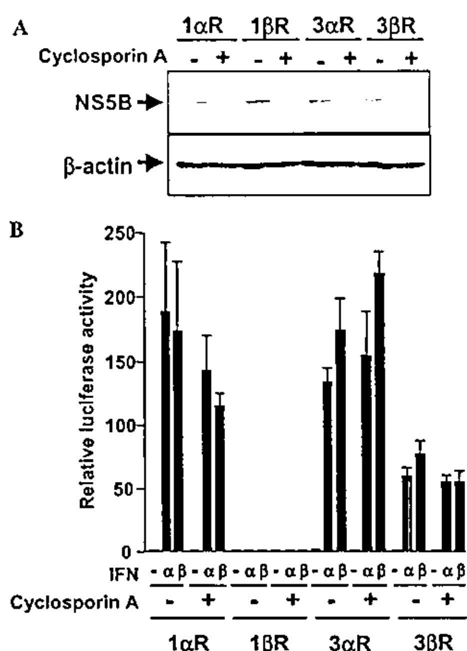

Fig. 8. Characterization of cured cells obtained from 1αR, 1βR, 3αR, and 3βR cells. (A) Western blot analysis. Regarding the four replicon cell lines possessing the IFN-resistant phenotype and their cured cell lines, NS5B was detected by immunoblot analysis using anti-NS5B antibodies. β-Actin was used as a control for the amount of protein loaded per lane. (B) Analysis for IFN signal transduction. Regarding the four replicon cell lines possessing the IFN-resistant phenotype and their cured cell lines, a dual luciferase reporter assay using pISRE(V2)-Luci [32] was performed as shown in Fig. 5.

IFN-resistant phenotype in at least the 1βR cells, although further experiments are needed to obtain conclusive results.

Discussion

In this study, we first established nine replicon cell lines possessing the IFN-resistant phenotype from 50-1 cells possessing an IFN-sensitive phenotype. Interestingly, we were able to divide these nine replicon cell lines into two types according to their IFN resistance. The first type included four cell lines of the αR series plus the αRmix cell line; treated with IFN-α alone, these lines showed a partially resistant phenotype against both IFN-α and IFN-β. The second type included four cell lines of the βR series treated with IFN-α and IFN-β; these lines showed a severely resistant phenotype against both IFN-α and IFN-β. Therefore, these findings suggest that these two IFN-resistant phenotypes were caused by different mechanisms. To clarify these mechanisms, it will be important to determine which viral and cellular factors contribute to the acquisition of IFN resistance of the replicons.

To identify such viral factors, genetic analysis found that all of these newly established replicons had one common amino acid substitution (Q1737H) in the NS4B, and several amino acid substitutions were found in the NS5A and NS5B of the βR series. Since these amino acid substitutions did not appear during the long-term culture (at least 12 months) of 50-1 cells, the genetic alterations observed in the replicons established in this study are considered to have appeared during the prolonged IFN treatment, and may induce the replicons' IFN resistance. NS4B possesses four fixed and one flexible transmembrane (TM) structures, located on the endoplasmic reticulum [37]. Since it has been proposed that the amino-terminal region of the first TM may play an important role not only for the topology of NS4B but also for the efficiency of HCV replication [37], the Q1737H substitution in this region may affect the function of NS4B and contribute to the acquisition of IFN resistance. However, even if this hypothesis were correct, additional factors would be necessary to acquire severe IFN resistance, because the IFN resistance of the αR series possessing the Q1737H mutation was weaker than that of the βR series possessing that mutation. Such factors that might be involved in the acquisition of severe IFN resistance are the additional cell-line-specific amino acid substitutions observed in NS5A and NS5B of the βR series. Although such cell-line-specific amino acid substitutions were not found in 5βR (Fig. 7), three clones of 5βR each possessed S1269Y, K1270R, and R1135K substitutions in NS3, which were not observed in any of the three clones of 5αR. Such amino acid substitutions may contribute to the acquisition of IFN resistance. To clarify these points, further analysis, such as the characterization of HCV replicon cells re-established by the transfection of these HCV replicon RNAs to Huh-7 cells, will be necessary.

To date, the IFN sensitivity-determining region (ISDR; amino acids 2209–2248 in the HCV-1b genotype), in which substitutions correlate well with IFN sensitivity in patients with CH, has been known as a good prediction factor for current IFN therapy [38,39]. Contrary to this phenomenon, all HCV replicons established thus far show high sensitivity to IFNs via unknown mechanisms. Nevertheless, most HCV replicons, including the 50-1 replicon, possess the IFN-resistant type of ISDR sequence, according to Enomoto's criteria. Interestingly, ISDR sequences of all HCV replicons except 4βR were barely altered, suggesting that unknown factors other than ISDR can regulate the IFN sensitivity in an HCV replicon system. Since it has been thought that NS5A blocks a signal of IFNs by interacting with PKR, a double-strand RNA-dependent protein kinase [40,41], amino acid substitutions in the NA5A protein found in the βR series may exert the function of PKR.

Although several genetic mutations were observed in the HCV replicons established in this study, the possibility is also considered that some cellular factors,

sequences were compared with those of the original 50-1 replicon and with 50-1 replicons after 6 and 12 months in cell culture.

Regarding the first 2.0 kb fragment of the replicon RNA, none of the common mutations were found among any of the replicons obtained from the cells possessing the IFN-resistant phenotype (data not shown). No αR-series-specific or βR-series-specific mutations were found either, although several sporadic mutations or deletions were observed in the nonfunctional region upstream of the encephalomyocarditis virus internal ribosome entry site or in the Neo^R region.

Contrary to the first 2 kb fragment of replicon RNA, in the NS region (6.1 kb) we found that at position 5552 (the nucleotide number in the HCV genotype 1b genome), uridine was commonly exchanged for adenosine among all replicons obtained from the cells possessing the IFN-resistant phenotype. This mutation results in the substitution of histidine (H) for glutamine (Q) at amino acid position 1737 in the NS4B (Q1737H in Fig. 7). Furthermore, several amino acid substitutions were found in NS5A (M2174V for 1βR; T2319A and N for 3βR; and T2242N and F2256L for 4βR) and NS5B (A2752V for 3βR) in the βR series only, although no other common amino acid substitutions were found in the βR series. The amino acid substitutions we found did not appear in long-term culture (to at least 12 months) of 50-1 cells. These results suggest that the amino acid substitutions found may contribute to the acquisition of the IFN-resistant phenotype. In addition, although four amino acid substitutions (P1115L, K1609E, V1896F, and E1966A) were observed in the αR series, αRmix, βR series, and 50-1 replicon after six months in cell culture, it is interesting to note that two additional amino acid substitutions (I1686V and L1701R) found in the 50-1 replicon after six months in cell culture were barely detected in the αR series, αRmix, or βR series (Fig. 7).

Characterization of cured cells obtained from HCV replicon cells possessing the IFN-resistant phenotype

To further examine whether or not IFN resistance depends on the presence of IFN-selected HCV replicon RNAs, we prepared cured cells from established HCV replicon cells by treatment with cyclosporin A, which was recently found to be a potent inhibitor of HCV replication [33]. 1αR and 3αR cells possessing a partially IFN-resistant phenotype, and 1βR and 3βR cells possessing a completely IFN-resistant phenotype, were treated with cyclosporin A as described under Materials and methods. As shown in Fig. 8A, we demonstrated by Western blot analysis that NS5B proteins were no longer detected in 1αR, 1βR, 3αR, or 3βR cells after eight days of cyclosporin A treatment. We further confirmed by RT-PCR [19] for the detection of 5′-UTR that replicon RNAs were excluded from the cells (data not shown). Using these cured cells and their parental cells, we examined whether or not the cured cells' IFN responses were altered after elimination of the replicon RNAs. The results of the luciferase reporter assay shown in Fig. 5 revealed that IFN responses were not remarkably changed in the cured cells (Fig. 8B). Although both IFN-α and IFN-β were still transduced in 1αR, 3αR, and 3βR cells, IFN response was not restored in the cured cells obtained from 1βR cells. This result suggests that some host factor(s) rather than replicon RNA(s) contributed to the

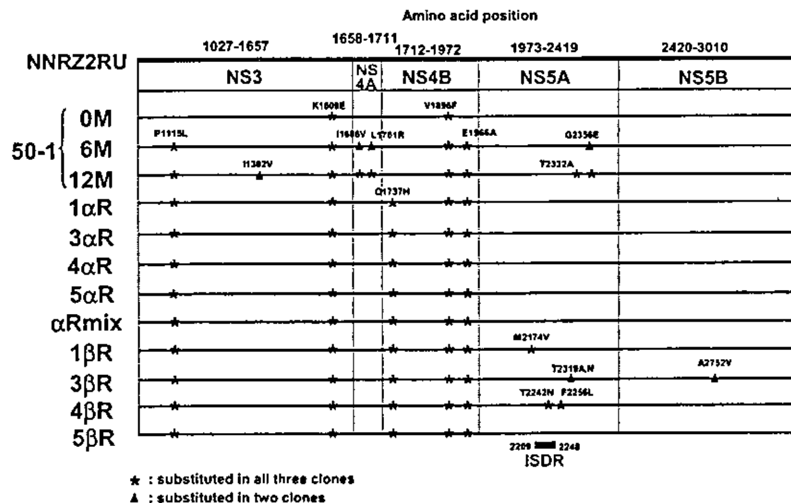

Fig. 7. Genetic analysis of the NS region of replicon RNAs in the established replicon cell lines possessing IFN-resistant phenotype. Compared with the amino acid sequences of NS region of the original replicon (NNRZ2RU), amino acid positions substituted in all thee clones and in two of three clones are indicated by asterisk and triangle, respectively. The results of genetic analysis of parental 50-1 replicon (0 M), and 50-1 replicons (6 and 12 M) after 6 and 12 months in culture are presented for comparison.

either alone or in combination with viral factors, contributed to the acquisition of IFN resistance. In an experiment to explore this possibility, we examined the IFN responses of cured cells from which replicon RNAs were eliminated by cyclosporin A. The obtained data suggested that some cellular factor(s) determined the IFN-resistant phenotype of at least the 1βR cells. It is considered that one reason why we have obtained HCV replicon cells that are deficient in IFN signaling (such as the 1βR cells) is their spontaneous appearance and their selection during prolonged IFN treatment. However, we are not able to exclude the possibility that persistent HCV replication induces some irreversible genetic mutations, which result in deficient IFN signaling, because it was recently reported that HCV replication induces a mutator phenotype that involves enhanced mutations of many somatic genes [43]. Therefore, it is important to evaluate these possibilities in future studies. Although the mechanism underlying the acquisition of IFN resistance is still ambiguous in the present study, our newly established HCV replicon cell lines possessing the IFN-resistant phenotype will be a very useful tool to further our understanding of molecular mechanisms for IFN resistance by HCV. Moreover, these replicon cells may be useful in the evaluation of combination therapies, such as IFN plus ribavirin.

