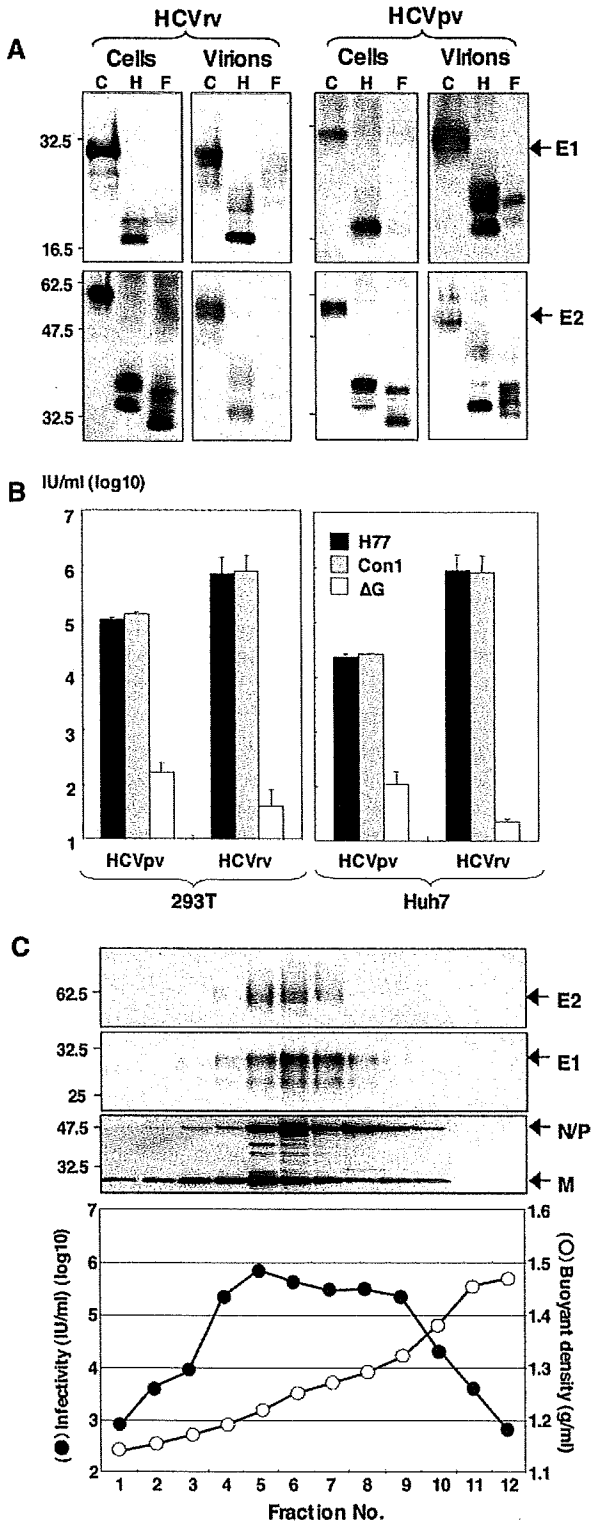

FIG. 3. Characterization of HCVrv and HCVpv. (A) The E1 and E2 proteins of the H77 strain expressed in 293T cells and incorporated into the particles of HCVrv and HCVpv were either untreated (C) or treated with endoglycosidase H (H) or peptide-*N*-glycosidase F (F). Following fractionation on sodium dodecyl sulfate-polyacrylamide gel gels, the glycoproteins were detected by immunoblotting with anti-E1 (BDI198) and anti-E2 (AP33) monoclonal antibodies. (B) The infectivities of HCVrv and HCVpv bearing HCV envelope proteins of genotypes 1a (H77 strain) and 1b (Con1 strain) generated in 293T or Huh7 cells were determined with Huh7 cells. The envelopeless VSV (ΔG) was used as a control. (C) (Top) CsCl gradient sedimentation of HCVrv produced in 293T cells. The supernatant was fractionated from the top of the gradient and analyzed by immunoblotting with anti-E2, anti-E1, and anti-VSV antibodies. (Bottom) The infectivity (filled circles) of each fraction was determined after the removal of CsCl with column purification. Fraction densities (open circles) are expressed in grams/milliliter.

