variety of gene cloning strategies have been established and utilized for the cloning of cDNAs to analyze the biological functions of their protein products. These cloning strategies can be divided into two major categories: the viral and non-viral methods [1,2]. In the viral methods of transducing cDNAs into mammalian cells, retroviral, adenoviral, adeno-associated viral and herpes simplex viral vectors are often used [3]. Retroviral vectors have an advantage in the transduction of cDNAs over the non-viral conventional methods, as they efficiently and stably integrate the cDNAs into the genome of the cells. The integrated cDNA is stably expressed, and the functions of its protein products can then be analyzed. A large repertoire of well-characterized-retroviral vectors has been developed during the last decade and used for the treatment of human diseases [4,5].

Ligation-dependent cloning is most commonly employed to introduce DNA fragments into vectors [6,7]. However, ligation-dependent cloning often requires multiple rounds of enzyme treatments and purification of both the inserts and vectors. Furthermore, the limited number of appropriate restriction enzyme sites for the insertion of the DNA into the vector DNA limits the flexibility of the vectors in constructing recombinant molecules. These processes hamper high-throughput cloning. In contrast, homologous recombination technologies enable the seamless insertion of any DNA fragment into any desired position [8,9]. Although the homologous recombination method offers several advantages for high-throughput cloning, it cannot be applied to vectors that contain homologous sequences within them, as homologous recombination preferentially occurs between those sequences, and the DNA of interest cannot be inserted [10]. Therefore, the homologous recombination method cannot be applied to retroviral vectors because they contain two homologous long terminal repeats (LTR).

In this study, we designed a modified retroviral vector into which PCR-amplified DNA fragments can be selectively and efficiently inserted using homologous recombination. We cloned TCR cDNA prepared from a large number of human single T cells into the retroviral vector using homologous recombination and analyzed the functions of these cDNAs in a high-throughput manner.

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