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Promotion of Microsatellite Instability by Hepatitis C Virus Core Protein in Human Non-neoplastic Hepatocyte Cells

Atsushi Naganuma, Hiromichi Dansako, Takashi Nakamura, Akito Nozaki, and Nobuyuki Kato

Department of Molecular Biology, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan

ABSTRACT

Hepatitis C virus proteins exert an effect on a variety of cellular functions, including gene expression, signal transduction, and apoptosis, and because they possess oncogenic potentials, they have also been suggested to play an important role in hepatocarcinogenesis. Although the mechanisms of hepatocarcinogenesis remain poorly understood, we hypothesized that the disease may arise because of a disturbance of the DNA repair system by hepatitis C virus proteins. To test this hypothesis, we developed a reproducible microsatellite instability assay system for mismatch-repair using human-cultured cells transducted with pCXpur retrovirus expression vector, in which the puromycin resistance gene was rendered out-of-frame by insertion of a $(CA)_{17}$ dinucleotide repeat tract immediately following the ATG start codon. Using several human cancer cell lines known to be replication error positive or negative, we demonstrated that this assay system was useful for monitoring the propensity for mismatch-repair in the cells. This assay system was applicable to non-neoplastic human PH5CH8 hepatocytes, which could support hepatitis C virus replication. Using PH5CH8 cells, in which hepatitis C virus proteins were stably expressed by the retrovirus-mediated gene transfer, we found that the core protein promoted microsatellite instability in PH5CH8 cells. Interestingly, such promotion by the core protein only occurred in cells having the core protein belonging to genotype 1b or 2a and did not occur in cells having the core protein belonging to genotype 1a, 2b, or 3a. This is the first report to demonstrate that the core protein may disturb the DNA repair system.

INTRODUCTION

Hepatitis C virus (HCV), discovered in 1989, is the major causative agent of parenteral non-A, non-B hepatitis worldwide (1). Following the development of a method of diagnosing HCV infection (2), it became apparent that HCV infection frequently causes chronic hepatitis, and the persistent infection with HCV is implicated in liver cirrhosis and hepatocellular carcinoma (HCC; 1–4). HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the family *Flaviviridae* (5, 6). The HCV genome shows remarkable genetic heterogeneity and at least six major HCV genotypes, further grouped into >50 subtypes, have been identified to date (7, 8). The HCV genome encodes a large polyprotein precursor of approximately 3,000 amino acid (aa) residues, and this precursor protein is cleaved by the host and viral proteinases to generate at least 10 proteins in the following order: NH_2-core-envelope 1 (E1); E2; p7; nonstructural protein 2 (NS2); NS3; NS4A; NS4B; NS5A; NS5B; and COOH (9–11). These HCV proteins not only play a role in viral replication but also affect a variety of cellular functions, including gene expression, signal transduction, and apoptosis (12, 13).

HCV replication and the viral protein expression have been observed in HCCs, but the molecular mechanism of HCV-associated hepatocarcinogenesis remains poorly understood. One major reason for this is the lack of reproducible and efficient HCV proliferation in cell culture (14). In HCV-related hepatocarcinogenesis, it has been speculated that repeated hepatocytic regeneration processes also occur in HCV-infected individuals to offset the damage caused by HCV multiplication and maintain sufficient liver function. Such a process of damage and regeneration probably enhances the likelihood of genetic alteration (15). In addition, it has also been reported that no significant differences were found in the number and type of chromosomal imbalances between hepatitis B virus- and HCV-infected HCCs (16). This finding is consistent with models suggesting that hepatitis B virus and HCV cause cancer through nonspecific inflammatory and regenerative processes (17). On the other hand, it has been demonstrated that HCV proteins significantly influence a variety of oncogenic processes. For example, the HCV core protein may cooperate with H-ras in the process of transforming the cells into malignant phenotypes (18), and the constitutive expression of core protein in transgenic mice has been shown to induce HCC (19). Furthermore, it has been reported that the HCV NS3, NS4B, and NS5A proteins also have oncogenic potential (20–22). Therefore, it is likely that HCV proteins contribute to the initiation or development of HCC.

We reported previously that PH5CH8 cells cloned from PH5CH cell line (23) could support HCV replication (24), although the level of HCV proliferation was fairly low. PH5CH cell line was established by immortalization with SV40 large T antigen using non-neoplastic liver tissue from a patient with HCV-related HCC (23). PH5CH8 cells are considered to be useful in examining the role of HCV proteins during the process of hepatocarcinogenesis. In addition, PH5CH8 cells possess wild type of p53 and Rb protein and show nonmalignant phenotype (23), although SV40 large T antigen would partially repress the function of p53. Then, we speculated that the DNA repair system of host cells may be one of the target sites of HCV proteins, because the constant operation of this system is crucial to the process of inflammation and regeneration of hepatic lesions in patients with chronic hepatitis C. Although DNA damages caused by such damaging agents as X-rays, UV light, and alkylating agents are repaired by base excision, nucleotide excision, recombinational repair, and so forth, the mismatch-repair (MMR) system is used to repair A-G or T-C mismatches, insertion, and deletion caused by the replication errors (RER) during the regenerative process (25). In addition, studies on genetic instability using clinical specimens from patients with HCC have revealed that microsatellite instability (MSI) was found in approximately 20% of the patients examined (26, 27), whereas no MSI was found in the histologically normal liver (26). In this study, we focused on the MMR system to examine the effects of HCV proteins. For this purpose, we developed a novel MSI assay system in human cultured cells using the retrovirus expression vector containing the (CA) repeat sequence. Our results indicate that the core protein may promote MSI in PH5CH8 cells.

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Notes: A. Naganuma and H. Dansako contributed equally to this work. The present address for A. Naganuma is First Department of Internal Medicine, Gunma University School of Medicine, Maebashi 371-8511, Japan.

Requests for reprints: Nobuyuki Kato, Department of Molecular Biology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. Phone: 81-86-235-7385; Fax: 81-86-235-7392; E-mail: nkato@md.okayama-u.ac.jp.

MATERIALS AND METHODS

Cell Lines. The human colon cancer cell lines LS174T, LoVo, and SW480 were provided by the Cell Resource Center of the Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University). LS174T, LoVo, and SW480 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), F12 supplemented with 20% FBS, and RPMI 1640 supplemented with 10% FBS, respectively. The additional human colon cancer cell line HCT116 was a kind gift of K. Shimizu (Okayama University) and was maintained in DMEM supplemented with 10% FBS. Cells of the human cervical carcinoma cell line HeLa were maintained in DMEM supplemented with 10% FBS. PH5CH8 hepatocytes were maintained as described previously (23). Bosc 23 cells were also maintained in DMEM supplemented with 10% FBS.

Construction of Expression Vectors. pCX retroviral vector pCXpur (28), a kind gift of T. Akagi (Osaka Bioscience Institute), which contains the resistance gene for puromycin, was used for the construction of pCXpur containing 17 CA repeats [pCXpur/$(CA)_{17}$/out-of-frame and pCXpur/$(CA)_{17}$/in-frame]. pCXpur/$(CA)_{17}$/out-of-frame was generated by inserting a fragment that was ligated with *Sal*I sites of the PCR products of two primer sets (primers PU-NotI5′ and FCA3′; primers TA5′ and PU-StuI3′; Table 1) using pCXpur as a template, into the *Not*I-*Stu*I sites of pCXpur. A schematic of the construction of the pCXpur/$(CA)_{17}$/out-of-frame retrovirus vector is shown in Fig. 1. pCXpur/$(CA)_{17}$/in-frame was also generated by inserting a fragment that was ligated with *Sal*I sites of the PCR products of two primer sets (primers PU-NotI5′ and FCA3′; primers PU-5′ and PU-StuI3′; Table 1) using pCXpur as a template, into the *Not*I-*Stu*I sites of pCXpur. The nucleotide sequences of the obtained pCXpur/$(CA)_{17}$/out-of-frame and pCXpur/$(CA)_{17}$/in-frame retrovirus vector were confirmed by Big Dye termination cycle sequencing using an Applied Biosystem 310 automated sequencer (Applied Biosystems, Norwalk, CT).

Retrovirus Infection of the Recipient Cell Lines. Transient transfections were performed with 2 μg of DNA and 6 μl of FuGENE6 (Boehringer Mannheim, Mannheim, Germany) in 6-well plates according to the manufacturer's instructions. Retrovirus infection was performed by a method described previously (29, 30). At 3 h postinfection, the cells were cultured with fresh medium until reaching confluence (approximately 3 days), and then the cells were used for the selection with puromycin (5 μg/ml) or blasticidin (20 μg/ml).

MSI Assay Using Human Cultured Cells. Various human-cultured cells (2×10^5 cells) were plated on a 6-well plate for 24 h before retrovirus infection and were infected with the retrovirus pCXpur/$(CA)_{17}$/out-of-frame or pCXpur/$(CA)_{17}$/in-frame. Mock infection was also performed as a control. At 3 days postinfection, cells were passaged to a 10-cm dish and further cultured for 2 days, and then aliquots of 1×10^5 cells were placed in 10-cm dishes for selection by puromycin (5 or 10 μg/ml) and grown for 14 days with a medium change every 3–4 days. In some experiments, the culture period from retrovirus infection to addition of puromycin was changed to 9–21 days from 5 days. The obtained puromycin-resistant (pur^R) colonies were stained with Coomassie Brilliant Blue (0.6g/liter in 50% methanol-10% acetic acid) and were automatically counted using a ChemiImager 4000 (α Innotech Corp.). Triplicate infection experiments were repeated at least three times to verify the reproducibility of the results. To examine the efficiency of retrovirus infection, the retrovirus pCLMFG-LacZ (31) was used, and at 2 days postinfection, the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Preparation of PH5CH8 Cells Stably Expressing HCV Proteins. PH5CH8 cells were infected with retrovirus pCXbsr encoding various HCV proteins, as described above. Because the HCV core protein constructs, pCXbsr/core(1b-M) (32), pCXbsr/core(1b-P) (32), pCXbsr/core(1b-HCC) (33), pCXbsr/core(1a) (30), pCXbsr/core(2a) (30), pCXbsr/core(2b) (30), and pCXbsr/core(3a) (30), which are able to express core(1b-M), core(1b-P), core(1b-HCC), core(1a), core(2a), core(2b), and core(3a) proteins, respectively (the numbers and letters in parentheses indicate the HCV genotype), were used to obtain the PH5CH8 cells stably expressing the core protein. Because the other HCV protein constructs, pCXbsr/E1(1b-P), pCXbsr/E2(1b-P), and pCXbsr/NS5A(1b-P), which are able to express E1(1b-P), E2(1b-P), and NS5A(1b-P) proteins, respectively, were also used to obtain the PH5CH8 cells stably expressing E1(1b-P), E2(1b-P), and NS5A(1b-P) proteins, respectively. pCXbsr was used as a control. At 2 days postinfection, the PH5CH8 cells were changed with fresh medium containing blasticidin (20 μg/ml), and the culture was continued for 7 days to select the cells expressing HCV proteins.

MSI Assay Using PH5CH8 Cells Stably Expressing HCV Proteins. The PH5CH8 cells (2×10^5 cells) stably expressing HCV protein [core, E1, E2, or NS5A] were used for the MSI assay as described above. The PH5CH8 cells, which became resistant to blasticidin after infection of the retrovirus pCXbsr, were used as a control. These cells were further infected with the retrovirus pCXpur/$(CA)_{17}$/out-of-frame, and then an MSI assay was performed according to the method described above, with the exception that double-selections were performed using both blasticidin (20 μg/ml) and puromycin (5 or 10 μg/ml).