Huh7 cells. To further determine the cell tropism for virus propagation, HCVrv was generated in various cell lines, and replication was assessed during incubation for up to 6 days (Fig. 4C, left). The growth kinetics of the wild-type VSV revealed an efficient replication of VSV in all the cell lines examined (Fig. 4C, right). Huh7 cells exhibited the highest susceptibility to propagation of HCVrv, followed by Hep3B cells, and no propagation was detected in the other cell lines. These results indicate that various human cell lines are capable of producing HCVrv that is infectious to Huh7 cells and that Huh7 cells are highly permissive to the propagation of HCVrv.

Involvement of hCD81 in the infection with HCVpv and HCVrv. Among the candidates for entry receptor of HCV, hCD81 was shown to be most essential for the infection with HCVpp (5, 23) and HCVcc (27, 56, 60). The infection of Huh7 cells with HCVpv and HCVrv was inhibited by anti-hCD81 antibody, whereas no inhibition of VSVpv infection was observed (Fig. 5A). Treatment with siRNA targeted to hCD81 induced a reduction of hCD81 expression on the surface of Huh7 cells (Fig. 5B), and the susceptibility of hCD81-knockdown cells to infection with HCVpv and HCVrv, but not to that with VSVpv, was clearly reduced (Fig. 5C). To further determine the involvement of hCD81 in the infectivity of HCVpv and HCVrv, hCD81-negative HepG2 cells stably expressing hCD81 (HepCD81) were established, and fluorescence-activated cell sorter (FACS) analysis revealed that expression of hCD81 on the cell surface was higher than that of Huh7 cells (Fig. 5D). Although HCVpv and HCVrv are not infectious in HepG2 cells, HepCD81 cells were permissive to both HCVpv and HCVrv infection, and pretreatment with the anti-hCD81 antibody inhibited the infection of HepCD81 cells with HCVpv and HCVrv (Fig. 5E). These results indicate that hCD81 plays a crucial role in infection with HCVpv and HCVrv, as it has been reported to play in infection with HCVpp and HCVcc.

Infectivity of HCVpv and HCVrv in various cell lines. To further examine the cell tropism of the viruses, HCVpv and HCVrv of the H77 and Con1 strains generated in 293T or Huh7 cells and HCVpp of the H77 strain generated in 293T cells were inoculated into various cell lines and primary Hc (Table 1). As expected, the control VSVΔG exhibited no infectivity in any of the cells examined (data not shown). The HCVpv and HCVrv derived from both genotypes were highly infectious in Huh7 cells, followed by HepCD81 and Hep3B cells, and weakly infectious in PLC/PRF/5, 293T, and Vero cells. No infectivity was detected in the other cell lines examined. The cell tropisms of the HCVpp were similar to those of HCVpv and HCVrv. Although the ectopic expression of hCD81 in Chinese hamster ovary cells (CHOCD81) did not confer susceptibility to HCVpv, HCVrv, or HCVpp infection,

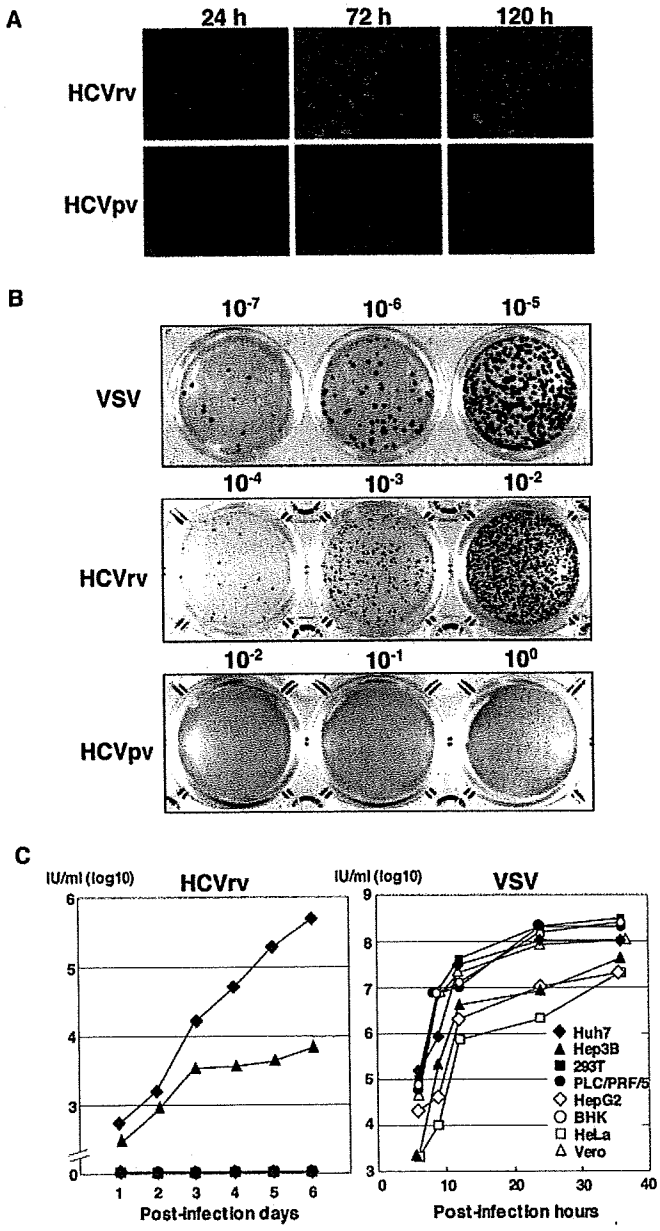

FIG. 4. Propagation of HCVrv. (A) Detection of viral proteins in Huh7 cells infected with HCVpv or HCVrv. Huh7 cells were infected with HCVpv or HCVrv at an MOI of 0.01. Twenty-four, 72, and 120 h after infection, cells were fixed and stained with monoclonal antibody to VSV N protein and Alexa 488-conjugated secondary antibody. Cell nuclei were stained by Hoechst 33258. Pictures were taken using a fluorescence microscope by double exposure of the same fields with filters for Alexa 488 or Hoechst 33258. (B) Focus formation of HCVpv, HCVrv, or VSV in Huh7 cells. Huh7 cells were infected with serial 10-fold dilutions of HCVpv, HCVrv, or VSV and incubated at 30°C for 72 h for HCVpv and HCVrv or 24 h for VSV in a culture medium containing 0.8% methylcellulose. Foci of infected cells were detected by immunohistochemical staining. (C) Kinetics of HCVrv (left) and VSV (right) propagation in various cell lines. HCVrv and VSV generated in Huh7 cells were used to infect cells at an MOI of 0.01. The culture supernatant was collected at the indicated time points and titrated by a focus-formation assay. Infectious titers are expressed in IU/milliliter.

the expression of hCD81 in HepG2 cells (HepCD81) (Fig. 5A and D) rendered them permissive to infection with all of the viruses. Furthermore, Hc were not susceptible to the infection with HCVpv, HCVrv, or HCVpp, despite the expression of hCD81. These results suggest that expression of hCD81 is essential for the infection with HCVpv and HCVrv, as reported for infection with HCVpp and HCVcc, but conditions with a lack of hCD81 are insufficient for the infection with HCVpv, HCVrv, and HCVpp.