Sequence Analysis of the (CA) Repeat-Surrounding Region Recovered from pur^R Colonies. The pur^R colonies (7–10 independent colonies) obtained in the MSI assay were randomly selected and grown to confluence in 6-well plates, and their genomic DNAs were prepared using an ISOGEN extraction kit (Nippon Gene Co., Toyama, Japan) as indicated by the manufacturer. These DNAs (each 1 μg) were used for the amplification of the (CA) repeat-surrounding region by PCR using a primer set (primers MIB5′ and MIE3′; Table 1). Thirty-five PCR cycles were performed, and each cycle consisted of annealing at 63°C for 45 s, primer extension at 68°C for 1 min, and denatur-

Table 1 *Oligonucleotides used in this study*

Oligonucleotide	Sequence	Direction
PU-NotI5′	aattaaGCGGCCGCGGTAACAATTGTTAACTAAC	Forward
FCA3′	attatGTCGACTGTGTGTGTGTGTGTGTGTGTGTGTGTG GTGGTCATGGTATTATCGTGTTTTTCAAAGGAAAACC ACGTCCCGTGGTTCGGGGGGCCTAGAC	Reverse
TA5′	attatGTCGACTAACCGAGTACAAGCCCACGGTG	Forward
PU-StuI3′	attatAGGCCTTCCATCTGTTGCTGCGCGGCCAG	Reverse
PU-5′	attatGTCGACACCGAGTACAAGCCCCACGGTG	Forward
MIB5′	attatGGATCCGCACATGCTTTACATGTGTTTAGTCG	Forward
MIE3′	attatGAATTCAGGCGCACCGTGGGCTTGTACTC	Reverse
hMLH1F	TTCTCAGGTTATCGGAGCCAGCAC	Forward
hMLH1R	CTTCGTCCCAATTCACCTCAGTGG	Reverse
hMSH2F	TACATGTCACAGCACTCACCACTG	Forward
hMSH2R	CTCCTCAAGTTCCAGGGCTTTCTG	Reverse
hMSH6F	ACAAGGGGCTGGGTTAGCAAAAG	Forward
hMSH6R	AGTCACCATTCTCTTCCGCTTTCG	Reverse
hPMS2F	CAGGTTTCATTTCACAATGCACGC	Forward
hPMS2R	TTACCTTCAACATCCAGCAGTGGC	Reverse
hMSH3F	GATGGCATTTTCACAAGGATGGG	Forward
hMSH3R	CTGGCGGATAATGGGTGACAAAC	Reverse
hPMS1F	GGCAACAGTTCGACTCCTTTCAAG	Forward
hPMS1R	TTGATACCCTCCCCGTTATCTCGC	Reverse
GAPDHF	GATGCTGGCGCTGAGTACGTCG	Forward
GAPDHR	GAGGAGACCACCTGGTGCTCAG	Reverse

The recognition sites of restriction enzymes are underlined.

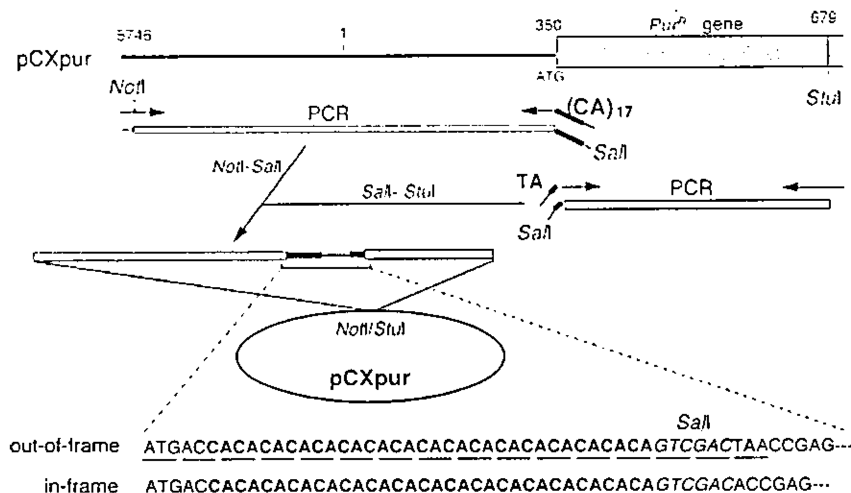

Fig. 1. Construction of pCXpur retrovirus vector containing the (CA) repeat sequence. The outline for the construction of the pCXbsr/$(CA)_{17}$/out-of-frame retrovirus vector is presented schematically. The nucleotide sequences of the (CA) repeat unit of pCXbsr/$(CA)_{17}$/out-of-frame and pCXbsr/$(CA)_{17}$/in-frame are shown (*bottom*), and each codon is *underlined*. pur^R, puromycin-resistant.

ation at 97°C for 45 s using proofreading KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). PCR products (186 bp) containing the (CA) repeat sequence were cloned into the *Bam*HI and *Eco*RI sites of pCRII-TOPO (Invitrogen, Carlsbad, CA). Plasmid inserts were sequenced in both the sense and antisense.

Western Blot Analysis. The preparation of cell lysates, SDS-PAGE, and immunoblotting analysis with a polyvinylidene difluoride membrane were performed as described previously (10). Anti-core monoclonal antibody (2ZCP9: Institute of Immunology Co., Tokyo, Japan), anti-E1 monoclonal antibody (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science), anti-E2 monoclonal antibody (34), and anti-NS5A antibody (a generous gift from A. Takamizawa, Osaka University) were used for the detection of core, E1, E2, and NS5A proteins, respectively. Anti-β-actin antibody (AC-15; Sigma) was also used for the detection of β-actin as an internal control. Immunocomplexes on the filters were detected by enhanced chemiluminescence assay (Renaissance; NEN, Boston, MA).

Reverse Transcription (RT)-PCR. Total cellular RNA was extracted using an ISOGEN extraction kit for the RT-PCR analysis. RT-PCR was performed by a method described previously (30). The sequences of hMLH1 (accession number U07418), hMSH2 (accession number U03911), hMSH6 (accession number U54777), hPMS1 (accession number U13695), hPMS2 (accession number U14658), hMSH3 (accession number U61981), and glyceraldehyde-3-phosphate dehydrogenase (accession number NM 002046) were used to design the primers listed in Table 1. Twenty-five cycles of PCR (20 cycles for glyceraldehyde-3-phosphate dehydrogenase only) were performed, and the amplified DNA was detected by staining with ethidium bromide after separation by 3% agarose gel electrophoresis.

RESULTS

Construction of the Retrovirus Vectors Containing the Microsatellite (CA) Repeat Sequence. The retrovirus expression vector pCXpur (28) contains a pur^R gene to select for transduced cells. Initially, we made a pCXpur/$(CA)_{17}$/in-frame, in which 42 nucleotides [AC +17 CA repeats + GTCGAC (*Sal*I site)] were inserted immediately following the ATG initiation codon of the pur^R gene, and examined the influence of this insert on the pur^R activity. We confirmed that the human colon cancer SW480 cells (35), which were known to possess RER− (MMR proficient) phenotype, infected with the retrovirus pCXpur/$(CA)_{17}$/in-frame were able to proliferate in the presence of puromycin (10 μg/ml; data not shown), indicating that the pur^R gene product from pCXpur/$(CA)_{17}$/in-frame is functional in the cells. We next constructed pCXpur/$(CA)_{17}$/out-of-frame, in which the pur^R gene was rendered out-of-frame by the insertion of 44 nucleotides [AC +17 CA repeats + GTCGAC (*Sal*I site) + TA (to make a TAA stop codon)] immediately following the ATG initiation codon, as shown in Fig. 1. By this modification, the pur^R gene product should not be produced from pCXpur/$(CA)_{17}$/out-of-frame. Using the SW480 cells (RER−), we confirmed that cells infected with the retrovirus pCXpur/$(CA)_{17}$/out-of-frame were also unable to survive in the presence of puromycin (10 μg/ml; data not shown), indicating that the pur^R gene product is not produced from pCXpur/$(CA)_{17}$/out-of-frame, as we expected (Fig. 1). With regard to the plasmid vector for MSI assay at the cell-culture level, to date, several similar vector systems using the neomycin resistance gene, hygromycin B phosphotransferase gene, or β-galactosidase gene have been reported (36–39), but there has been no system using the pur^R gene. Puromycin has an advantage for the fast (within a few days) and keen-edged selection of the cells. In the present study, none of the cells lines examined were able to survive in the presence of 1 μg/ml of puromycin.

Establishment of the MSI Assay System. In this assay, after the transduction of pCXpur/$(CA)_{17}$/out-of-frame [pCXpur/$(CA)_{17}$/in-frame as a positive control], the recipient cells were cultured for 5 days, and then the cells were selected with puromycin (5 or 10 μg/ml). In theory, although the cells transducted with pCXpur/$(CA)_{17}$/in-frame are able to proliferate in the presence of puromycin, the cells transducted with pCXpur/$(CA)_{17}$/out-of-frame should not be able to survive in the presence of puromycin, as we confirmed in RER− cells. However, if some frameshift mutations do occur in the vicinity of the $(CA)_{17}$ sequence during the 5 days of culture before addition of puromycin, such cells would become pur^R cells and grow up even in the presence of puromycin. As a consequence, we therefore considered the colonies to be pur^R colonies at about 2 weeks after addition of puromycin. Because the microsatellite insert puts the pur^R gene in the −1 reading frame, detectable dinucleotide frameshift mutations include the deletions of 2, 8, 14, 20, 26, or 32 bp and insertions of 4, 10, or 16 bp, and so forth. As the method of gene transduction, we used retrovirus infection because of its highly efficient gene transfer into cells. Recently, Zienolddiny *et al.*(38) also used a retrovirus infection system for MSI assay.

We initially verified our method using several human cell lines. It has been reported that HCT116 and LoVo cells exhibited marked dinucleotide repeat instability, because HCT116 cells possessed a nonsense mutation in exon 9 in *hMLH1* gene, and LoVo cells were *hMSH2*-deficient (deletion of exons 4–8; 40). LS174T cells have been also reported to possess RER+ (MMR deficient) phenotype by the analysis of 32 microsatellite loci (41). On the other hand, HeLa and SW480 cells are known to possess RER− phenotype because of accurately replication of repetitive DNA and correction of mismatches

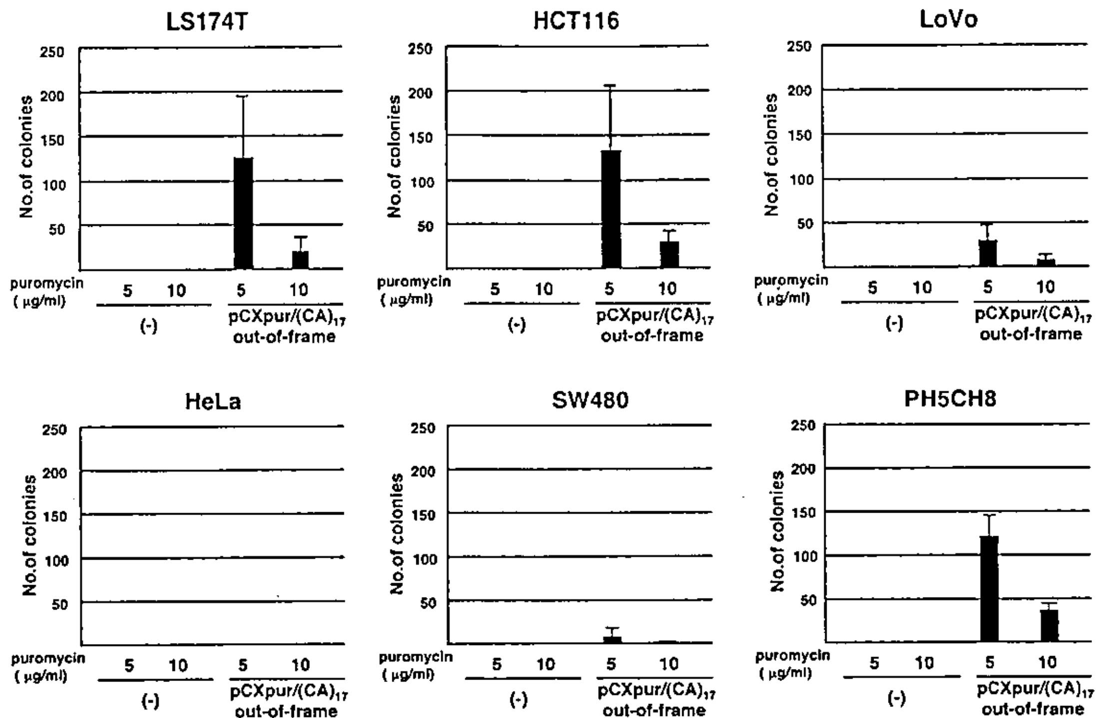

Fig. 2. Microsatellite instability assay using pCXbsr/(CA)$_{17}$/out-of-frame in various cell lines. The puromycin-resistant colonies stained with Coomassie Brilliant Blue were automatically counted by a ChemiImager 4000. *(-)*, mock infection.