Neutralization of HCVpv and HCVrv infection by antibodies to HCV envelope proteins and sera of HCV patients. It has been reported that HCVpp can be neutralized by several well-characterized E2-specific monoclonal and polyclonal antibodies (5, 23, 49). The neutralization activity of anti-E1 (AP21.010) and anti-E2 (AP33) monoclonal antibodies (49) and anti-E1 (R852) and anti-E2 (R646) rabbit polyclonal antibodies raised against the E1 and E2 proteins of the H77 strain on the infection with HCVpv and HCVrv was determined (Fig. 6A). The infections with both HCVpv and HCVrv bearing E1 and E2 proteins of the H77 strain were clearly inhibited by anti-E2 (AP33) antibody or anti-E2 (R646) rabbit serum, consistent with a previous report on the effect of these antibodies on HCVpp infection (49), whereas no neutralization by AP21.010 and R852 antibodies was observed. The infections with HCVpv and HCVrv bearing E1 and E2 proteins of the Con1 strain were also inhibited by AP33 and R646 antibodies (data not shown), suggesting that the infectivity of HCVpv and HCVrv was cross-neutralized by anti-E2 antibody, as reported for HCVpp (49). These results indicate that the E2 protein plays a crucial role in the infectivity of both HCVpv and HCVrv. Although the addition of naïve human sera (HD) inhibited infection with VSVpv, infection with HCVpv or HCVrv was clearly enhanced, as reported for HCVpp infection of Huh7 cells (28, 42). To assess the neutralization ability of these antibodies in patients, HCVpv and HCVrv were incubated with a 2% concentration of the sera of chronic HCV patients infected with genotype 1b HCV (Fig. 6B). All of the sera of patients of genotype 1b showed high levels of neutralization activity against infection with HCVpv and HCVrv bearing envelope proteins of genotype 1a, whereas they had no effect on the infectivity of VSVpv, in contrast to the inhibition achieved by the naïve sera. These results indicate that HCV patients elicit high levels of antibodies that are likely to cross-neutralize the infectivity of HCVpv and HCVrv.

Inhibition of HCVpv and HCVrv infection by bafilomycin A_1. Enveloped viruses enter target cells through two different pathways: one is a pH-independent direct fusion at the plasma membrane, and the other is a pH-dependent receptor-mediated endocytosis (58). Previous studies have revealed that both HCVpp and HCVcc were sensitive to the inhibitors of vacuolar acidification, such as ammonium chloride, concanamycin A, or bafilomycin A_1, suggesting that these viruses enter via a pH-dependent endocytosis into target cells (23, 61). To determine the entry pathway of HCVpv and HCVrv, Huh7 cells were pretreated with various concentrations of bafilomycin A_1, and then the cells were inoculated with HCVpv, HCVrv, VSVpv, and MLVpv (Fig. 7). As expected, the treatment did not affect the infection with MLVpv bearing an envelope protein of MLV that enters cells via a pH-independent pathway. In contrast, infection with VSVpv bearing the G protein of VSV,

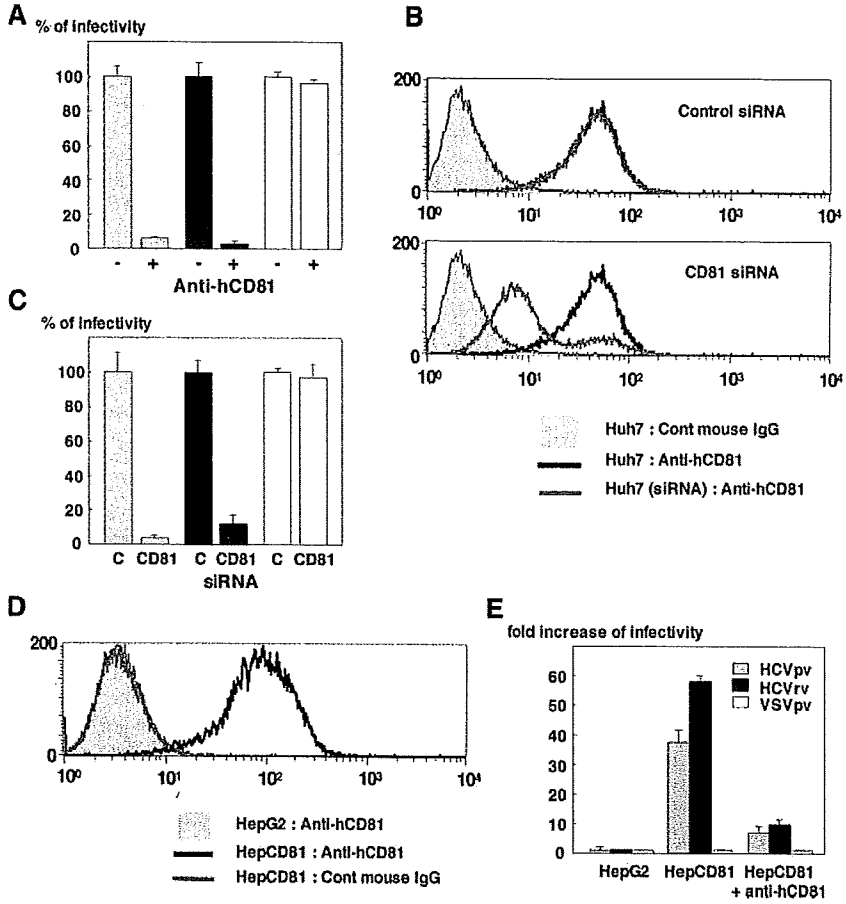

FIG. 5. Involvement of hCD81 in the infection of HCVpv and HCVrv. (A) Effect of anti-hCD81 antibody on the infectivity of HCVpv (gray-filled bars), HCVrv (black-filled bars), or VSVpv (open bars) in Huh7 cells. (B) Cell surface expression of hCD81 on Huh7 cells transfected with siRNA targeted to hCD81 or control siRNA was examined by FACS analysis after staining with anti-hCD81 antibody. (C) Effect of knockdown of hCD81 in Huh7 cells by siRNA targeted to hCD81 on the infection of HCVpv, HCVrv, or VSVpv. (D) Cell surface expression of hCD81 on HepG2 and HepCD81 cells was examined by FACS analysis after staining with anti-hCD81 antibody. (E) Infectivity of HCVpv, HCVrv, or VSVpv to HepG2 or HepCD81 cells and the effect of anti-hCD81 antibody on the infection of the viruses to HepCD81 cells. The results shown are from three independent assays, with the error bars representing the standard deviations.

which enters cells through pH-dependent endocytosis, was inhibited by the treatment with bafilomycin A_1 in a dose-dependent manner. Infection with HCVpv and HCVrv was also clearly inhibited by the treatment with bafilomycin A_1 in a dose-dependent manner, as with VSVpv. This suggests that low pH exposure is essential for the entry of HCVpv and HCVrv.

Effects of ER α-glucosidase inhibitors on HCVrv infection. Previous studies have shown that deoxynojirimycin (DNJ) and Nn-DNJ, a long-alkyl-chain iminosugar derivative of DNJ, inhibit the infection of flaviviruses such as Japanese encephalitis virus (JEV) and dengue virus in a dose-dependent manner (15, 64). Although the effects of glycosylation inhibitors on the folding and assembly of HCV envelope proteins in the N-glycosylation steps and the binding properties of HCV-LP produced in insect cells have been reported (11, 12), glycobiological analyses of HCV envelope proteins involved in virus infectivity have not been reported yet. To determine the effects of the inhibitor of Golgi mannosidase (DMJ) and of ER α-glucosidase (Nn-DNJ) on the infectivity of HCVrv, Huh7 cells were treated with these inhibitors. Treatment of Huh7 cells with Nn-DNJ but not with DMJ reduced the infectivity of HCVrv in a dose-dependent manner, and this reduction was more efficient than that in the infectivity of VSV (Fig. 8A, top). Although immunoblotting and Coomassie staining of the particles revealed that incorporation of the envelope proteins and generation of HCVrv and VSV particles recovered from cells treated with 100 μM of Nn-DNJ were severely impaired by the cytotoxic effects of Nn-DNJ (Fig. 8A, bottom left), treatment with 10 μM of Nn-DNJ selectively reduced the infectivity of HCVrv but not of VSV without any cytotoxic effect (Fig. 8A, top left). In contrast, Huh7 cells treated with more than 0.5 mM of DMJ exhibited a slight reduction of molecular sizes of E1 or VSVG proteins incorporated into the particles (Fig. 8A, bottom right); no effect on the incorporation of the envelope proteins into the viral particles and the infectivity was observed (Fig. 8A, top right). Next, we assessed the effects of the inhibitors on the propagation of the viruses. Focus formation of HCVrv was also inhibited by the treatment with Nn-DNJ but not with DMJ (Fig. 8B). To further confirm the effect of modification of the envelope glycoproteins by ER α-glucosidase on the infectivity of HCV, Huh7.5.1 cells were treated with the