(35). Therefore, HCT116, LoVo and LS174T were used as the RER+ cell lines, and HeLa and SW480 were used as the RER− cell lines. PH5CH8 cells were also used for the analysis using our method, although the state of MMR system has not yet been determined by the analysis of microsatellite loci.

All cell lines examined at 2 days postinfection with the retrovirus pCLMFG-LacZ were efficiently stained with 5-bromo-4-chloro-3-indolyl-b-D-galactropyranoside, although the level of staining and the percentage of stained cells in the LoVo and HeLa cell lines were somewhat lower than in the other cell lines (data not shown). At 2 weeks after the selection with puromycin, pur^R colonies were counted after staining with Coomassie Brilliant Blue. As shown in Fig. 2, a substantial number of colonies were obtained in the RER+ cell lines (LS174T, HCT116, and LoVo), whereas no or only a few colonies were obtained in RER− cell lines (HeLa and SW480). Because the growth rate of LoVo cells was rather lower than those of LS174T and HCT116 cells, it might cause the low number of pur^R colonies in LoVo cell line despite RER+ phenotype. In all cases, the number of pur^R colonies obtained in the presence of puromycin (10 μg/ml) was lower than that obtained in the presence of puromycin (5 μg/ml), suggesting that the colonies expressing *pur^R* gene at low level were not able to survive in the presence of puromycin (10 μg/ml). This phenomenon may be explained by the reason that the expression level of *pur^R* gene depends on the integration site of the retrovirus. All cell lines infected with the retrovirus pCXpur/(CA)$_{17}$/in-frame became fully confluent up to 2 weeks after the selection with puromycin (data not shown), and no colonies were obtained from any of the mock-infected cell lines (Fig. 2). These results revealed that the number of pur^R colonies obtained indicated a good correlation with the RER phenotypes. Interestingly, however, nonmalignant PH5CH8 cells showed the RER+ phenotype, because the number of pur^R colonies obtained in PH5CH8 cells was similar to that obtained in LS174T and HCT 116 cells showing the RER+ phenotype. In addition, the modification of the culture period (from 5 days to 14 or 21 days) before addition of puromycin in the MSI assay using LS174T cells revealed that the number of pur^R colonies increased in a time-dependent manner at both of two different concentrations (5 and 10 μg/ml) of puromycin (data not shown).

Sequence Analysis of the Integrated (CA) Repeat Unit in the pur^R Colonies. To further evaluate the reliability of our method, 7–10 independent pur^R colonies derived from LS174T, HCT116, and PH5CH8 cells were isolated and expanded. Using the pCXpur/(CA)$_{17}$/out-of-frame vector DNA, we initially confirmed that KOD-plus DNA polymerase was superior to nonproofreading *Taq*DNA polymerases, as described previously (33), because 3 of 10 clones obtained by *Taq*DNA polymerases showed deletions of 1–3 nucleotides, whereas all 10 clones obtained by KOD-plus DNA polymerase showed the exact (CA)$_{17}$ sequence. Therefore, using the genomic DNA from each colony, a fragment of 186 bp containing the CA repeat unit was amplified by proofreading KOD-plus DNA polymerase and was cloned into pCRII-TOPO for sequencing analysis. In most cases, four-independent clones were obtained from each pur^R colony and sequenced. Table 2 provides a summary of all of the sequenced clones. As can be seen, at least one clone, which became in-frame by the deletion of 2 bp (CA) from (CA)$_{17}$, was obtained from all pur^R colonies examined. In addition to (CA)$_{16}$, (CA)$_{13}$ resulting in in-frame was obtained from one colony in LS174T cells, and (CA)$_{19}$, (CA)$_{10}$, and (CA)$_7$ resulting in in-frame were obtained from four colonies in HCT116 cells. One interesting additional sequence, (CA)$_{17}$A, which resulted in in-frame was also obtained from one colony in HCT116 cells. Although all of the clones obtained from HCT116-derived colonies showed the expected pattern of frameshift mutation resulting in in-frame, a single clone possessing the original (CA)$_{17}$ without mutation was also obtained from 4 LS174T-derived colonies. Because each of the remaining three clones from these four colonies possessed (CA)$_{16}$ resulting in in-frame, it is suggested that more than two copies including the retrovirus possessing (CA)$_{17}$ sequence were infected and integrated in a single target cell. Com-

Table 2 *Sequence analysis of (CA) repeat region obtained from the* pur^R *colonies*

The numbers in the table indicate the actual number of plasmid clones obtained and sequenced.

PCR product		Colony no. 1	2	3	4	5	6	7	8	9	10
LS174T (resistant to puromycin 10 μg/ml)											
$(CA)_{17}$	out-of-frame	1	1			1					1
$(CA)_{16}$	in-frame	3	3	4	4	3	4	3	4	4	3
$(CA)_{13}$	in-frame							1			
HCT116 (resistant to puromycin 10 μg/ml)											
$(CA)_{19}$	in-frame				3						
$(CA)_{17}A$	in-frame					3					
$(CA)_{16}$	in-frame	3	4	3	1	1	4	2			
$(CA)_{10}$	in-frame							2			
$(CA)_{7}$	in-frame	1		1							
PH5CH8 (resistant to puromycin 10 μg/ml)											
$(CA)_{17}$	out-of-frame							1	1	1	
$(CA)_{16}$	in-frame	4	4	3	2	2	4	3	2	1	2
$(CA)_{15}$	out-of-frame								1		
$(CA)_{14}$	out-of-frame									2	
$(CA)_{13}$	in-frame			1	2						
$(CA)_{9}$+CC	in-frame										2
$(CA)_{7}$	in-frame					2					

pared with the results from LS174T and HCT116 cells, PH5CH8-derived colonies showed a variety of mutation patterns. Although the $(CA)_{16}$ sequence was obtained from all colonies, $(CA)_{13}$ and $(CA)_7$ resulting in in-frame were obtained from two colonies and one colony, respectively, and $(CA)_9$CC resulting in in-frame was also obtained from one additional colony. In addition, $(CA)_{15}$ and $(CA)_{14}$ resulting in out-of-frame were obtained from a single colony, respectively, and the original $(CA)_{17}$ without mutation was also obtained from the three colonies. These results suggest that at least three copies of retrovirus were initially infected and integrated in a single target cell. In summary, sequence data on the (CA) repeat region indicated that the pur^R colonies possessed the frameshift mutation (2-bp deletion) resulting in in-frame in the open reading frame of pur^R gene. Taken together with these results, we concluded that our method can be used as an MSI assay at the cell-culture level.

HCV Core Protein Promoted MSI in PH5CH8 Cells. Because PH5CH8 cells did not show any tumorigenic potential when inoculated s.c. into thymic nude mice (23), we were surprised by the result that PH5CH8 cells showed the RER+ phenotype, as did the human colon cancer cell lines. Although the mechanism responsible for this finding is unclear, we speculate that HCV proteins may have further promoted MSI in PH5CH8 cells. Therefore, to evaluate this possibility, we initially prepared PH5CH8 cells stably expressing HCV protein [core(1b-P), E1(1b-P), E2(1b-P), or NS5A(1b-P)] as the recipient cells for the pCXpur/$(CA)_{17}$/out-of-frame retrovirus infection, by the pCXbsr/core(1b-P), pCXbsr/E1(1b-P), pCXbsr/E2(1b-P), or pCXbsr/NS5A(1b-P) retrovirus infection and following selection with blasticidin. As control recipient cells, we prepared PH5CH8 cells infected with retrovirus pCXbsr and selected with blasticidin. After retrovirus infection and following selection with blasticidin for 7 days, we monitored the growth curve of these blasticidin-resistant PH5CH8 cells, and we observed that the growth rates of these cells were almost the same (data not shown). We also confirmed by Western blot analysis the stable expression of core(1b-P), NS5A(1b-P), E1(1b-P), and E2(1b-P) proteins in PH5CH8 cells at day 10 and day 19 post-infection with retrovirus pCXbsr encoding HCV proteins (data not shown). Using these PH5CH8 cells, we performed an MSI assay, and found that the number of pur^R colonies obtained from the cells expressing the core(1b-P) protein was approximately 1.5-fold (selection with 5 μg/ml of puromycin) and approximately 2.5-fold (selection with 10 μg/ml of puromycin) higher than that from the control cells, as shown in Fig. 3. As compared with the core(1b-P) protein, the E1(1b-P), E2(1b-P), and NS5A(1b-P) proteins did not increase the number of pur^R colonies, although NS5A(1b-P) protein slightly decreased the number of pur^R colonies. Because the increase of pur^R colonies in PH5CH8 cells expressing the core(1b-P) protein was reproducibly observed, it was suggested that core(1b-P) protein was able to further promote the MSI in PH5CH8 cells.

Promotion of MSI by the Core Protein Depends on HCV Genotype or Strain. Because the core protein is known to show some aa sequence heterogeneity among HCV genotypes (7, 8), we examined whether or not HCV core proteins other than the core(1b-P) protein are able to promote the MSI, using pCXbsr/core(1a), pCXbsr/core(2a), pCXbsr/core(2b), and pCXbsr/core(3a) retrovirus vectors

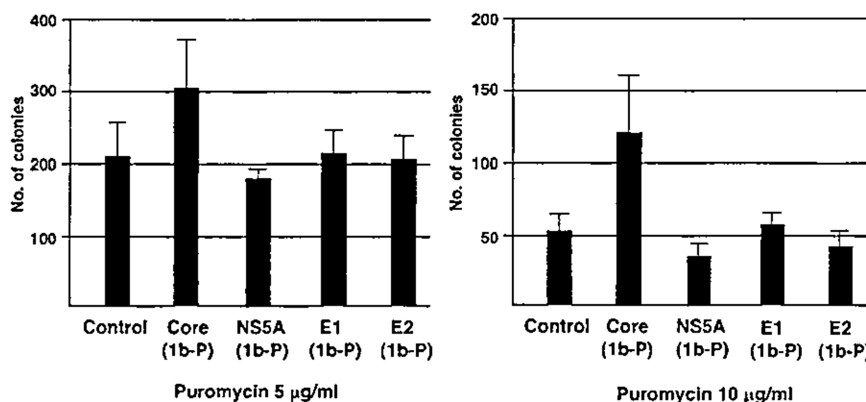

Fig. 3. Hepatitis C virus core protein promoted microsatellite instability in PH5CH8 cells. Microsatellite instability assay using pCXbsr/$(CA)_{17}$/out-of-frame was carried out in PH5CH8 cells stably expressing core(1b-P), NS5A(1b-P), E1(1b-P), or E2(1b-P) protein. The culture period from retrovirus infection to addition of puromycin was 9 days. The puromycin-resistant colonies were counted by the method described in Fig. 2. *Control*, PH5CH8 cells infected with retrovirus pCXbsr.

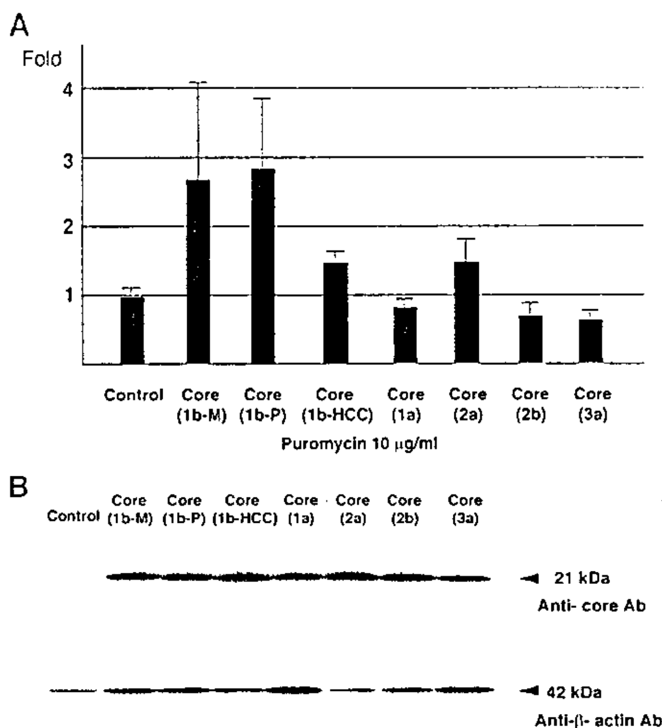

Fig. 4. *A*, promotion of microsatellite instability by the core protein depends on hepatitis C virus genotype. Microsatellite instability assay using pCXbsr/$(CA)_{17}$/out-of-frame was carried out in PH5CH8 cells stably expressing the core protein derived from various hepatitis C virus genotypes. The culture period from retrovirus infection to addition of puromycin was 9 days. The puromycin-resistant colonies were counted by the method described in Fig. 2. *Control*, PH5CH8 cells infected with retrovirus pCXbsr. *B*, stable expression of the core protein in PH5CH8 cells. PH5CH8 cells were infected with retrovirus pCXbsr encoding the core protein belonging to various genotypes, and at 19 days postinfection, the lysate of cells was used for the detection of core protein and β-actin by Western blot analysis. *Control*, PH5CH8 cells infected with retrovirus pCXbsr.

encoding the core(1a), core(2a), core(2b), and core(3a) protein, respectively. In addition, pCXbsr/core(1b-HCC) was also used as a retrovirus vector encoding the core(1b-HCC) protein, which was derived from a cancerous HCC lesion. The pCXbsr/core(1b-M) retrovirus vector (32) encoding core(1b-M) protein, which possessed the consensus sequence of genotype 1b, was also used for the MSI assay. The core(1b-P), core(1b-HCC), core (1a), core(2a), core(2b), and core(3a) proteins differed by 1, 6, 3, 14, 22, and 17 aa from the core(1b-M) protein, respectively (30). Using these retrovirus vectors, including pCXbsr as a control vector, we initially prepared PH5CH8 cells stably expressing the core(1b-M), core(1b-P), core(1b-HCC), core(1a), core(2a), core(2b), and core(3a) proteins, respectively. We performed an MSI assay using these core protein-expressing cells. As shown in Fig. 4*A*, the results revealed that the number of pur^R colonies obtained from the cells expressing the core(1b-M), core(1b-P), core(1b-HCC), or core(2a) protein was 1.5- to 2.8-fold higher than that obtained in the control, whereas the number of pur^R colonies obtained from the cells expressing the core(1a), core(2b), or core(3a) protein was similar to that obtained in the control. Western blot analysis confirmed that these core proteins were stably and equally expressed in PH5CH8 cells at day 19 postinfection with the retrovirus pCXbsr expressing the core protein (Fig. 4*B*). These results suggest that the effectiveness of the core protein in promoting MSI, that is, in down-regulating MMR, is dependent on the HCV genotype or strain.

Expression Level of MMR-Related Genes in PH5CH8 Cells Expressing the Core Protein. To investigate the possibility that the core protein represses the expression of genes functioning in MMR, we examined the effect of the core protein on the expression level of MMR-related genes, including *hMLH1* and *hMSH2*, the frequent genetic mutations of which have been observed in the hereditary nonpolyposis colorectal cancer and a variety of sporadic cancers (25). As shown in Fig. 5, we were not able to find any significant differences in the expression level of *hMLH1*, *hMSH2*, *hMSH6*, *hPMS2*, *hMSH3*, and *hPMS1* genes between PH5CH8 cells expressing the core(1b-P) or NS5A(1b-P) protein, and PH5CH8 cells infected with retrovirus pCXbsr. This result suggests that the down-regulation of MMR by the core protein occurs by an as yet unknown mechanism other than the repression of MMR-related genes.

DISCUSSION

In this study, we first demonstrated that HCV core proteins were able to further repress the down-regulation of MMR activity in cultured human non-neoplastic hepatocytes, by a newly developed MSI assay system using a microsatellite sequence consisting of $(CA)_{17}$.

Regarding the MSI assay system developed in this study, we used retrovirus infection as a method for transduction of a microsatellite (CA) repeat sequence to the cells. However, it remains possible that the RER of pCXpur/$(CA)_{17}$/out-of-frame occurs in the packaging of Bosc23 cells and results in the production of the retrovirus possessing the (CA) repeat sequence altered in-frame. Although we cannot absolutely exclude this possibility, it is unlikely that such an event occurs in Bosc23 cells, because we observed a good correlation between the RER+ and RER− phenotypes of the examined cell lines with respect to the number of pur^R colonies obtained. In addition, we observed that the number of pur^R colonies increased in a culture-time-dependent manner. Therefore, the MSI assay developed in this study will be a useful method at the cell culture level.

The fact that non-neoplastic PH5CH8 cells showed remarkable RER+ phenotype was an unexpected result. Although the PH5CH8 cell line was cloned from PH5CH cells as an HCV-susceptible clone (24), we observed that not only the PH5CH8 cells but also the parental PH5CH cells showed the RER+ phenotype (data not shown). PH5CH cells were established from the non-neoplastic liver as a SV40 large T antigen-immortalized cell line and express hepatocyte characteristics (23). Therefore, the activity of p53 and pRb, two tumor suppressor

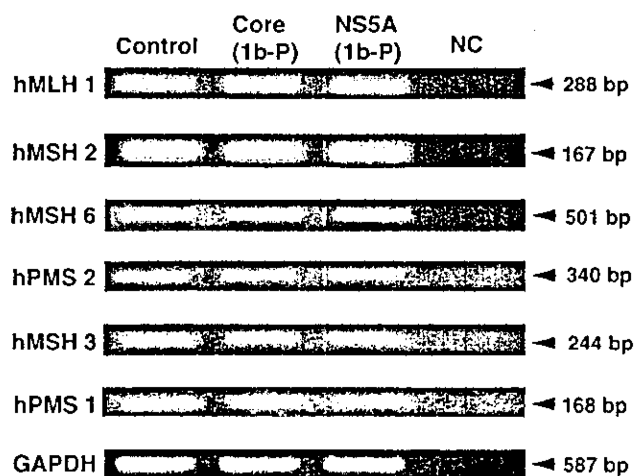

Fig. 5. Effect of the core protein on the expression level of mismatch-repair-related genes in PH5CH8 cells. PH5CH8 cells were infected with retrovirus pCXbsr/core(1b-P) or pCXbsr/NS5A(1b-P), and the cells were used for reverse transcription-PCR analysis of mismatch-repair-related genes. As a control, PH5CH8 cells infected with retrovirus pCXbsr were also used for the analysis. *Control*, PH5CH8 cells infected with retrovirus pCXbsr; *Core(1b-P)*, PH5CH8 cells stably expressing core(1b-P) protein; *NS5A(1b-P)*, PH5CH8 cells stably expressing NS5A(1b-P) protein; *NC*, no RNA.

proteins, in PH5CH cells should be partially repressed by the physical binding of the SV40 large T antigen (42). By complex with p53, the SV40 large T antigen blocks the apoptotic function of p53 and allows proliferation (43), and by binding pRb, the SV40 large T antigen induces the release of the E2F transcription factor, which activates the promoters of genes required for the S-phase transition (44). The functional repression of p53 or pRb may be involved in the repression of MMR activity, although no data suggesting such a relation has yet been reported. As an alternative possibility, the SV40 large T antigen may bind and repress some proteins that function in the MMR system, because it was reported recently that the SV40 large T antigen bound MRE11-NBS1-RAD50 complex, which was involved in homologous recombination, and, as a consequence, perturbed the double-strand break repair (45). Preliminary experiments using NKNT-3 cells (SV40-large T antigen immortalized non-neoplastic human hepatocytes) derived from primary normal human hepatocytes (46) and Saos-2 cells (derived from p53-deficient human osteogenic sarcoma; Ref. 47) revealed that NKNT-3 cells, like PH5CH8 cells, also showed the RER+ phenotype, but Saos-2 cells showed the RER− phenotype in our MSI assay. These results suggest that the activity of MMR is influenced by the SV40 large T antigen but not by p53; however, in addition to PH5CH8 cells, the analysis of cell lines derived from HCV-related HCC cases will be necessary to clarify the reason that PH5CH8 cells show the RER+ phenotype.

Although we found that the core protein promoted MSI in PH5CH8 cells, it is difficult to prove our findings in an HCV replication system because of the lack of a sufficiently reproducible and efficient HCV proliferation system (14). Alternatively, several HCV subgenomic replicons containing NS3-NS5B regions have been established using a human hepatoma cell line Huh-7 (48–50). These subgenomic replicon systems may be useful for the functional evaluation of the core protein. However, our preliminary results revealed that these subgenomic replicon cells showed the RER− phenotype and that no pur^R colonies were obtained from these subgenomic replicon cells stably expressing the core(1b-P) protein. These results suggest that these replicon cells have an intact MMR system that is not influenced by the core protein. To reproduce the promotion of MSI by the core protein in cells in which the HCV genome is replicated, we are currently establishing an HCV subgenomic replicon using PH5CH8 cells.