TABLE 1. Infectivity of HCVpv, HCVrv, or HCVpp in various cells

Target cells	Cell surface expression of[a]:		Virus, producer cells, and strain (genotype)[b]								HCVpp virus produced in 293T cells and of strain H77 (genotype 1a)[b]
			HCVpv				HCVrv				
			293T		Huh7		293T		Huh7		
	hCD81	SR-BI	H77 (1a)	Con1 (1b)	H77 (1a)	Con1 (1b)	H77 (1a)	Con1 (1b)	H77 (1a)	Con1 (1b)	
Huh7	++	++	+++	+++	++	++	+++	+++	+++	+++	+++
HepG2	−	++	−	−	−	−	−	−	−	−	−
HepCD81	++	++	++	++	+	+	+++	+++	+++	+++	++
Hep3B	++	+	++	++	+	+	+++	+++	+++	+++	++
PLC/PRF/5	++	+	+	+	−	−	+	+	+	+	−
FLC4	−	++	−	−	−	−	−	−	−	−	−
Hc	++	−	−	−	−	−	−	−	−	−	−
HeLa	+	+	−	−	−	−	−	−	−	−	−
293T	++	+	+	+	−	−	+	+	+	+	−
Vero	−	−	+	+	−	−	+	+	+	+	−
BHK	−	−	−	−	−	−	−	−	−	−	−
CHOK1	−	−	−	−	−	−	−	−	−	−	−
CHOCD81	++	−	−	−	−	−	−	−	−	−	−

[a] Cell surface expression of receptor candidates was examined by FACS analyses with specific antibodies. Mean fluorescence intensity shifts of less than 1, between 1 and 2, and between 2 and 3 are indicated as −, +, and ++, respectively.

[b] Infectious titers higher than 5×10^4 IU/ml, between 5×10^3 and 5×10^4 IU/ml, between 5×10^2 and 5×10^3 IU/ml, and lower than 5×10^2 IU/ml are indicated as +++, ++, +, and −, respectively. The results were derived from at least three independent experiments, and the standard deviations did not exceed 30% of the mean values.

inhibitors, and infectivity of HCVcc was determined (Fig. 8C, top). Treatment with Nn-DNJ clearly inhibited the infection with HCVcc in a dose-dependent manner, as it did the infection with HCVrv. Focus formation of HCVcc was also inhibited by the treatment of Huh7.5.1 cells with Nn-DNJ (Fig. 8C, bottom). These results indicate that modification of the glycans of HCV E1 and E2 proteins in the ER by α-glucosidase rather than that in the Golgi is crucial for the infectivity of both HCVrv and HCVcc.

DISCUSSION

In general, enveloped viruses attach to host target cells and enter into cells through the interaction between viral envelope proteins and cell surface receptors and coreceptors. Due to the lack of a robust cell culture system to support the replication of various HCV genotypes, surrogate systems have been developed to examine the mechanisms of HCV infection. Although in vitro binding assays have identified several candidate receptors for HCV (4), the final determination of a true entry receptor or coreceptor capable of internalizing HCV particles has to be made by an infection assay. Toward this end, pseudotype virus systems based on VSV (27, 39) and retroviruses (5, 23) have been established. Both VSV and retroviruses normally bud from the plasma membrane, and therefore foreign envelope proteins expressed on the cell surface have been believed to incorporate into the pseudotype particles. HCV E1 and E2 proteins form heterodimers that have static ER retention signals in their C-terminal transmembrane region (17) and pulse-chase experiments and endoglycanase treatment of the intracellular forms of the proteins or those incorporated into the HCVpp have revealed that only a small fraction of the HCV envelope proteins are translocated to the plasma membrane and modified to the complex-type glycans (48). In addition, it was demonstrated that recruitment of the foreign envelope proteins by MLV and the lentivirus core protein does not occur at the cell surface but takes place intracellularly in the endosomal pathway (55, 56). Production of pseudotype VSVs bearing unmodified envelope glycoproteins of bunyaviruses has also been reported, in spite of the static retention of the envelope glycoproteins in the intracellular compartment and the lack of translocation into the plasma membrane (46). Therefore, cell surface expression of HCV envelope glycoproteins may not necessarily be a prerequisite for generation of pseudotype particles based on VSV or retroviruses.