Our observation that the core proteins belonging to genotypes 1b and 2a, but not those belonging to genotypes 1a, 2b, and 3a, may promote MSI in human hepatocytes is interesting. Although it is not yet defined which region of the core protein is responsible for the promotion of MSI, comparison of aa sequences among these core proteins revealed that aa position 91 was a Cys residue in the core(1a), core(2b), and core(3a) proteins, whereas this position was a Leu residue in the core(1b-M), core(1b-P), and core(2a) proteins and a Met residue in the core(1b-HCC) protein. Only this aa position showed good correlation with the effect of the core proteins in the MSI assay. To clarify whether or not aa position 91 is important to promote MSI, further analysis using chimeric core proteins will be necessary. On the other hand, several studies have described an increased risk of HCC in patients infected with HCV genotype 1b (51, 52), although the contradictory result has also been reported (53). The fact that the core protein belonging to genotype 1b was most effective at promoting the MSI in hepatocyte cells may be related to the increased risk of HCC in patients infected with HCV genotype 1b. To examine this possibility, further MSI analysis using various core proteins derived from many HCV strains belonging to different genotypes will be needed. In addition, our preliminary experiment showed that the number of pur^R colonies in PH5CH8 cells increased approximately 1.5-fold in the presence of $FeSO_4$ (100 μM), suggesting that the Fe(II) compound promotes microsatellite mutations. Although the mechanism of this phenomenon has not yet been clarified, it has been reported that Nickel(II) also induces microsatellite mutations in human lung cancer cell lines (39). Future studies on the relationship between the core protein and these cation compounds will also be important to clarify their roles during the process of hepatocarcinogenesis.

Because we could find no effect of the core protein on the expression level of MMR-related gene, the mechanism by which the core protein promotes MSI in human hepatocytes is still unclear. However, it remains possible that the core protein directly interacts with these components involved in MMR and then suppresses their functions. An alternative possibility—that the core protein affects the functions of the other proteins involved in MMR, including DNA polymerase δ/ϵ, exonuclease I, and endonuclease FEN1—remains to be examined. Future analyses to evaluate these possibilities may clarify the mechanism of the down-regulation of the MMR system by the core protein.

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Reduced Numbers and Impaired Ability of Myeloid and Plasmacytoid Dendritic Cells to Polarize T Helper Cells in Chronic Hepatitis C Virus Infection

Tatsuya Kanto,[1,2] Michiyo Inoue,[1,2] Hideki Miyatake,[1] Aki Sato,[1] Mitsuru Sakakibara,[1] Takayuki Yakushijin,[1] Chika Oki,[1] Ichiyo Itose,[1] Naoki Hiramatsu,[1] Tetsuo Takehara,[1] Akinori Kasahara,[3] and Norio Hayashi[1]

Departments of [1]Molecular Therapeutics, [2]Dendritic Cell Biology and Clinical Application, and [3]General Medicine, Osaka University Graduate School of Medicine, Osaka, Japan

Hepatitis C virus (HCV) infection induces a wide range of chronic liver injuries. The mechanism by which HCV evades the immune surveillance system remains obscure. Blood dendritic cells (DCs) consist of myeloid and plasmacytoid subsets that play distinct roles in the regulation of antivirus immune responses; however, their roles in the pathogenesis of HCV infection are yet to be determined. We compared the numbers and functions of myeloid and plasmacytoid DCs between 43 patients with chronic hepatitis and 26 age-matched healthy volunteers. Absolute numbers of myeloid DCs, plasmacytoid DCs, and DC progenitors in the periphery were significantly lower in patients with chronic hepatitis than in healthy volunteers. Myeloid and plasmacytoid DCs from the patients had impaired abilities to stimulate allogeneic CD4 T cells and to produce interleukin (IL)–12 p70 and interferon-α, compared with those from healthy volunteers. After exposure to naive CD4 T cells, myeloid DCs from the patients were less able to drive the T helper type 1 response, whereas myeloid and plasmacytoid DCs from the patients primed more IL-10–producing cells than did those from healthy volunteers. In conclusion, in chronic HCV infection, both types of blood DCs are reduced and have an impaired ability to polarize T helper cells.

Hepatitis C virus (HCV) is a double-stranded RNA virus that causes a wide range of chronic liver diseases in humans, from mild or active chronic hepatitis to liver cirrhosis and hepatocellular carcinoma [1]. Epidemiological studies have revealed that the incidence of HCV-related hepatocellular carcinoma is increasing in general, not only in areas where HCV is endemic, showing that HCV infection is a worldwide health problem [2]. To prevent the progression of liver injury and the subsequent occurrence of hepatocellular carcinoma, HCV should be eradicated. At present, a combination of pegylated interferon (IFN)–α and ribavirin is used as the standard treatment for chronic HCV infection [3]. This protocol has significantly improved the rate of HCV eradication, compared with that attained by IFN-α monotherapy; however, more than half of the patients are refractory to the combination therapy [3]. To improve the prognosis of chronically infected patients, the reason for HCV persistently infecting humans and resisting clearance, even with repeated administration of antiviral agents, needs to be elucidated. It has been suggested that HCV has strategies for escaping from the immune surveillance system—for example, by disabling antigen-presenting cell (APC) function [4] or inhibiting CD4 and CD8 T cell responses [5, 6].

Dendritic cells (DCs) are professional APCs that stim-

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Reprints or correspondence: Dr. Tatsuya Kanto, Dept. of Dendritic Cell Biology and Clinical Application, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan (kantot@moltx.med.osaka-u.ac.jp) or Dr. Norio Hayashi, Dept. of Molecular Therapeutics, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan (hayashin@moltx.med.osaka-u.ac.jp).

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ulate innate and adaptive immune reactions by priming other types of blood cells [7]. The existence of ontogenetically distinct DC subsets has been reported in humans and mice, and the subsets are grouped into at least 2 types—myeloid DCs and lymphoid (i.e., plasmacytoid) DCs [8]. In general, in humans, myeloid DCs produce interleukin (IL)–12 or tumor necrosis factor–α when stimulated and induce Th1 polarization, whereas plasmacytoid DCs produce a considerable amount of type-I IFN after viral infection and mainly induce Th2 polarization [8, 9]. However, the properties of each DC subset are flexible in vivo and vary according to their activation state or the nature of the maturation stimuli given to DCs [8, 9]. Several reports have focused on DC dysfunction and its involvement in the pathogenesis of a variety of disorders [10–12]. We and other groups have shown that, in chronic HCV infection, DCs have impaired allostimulatory capacities [4, 13], suggesting that DCs themselves are a reciprocal target of HCV-induced immune dysfunction.

Recently, the roles of blood DC subsets in HIV infection have been studied extensively [11, 14, 15]. A clear correlation was reported between plasmacytoid DC counts and HIV quantity in HIV-infected patients [14]. More importantly, plasmacytoid DC counts decrease in parallel with the progression of HIV-related disorders [15], suggesting that plasmacytoid DCs protect against the development of disease. To clarify the roles of DC subsets in HCV infection, we compared the numbers and functions of blood DCs between patients with chronic hepatitis and healthy volunteers. We were able to demonstrate that, in chronic HCV infection, both types of DCs are numerically and functionally impaired. Of note is the finding that myeloid DCs from patients with chronic hepatitis show impaired ability to induce Th1 polarization and that myeloid and plasmacytoid DCs from patients with chronic hepatitis can prime more IL-10–producing cells than can those from healthy volunteers.

SUBJECTS, MATERIALS, AND METHODS

Subjects. Among the patients with chronic hepatitis monitored at Osaka University Hospital (Osaka, Japan), 43 (27 men and 16 women; mean ± SD age, 48 ± 8 years) were enrolled in the present study after their written, informed consent had been obtained. Patients were confirmed to be positive for both serum anti-HCV antibody (Ab) and HCV RNA but were negative for other viral infections, including hepatitis B virus (HBV) and HIV. None of them had been treated with antiviral agents, including IFN-α or ribavirin. All patients had shown persistent or fluctuating alanine aminotransferase (ALT) abnormalities at enrollment (mean ± SD ALT levels, 91 ± 72 IU/L). The presence of other causes of liver disease, such as autoimmunodeficiency, alcohol abuse, and metabolic disorders, was excluded by use of laboratory and imaging analyses. In all patients, a combination of biochemical testing and ultrasonography or computer tomography scan analysis ruled out the presence of cirrhosis and tumors in the liver. As control subjects, 26 age-matched, healthy volunteers who were all negative for HCV, HBV, and HIV were examined (20 men and 6 women; mean ± SD age, 45 ± 10 years). HCV RNA quantity was assayed by use of a branched DNA probe assay (Chiron HCV-RNA). HCV serotyping was performed as described elsewhere [16]. The median HCV RNA titer in patients was 5.7 million genome equivalents/mL (Meq/mL) (range, 0–37 Meq/mL). Thirty-three patients had HCV serotype 1, and 4 patients had HCV serotype 2; HCV serotype was not determined for the remaining 6 patients.

Reagents. Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 were provided by Kirin Brewery or were purchased from PeproTech. Recombinant human IL-3 was purchased from R&D Systems. Recombinant human IL-2 was purchased from Genzyme-Techne. IFN-γ was purchased from Strathman-Biotech. Human lymphoblastoid IFN-α was provided by Sumitomo Pharmaceuticals.

Analysis of myeloid DCs, plasmacytoid DCs, and DC progenitors in the periphery. Twenty milliliters of heparinized venous blood was drawn from patients and volunteers. Peripheral blood mononuclear cells (PBMCs) were collected by use of density-gradient centrifugation on a ficoll-hypaque cushion. After the live PBMCs had been counted, the cells were subsequently stained with Abs. Blood DCs were defined as lineage marker (Lin; CD3, CD14, CD16, CD20, and CD56)–negative and HLA-DR^+ cells. After setting the gate on these cells, myeloid and plasmacytoid DCs were further defined by the pattern of CD11c and CD123 expression. Myeloid DCs are Lin^-, HLA-DR^+, $CD11c^+$, and $CD123^{low}$ cells, and plasmacytoid DCs are Lin^-, HLA-DR^+, $CD11c^-$, and $CD123^{high}$ cells. From the percentages of these cells determined by use of fluorescence-activated cell sorter (FACS; Becton Dickinson Immunocytometry Systems) analyses, the absolute numbers of the DC subset in the periphery were calculated by multiplying the PBMC counts.

Recent reports have shown that myeloid and plasmacytoid DCs develop from $CD34^+$ cells with the aid of various hematopoietic factors [17, 18]. Throughout the process, intermediate plasmacytoid DC progenitors are present in the periphery, and their phenotypes were determined [18]. We defined CD34 progenitors as Lin^-, HLA-DR^+, $CD123^+$, and $CD34^+$ cells. The early and late progenitors of DCs were defined as Lin^-, $CD34^+$, $CD123^+$, and $CD45RA^-$ cells and Lin^-, $CD34^+$, $CD123^+$ and $CD45RA^+$ cells, respectively. The numbers of these cells in the periphery were calculated from their percentages in PBMCs, as described above.

Separation of DC subsets from the blood. For the functional analyses, myeloid and plasmacytoid DCs were separated from PBMCs or buffy coat (provided by the Osaka Red Cross Blood

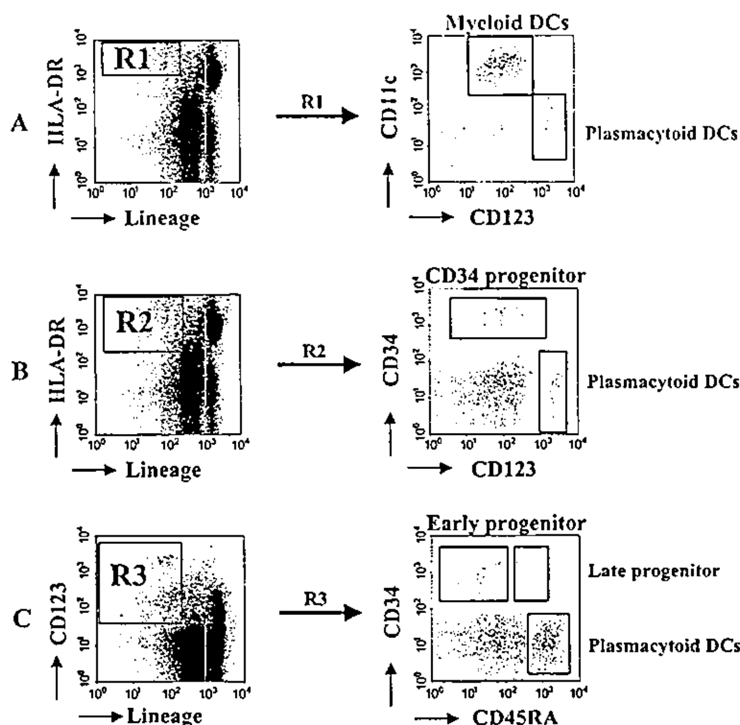

Figure 1. Identification of blood dendritic cell (DC) subsets, CD34 progenitors, and early and late progenitors of plasmacytoid DCs in peripheral blood mononuclear cells. *A*, Myeloid DCs are lineage marker–negative (Lin^-), $HLA\text{-}DR^+$, $CD11c^+$, and $CD123^{low}$; plasmacytoid DCs are Lin^-, $HLA\text{-}DR^+$, $CD11c^-$, and $CD123^{high}$. *B*, CD34 progenitors are Lin^-, $HLA\text{-}DR^+$, $CD34^+$, and $CD123^+$. *C*, Early progenitors of plasmacytoid DCs are Lin^-, $CD123^+$, $CD34^+$, and $CD45RA^-$; late progenitors are Lin^-, $CD123^+$, $CD34^+$, and $CD45RA^+$.

Center, Osaka, Japan) by use of blood DC antigen (BDCA)–1 and BDCA-4 separation kits (Miltenyi Biotec) with some modifications [19]. Alternatively, after Lin^+ cells were depleted magnetically with the cocktail Abs containing CD3, CD14, CD16, CD20, CD56, and glycophorins (StemCell Technologies), myeloid and plasmacytoid DCs were sorted separately by use of a FACS Vantage SE (Becton Dickinson Immunocytometry Systems). The purity of myeloid and plasmacytoid DCs was >95%, as assessed by FACS analysis.

DC culture. Sorted myeloid DCs were cultured in DC media (DCM) (Isocove's modified Dulbecco's medium supplemented with 10% fetal calf serum, 100 μg/mL streptomycin, 100 U/mL penicillin, 2 mmol/L L-glutamine, 5 mmol/L HEPES, and 5 mmol/L nonessential amino acid) containing 50 ng/mL GM-CSF and 10 ng/mL IL-4 for 3–5 days at 37°C in 5% CO_2. To analyze cytokine production, day-3 myeloid DCs (i.e., DCs that had been incubated for 3 days) were stimulated with the same numbers of human CD40 ligand (CD40L)–transfected L cells. The culture supernatants were collected 24 h after stimulation and were used for the cytokine ELISA. Plasmacytoid DCs were cultured in DCM in the presence of 50 ng/mL IL-3 for 2–3 days. To stimulate plasmacytoid DCs to produce IFN-α, we added 5 μmol/L stimulatory cytosine-phosphodiester-guanine oligodeoxynucleotide 2216 [20] to the freshly collected plasmacytoid DCs, and the supernatants were collected after 24 h.

Flow cytometric analysis. The expression of surface molecules on DCs was analyzed by use of a FACSCaliber (Becton Dickinson Immunocytometry Systems). At each step of the staining, 5×10^5 cells were stained with specific Abs for 30 min at 4°C in PBS containing 2% bovine serum albumin and 0.1% sodium azide. We used fluorescein isothiocyanate (FITC)–, phycoerythrin (PE)–, peridinin chlorophyll protein (Per-CP)–, cytochrome (Cy-chrome)–, or allophycocianin-labeled mouse monoclonal Abs for the staining of HLA-DR (L243), lineage cocktail (CD3, CD14, CD16, CD19, and CD56), CD3 (HIT3a), CD4 (13B8.2), CD11c (KB90), CD14 (rmC5-3), CD34 (581), CD40 (5C3), CD45RA (HI100), CD80 (L307.4), CD83 (HB15a), CD86 (IT2.2), and CDw123 (IL-3 receptor α-chain and 7G3). FITC- or PE-labeled mouse IgG was substituted for specific Abs, to obtain negative controls. All Abs except CD11c (DAKO) and CD83 (Coulter Immunotech) were purchased from BD Pharmingen.

Allogeneic mixed lymphocyte reaction (MLR). Responder naive CD4 T cells ($CD4^+$, $CD45RO^-$, and $CD45RA^+$ cells) were separated from PBMCs of healthy volunteers by use of the StemSep system (StemCell Technologies). The purity of naive CD4 T cells was >95%. The graded numbers of day-3 myeloid

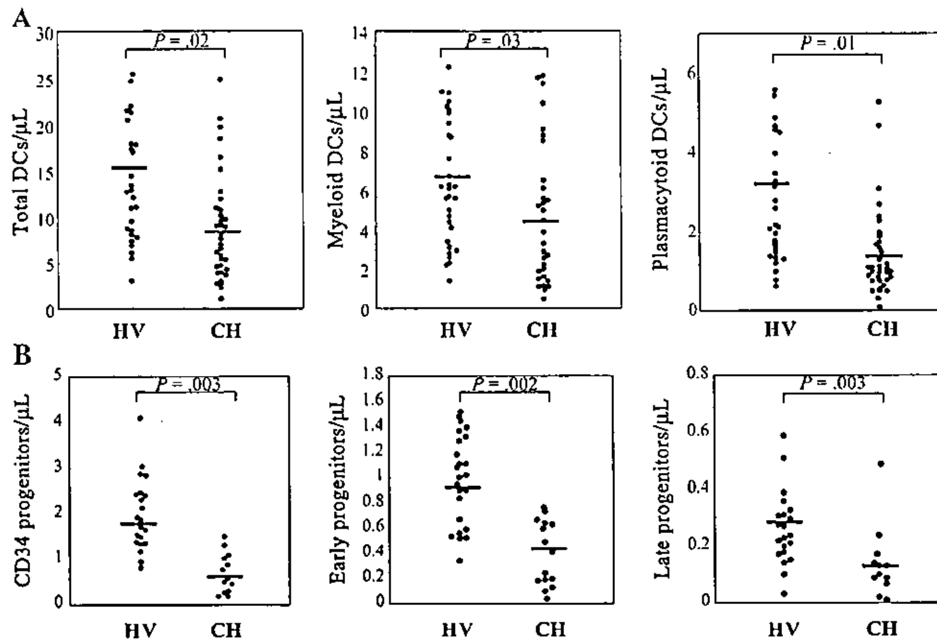

Figure 2. Reduction of blood dendritic cells (DCs) and their progenitors in patients with chronic hepatitis (CH). Absolute nos. of blood DCs *(A)* and progenitors *(B)* were determined from their frequencies in peripheral blood mononuclear cells. *Horizontal bars,* median. Statistical significance was analyzed by use of the Mann-Whitney *U* test. HV, healthy volunteers.

or plasmacytoid DCs were cultured for 5 days with 2×10^4 allogeneic naive CD4 T cells/well. In the final 16–20 h of culture, 1 μCi/well of [^{3}H]-thymidine (ICN Biomedicals) was pulsed. The uptake of [^{3}H]-thymidine to T cells was measured by use of a β-counter (Wallac).

Analysis of T helper cell polarization by DC subsets. After myeloid or plasmacytoid DCs were cultured in the presence of cytokines for 3 days, 1×10^4 DCs/well were cultured for an additional 7 days with 1×10^5 allogeneic naive CD4 T cells/well. On day 4 of the coculture, 5 ng/mL IL-2 was added. On day 7, the cells were stimulated with 50 ng/mL PMA and 1 μg/mL ionomycin and incubated for 24 h. The supernatants of the stimulated cells were collected and used for cytokine ELISA.

ELISA. The cytokine concentration was measured by use of ELISA with matched Ab pairs for human IFN-γ, IL-4, IL-10, and IL-12 p70 (Endogen), according to the manufacturer's instructions. IFN-α was assayed by use of the ELISA kit (BioSource). The range of the limits of detection of the assay was 15–1000 pg/mL.

Statistical analysis. The differences of various parameters between the healthy volunteers and the patients were analyzed by use of the Mann-Whitney *U* test. The nonparametric Spearman's test was used to explore correlations. All analyses were performed with StatView software (version 5; SAS Institute). $P < .05$ was considered to be statistically significant.

RESULTS

Reduction of blood DCs and their progenitors in patients with chronic hepatitis. After setting the gate on Lin$^-$ and HLA-DR$^+$ DCs, myeloid and plasmacytoid DCs in the periphery were clearly identified by the expression pattern of CD11c and CD123 (figure 1*A*). Total DC, myeloid DC, and plasmacytoid DC counts in the patients with chronic hepatitis were lower than those in healthy volunteers (figure 2*A*). To exclude the possibility that the reduction of DC subsets was due to a decrease in the total PBMC count in patients with chronic hepatitis, we compared the numbers of PBMCs, CD4, and monocytes among the groups and found no differences (data not shown). Therefore, the low blood DC counts are not simply due to low PBMC counts; DCs are selectively reduced in patients with chronic hepatitis. Furthermore, in the patients with chronic hepatitis, no correlation was found between DC counts and the serum ALT levels or HCV RNA titers (data not shown).