Recombinant VSV encoding foreign viral envelope proteins in place of the G protein has been shown to be a powerful tool for the investigation of viral infection and the development of vaccines for diseases caused by infection with viruses such as influenza virus, human immunodeficiency virus, respiratory syncytial virus, human papillomavirus, and filoviruses (20, 31). Although recombinant VSV encoding HCV envelope proteins has been generated as a surrogate model for HCV infection and a vaccine vector (9, 35), recombinant VSV generated in rodent cells possessing the chimeric E1 and/or E2 proteins has been shown to be noninfectious in a human hepatoma cell line that is susceptible to HCVpp infection (9). In this study, we successfully generated infectious recombinant and pseudotype VSVs incorporating unmodified E1 and E2 proteins in hepatic and nonhepatic human cell lines. The previously observed lack of infectivity of the recombinant VSV carrying the chimeric HCV envelope proteins might be attributable to the production of viral particles in rodent (BHK) cells (9), because in this study the HCVrv generated in BHK cells exhibited no infectivity in the target cells in spite of a sufficient amount of incorporation of the HCV envelope proteins. These results suggest that posttranslational modification or host factor(s) specific to human cells might be involved in the endowment of infectivity of recombinant VSVs. Furthermore, HCVrv can be produced in various cell lines upon infection with the G-complemented particles, which are known to exhibit infectivity in several cell lines, in contrast to the pseudotype viruses, infec-

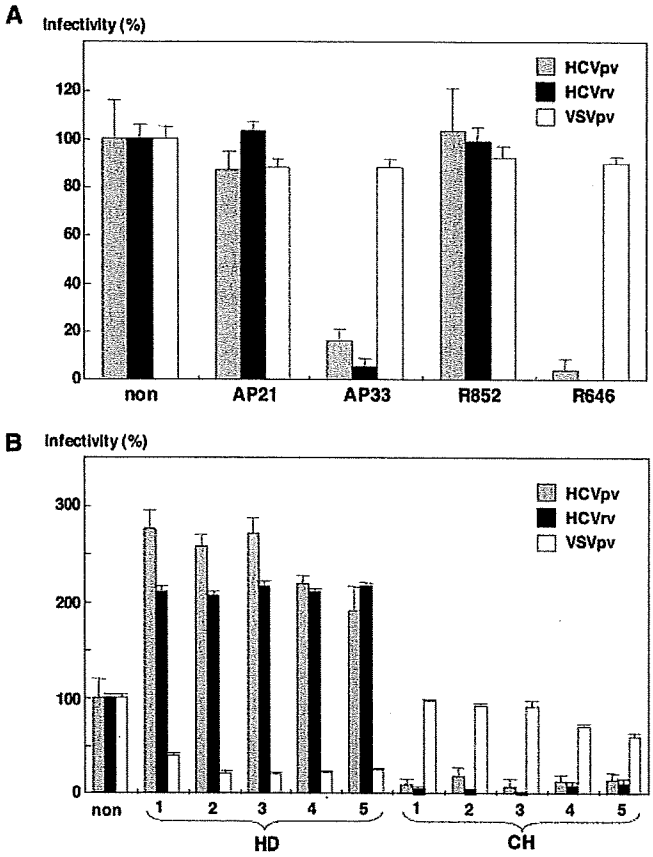

FIG. 6. Neutralization of HCVpv and HCVrv infection by antibodies to HCV envelope proteins and sera of HCV patients. (A) Effect of anti-E1 (AP21.010) and anti-E2 (AP33) monoclonal antibodies and anti-E1 (R852) and anti-E2 (R646) rabbit sera on the infectivity of HCVpv (gray-filled bars), HCVrv (black-filled bars), or VSVpv (open bars) to Huh7 cells. The viruses were preincubated for 1 h at room temperature with the antibodies before infection of Huh7 cells. (B) Effects of human sera from healthy donors and HCV patients on the infection of HCVpv, HCVrv, or VSVpv. The viruses were preincubated for 1 h at room temperature with five different healthy human sera (HD) or chronic HCV patient sera (CH) diluted 1:50 before infection of Huh7 cells.