Next, we analyzed the progenitor and precursor populations of DC subsets. In accordance with the phenotypes reported by Blom et al. [18], we identified CD34 progenitors (Lin$^-$, HLA-DR$^+$, CD123$^+$, and CD34$^+$), early progenitors (Lin$^-$, CD123$^+$, CD34$^+$, and CD45RA$^-$), and late progenitors (Lin$^-$, CD123$^+$, CD34$^+$, and CD45RA$^+$) in the PBMCs (figure 1*B* and 1*C*). The numbers of CD34 progenitors and early and late progenitors

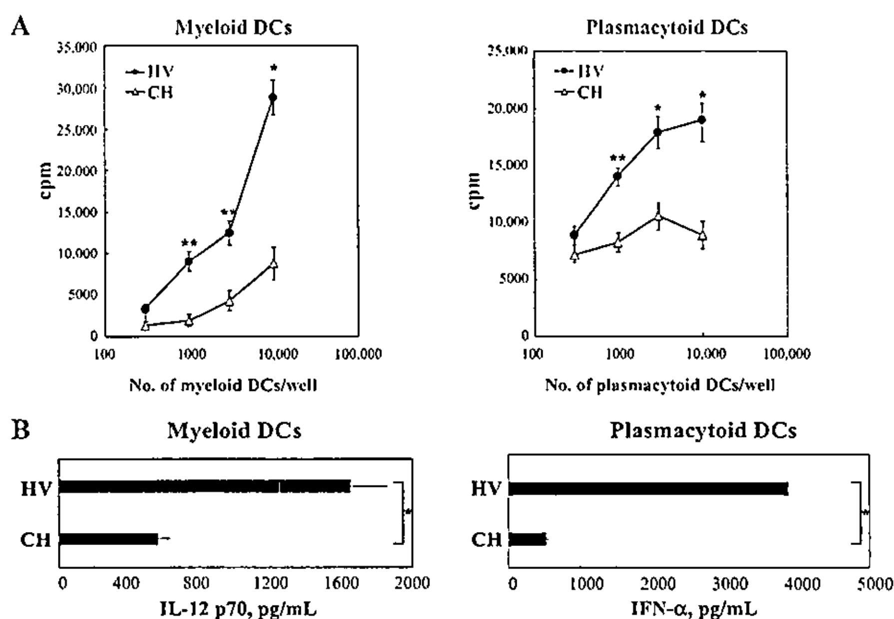

Figure 3. Functional impairment of both types of blood dendritic cells (DCs) from hepatitis C virus–infected patients. *A*, Allogeneic mixed lymphocyte reaction. *Vertical bars*, mean ± SD. *$P<.01$ and **$P<.05$, vs. patients with chronic hepatitis (CH). *B*, Day-3 myeloid DCs were stimulated with equal nos. of CD40L-expressing L cells for 24 h. Day-1 plasmacytoid DCs were cultured for 24 h in the presence of 5 μmol/L cytosine-phosphodiester-guanine oligodeoxynucleotide 2216. The supernatants were collected from both cultures and examined for interleukin (IL)–12 p70 and interferon (IFN)–α by use of ELISA. *Bars*, mean + SD. *$P<.05$ (Mann-Whitney *U* test). HV, healthy volunteers.

in patients with chronic hepatitis were significantly lower than those in healthy volunteers (figure 2*B*). In addition, a positive correlation was observed between Lin^- and $HLA\text{-}DR^+$ DC counts and those of $CD34^+$ progenitors or early progenitors (DCs vs. $CD34^+$ progenitors, $R^2 = 0.535$, and $P<.05$; DCs vs. early progenitors, $R^2 = 0.533$, and $P<.05$). These results suggest that the low number of blood DCs in patients with chronic hepatitis is due, in part, to the reduction in DC progenitors.

Functional impairment of blood DC subsets in patients with chronic hepatitis. After myeloid and plasmacytoid DCs had been sorted magnetically and had been cultured in the presence of relevant cytokines, the phenotypes of these cells were examined. On both myeloid and plasmacytoid DCs, the expressions of CD40, CD80, CD83, CD86, and HLA-DR were not different between patients with chronic hepatitis and healthy volunteers (data not shown).

To compare the allostimulatory capacity of day-3 DCs from each group, we performed MLR. The proliferative responses of CD4 T cells with myeloid or plasmacytoid DCs from patients with chronic hepatitis were lower than those from healthy volunteers (figure 3*A*).

We analyzed the production of IL-12 p70 by myeloid DCs and that of IFN-α by plasmacytoid DCs. After stimulation, myeloid and plasmacytoid DCs from patients with chronic hepatitis released lower amounts of these cytokines than did DCs from healthy volunteers (figure 3*B*). Therefore, blood DC subsets from patients with chronic hepatitis were functionally impaired in the stimulation of allogeneic T cells and cytokine production.

Decreased ability of myeloid DCs from patients with chronic hepatitis to induce Th1 polarization and higher capacity of myeloid DCs and plasmacytoid DCs to induce IL-10–producing cells. DCs prime naive CD4 T cells to differentiate into Th1 and Th2 or regulatory T cells. In our system, myeloid DCs predominantly induced IFN-γ–producing Th1 cells, whereas plasmacytoid DCs primed Th1- and IL-4–positive Th2 cells and IL-10–producing cells. Myeloid DCs from patients with chronic hepatitis were less able to drive the Th1 response than were myeloid DCs from healthy volunteers (figure 4). Plasmacytoid DCs from patients with chronic hepatitis induce IFN-γ or IL-4–producing cells at levels comparable to those induced by plasmacytoid DCs from healthy volunteers (figure 4). Both types of DCs from patients with chronic hepatitis primed more IL-10–producing cells than did those from healthy volunteers, although the levels of IL-10 were much higher in plasmacytoid DC–primed CD4 T cells than in myeloid DC–primed CD4 T cells (figure 4).

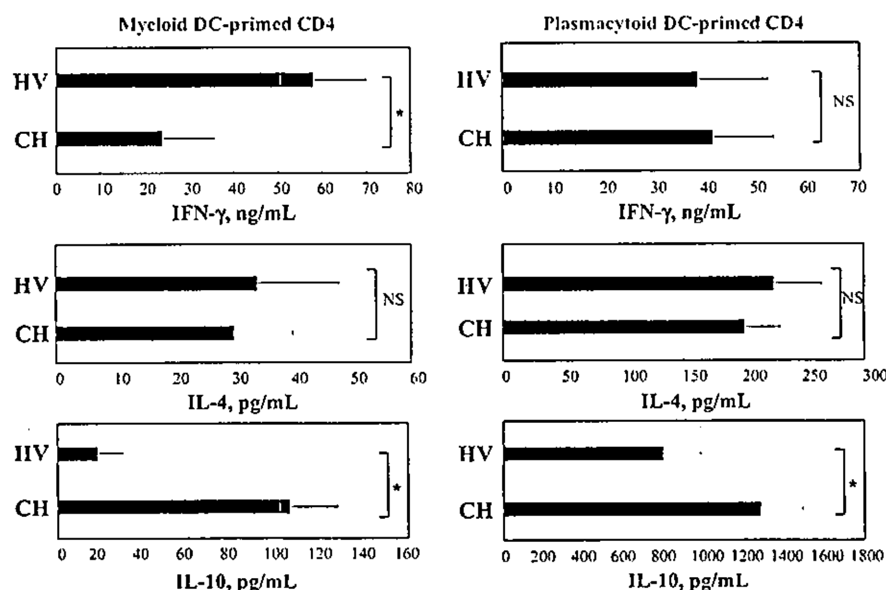

Figure 4. Inability of myeloid dendritic cells (DCs) from patients with chronic hepatitis (CH) to drive Th1-polarization. Myeloid and plasmacytoid DCs from patients with CH can prime more interleukin (IL)–10 producing cells than do those from healthy volunteers (HV). Day-3 myeloid or plasmacytoid DCs were cultured with allogeneic naive CD4 T cells for an additional 7 days. CD4 T cells were stimulated with PMA and ionomycin for 24 h, and the supernatants were collected for cytokine ELISA. *Bars*, mean + SD. $^*P < .05$ (Mann-Whitney *U* test). NS, not significant.

DISCUSSION

DCs are well known central regulators in both innate and adaptive immune responses against viruses [7, 8]. Several studies, including our own [4], have demonstrated that dysfunction of monocyte-derived DCs occurs during HCV infection and is implicated in the persistence of HCV [13]. In the present study, we have determined the reduction in and dysfunction of blood DC subsets in patients with chronic hepatitis.

The effect of DC counts on the pathogenesis of virus-related disorders has been well documented in HIV infection. Plasmacytoid DC counts decrease in parallel with an increase in HIV quantity and with the progression of disease severity [14]. On the other hand, long-term suppressors, who have been healthy despite HIV infection, exhibit higher plasmacytoid DC counts than do control subjects [15]. These observations strongly suggest that plasmacytoid DCs are able to control replication of HIV and HIV-related disorders. In contrast, no correlation was observed between DC counts and HCV RNA titers in patients with chronic hepatitis, implying that DC count is not a primary factor influencing HCV replication. In addition, the lack of correlation between DC counts and ALT levels suggests that the decrease in DC counts is not simply due to liver inflammation. In patients who cleared HCV after receiving IFN-α–based therapy, DC and progenitor counts were comparable to those of healthy volunteers (authors' unpublished data), showing that the presence of HCV principally contributes to the reduction in DCs.

There are several possible explanations for the mechanisms that cause DC deficiency during HCV infection. One hypothesis is that HCV aims to infect DCs and enhances their susceptibility to apoptosis. By means of polymerase chain reaction, the HCV genome has been detected in myeloid and plasmacytoid DCs recovered from some chronically infected patients, although the replication level in cells appeared to be low [13, 21]. The next possible explanation for the low numbers of circulating DCs is their enhanced mobilization to other tissues. In patients with systemic lupus erythematoses or tuberculosis, plasmacytoid DCs accumulate in cutaneous or granulomatous lesions, resulting in reduction of the circulating DC pool [22, 23]. Thus, during HCV infection, DCs may disappear from the periphery to move into the inflamed liver or lymphoid tissues. Finally, the reduction of DC progenitors and/or precursors may be involved. It is generally accepted that DCs are derived from hematopoietic stem cells or $CD34^+$ progenitors [17]. Blom et al. reported that myeloid and plasmacytoid DCs were generated from $CD34^+$ cells with the aid of hematopoietic factors, such as fms-like tyrosine kinase 3 ligand [17, 18]. The present study has demonstrated that numbers of $CD34^+$ and early progenitor cells are decreased in patients with chronic hepatitis, a fact that correlates well with the results of numerical DC analyses. Therefore, the development of DCs may be impeded in chronically patients with chronic hepatitis, and the mechanism needs to be further investigated.

An imbalance between Th1 and Th2 subsets is crucially in-

volved in the pathogenesis of chronic hepatitis [24, 25]. Several lines of evidence have clearly demonstrated that a Th1 response, either spontaneous or IFN-α induced, is required for eradication of HCV [26–28]. One of the possible mechanisms causing defective Th1 responses by myeloid DCs is the lower amount of IL-12 produced by them, since a critical role of IL-12 in Th1 polarization has been reported elsewhere [29]. Direct HCV infection of DCs may be involved in the impaired production of IL-12, which is supported by the finding that the expression of HCV proteins in monocyte-derived DCs suppresses the release of IL-12 [30]. In addition to dysfunction of myeloid DCs, the impaired capacity of plasmacytoid DCs to produce IFN-α plays a role in inducing the low-grade Th1 response in HCV infection. It has been reported that plasmacytoid DC–derived IFN efficiently promotes Th1 by stimulating monocytes to produce Th1-attracting chemokines [31]. Thus, plasmacytoid DCs may be less able to offer a Th1-inducing environment for the shortage of interaction with other cells via IFN-α.

Another feature of the DC system in HCV infection is the profound ability of both types of DCs to prime IL-10–producing T cells. IL-10 either inhibits the antigen-specific T cell response by suppressing APC function [32] or directly suppresses the Th1-polarizing ability of myeloid DCs. The mechanisms that allow plasmacytoid DCs to prime more IL-10–producing cells are yet to be identified. Kadowaki et al. reported that herpes simplex virus–activated plasmacytoid DCs induce IL-10– and IFN-γ–producing cells [33], whereas CD40L-activated plasmacytoid DCs can prime IL-10–producing regulatory CD8 T cells [34]. These observations show that plasmacytoid DCs alter their T cell–priming abilities in response to a variety of maturation stimuli. Thus, it is conceivable that plasmacytoid DCs in patients with chronic hepatitis are already primed by certain HCV-derived signals.

In summary, we have demonstrated that, in patients with chronic hepatitis, blood DC counts are reduced, and DCs are functionally impaired. Most notably, myeloid DCs from such patients are less able to induce Th1, and both myeloid and plasmacytoid DCs from these patients can prime more IL-10–producing cells than can those from healthy volunteers, thus favoring HCV persistence.

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