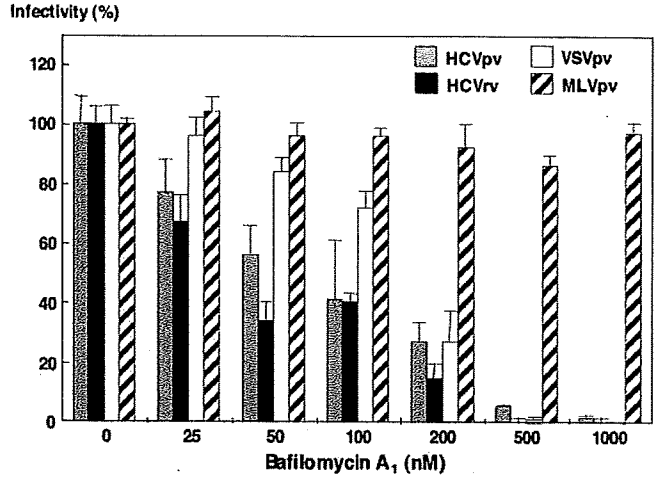

FIG. 7. Inhibition of HCVpv and HCVrv infection by bafilomycin A_1. HCVpv (gray-filled bars), HCVrv (black-filled bars), VSVpv (open bars), or MLVpv (striped bars) were inoculated to Huh7 cells after treatment with various concentrations of bafilomycin A_1. The results shown are from three independent assays, with the error bars representing the standard deviations.

tious particles of which were recovered only in cells exhibiting a high competency of transfection, such as 293T cells. Therefore, generation of HCVrv in various human cells, including nonhepatic cells such as B cells, might be useful for investigating the cell-specific modification and/or factors determining the cell tropism of HCV infection.

Overwhelming evidence that hCD81 facilitates the entry of HCV into Hc via interaction with the E2 protein has been accumulated not only by surrogate models, such as purified E2 proteins, HCV-LP, and HCVpp, but also by authentic HCV particles and HCVcc of genotype 2a (4). In this study, both HCVpv and HCVrv were shown to be infectious in Huh7 cells, and this infectivity was shown to be mediated through the interaction with hCD81. Although overexpression of hCD81 in HepG2 cells which lack endogenous expression of hCD81 renders them susceptible to infection by surrogate viruses, primary human Hc and HeLa cells expressing hCD81 and the rodent CHO cells stably expressing hCD81 (CHOCD81 cells) were resistant to infection by HCVrv and HCVpv (Table 1) (5, 14, 67), suggesting that hCD81 is one of the important factors for HCV entry but is not sufficient for infectivity of HCV in target cells. Recently, it was shown that participation of hCD81 in the infection of HCVpp or HCVcc bearing HCV envelope proteins isolated during chronic HCV infection was reduced, suggesting that the affinity of HCV envelope proteins to hCD81 was reduced and HCV utilizes receptors other than hCD81 (62, 69). HCVrv is useful for studies of the generation of various genotypes of escape variants under pressure of neutralization antibody or antagonist against HCV receptor candidates. Further studies of the functional relevance of hCD81 and other receptor candidates in the entry steps of HCV, such as binding, endocytosis, and membrane fusion, are needed.

Bafilomycin A_1, an H^+-ATPase inhibitor, was shown to reduce the infectivities of both HCVpv and HCVrv in a dose-dependent manner, as it did for the infectivities of both HCVpp and HCVcc (6, 23, 29, 61), suggesting that these viruses require low-pH-induced conformational changes of the envelope proteins upon entry. Furthermore, as with HCVcc (40, 61), preexposure of HCVpv and HCVrv to acidic pH did not reduce their infectivity (data not shown), indicating that additional factors are required for the internalization of the viruses. Recently, entry of HCVpp was shown to depend on the clathrin-mediated endocytosis through the knockdown of clathrin heavy chain by siRNA or chlorpromazine (8, 40), and dominant-negative mutants of Rab5 or Rab7, which are involved in the transport of clathrin-coated vesicles, revealed that entry of HCVpp requires delivery to early but not to late endosomes (40). N-linked glycosylation processing events in the ER are important for the secretion of several enveloped viruses. ER α-glucosidase I and II are involved in the trimming of terminal glucose on the core oligosaccharides, and the resulting monoglucosylated glycoproteins are able to bind to the ER chaperones calnexin (CNX) and/or calreticulin (CRT). ER α-glucosidase inhibitors, DNJ or castanospermine, which block

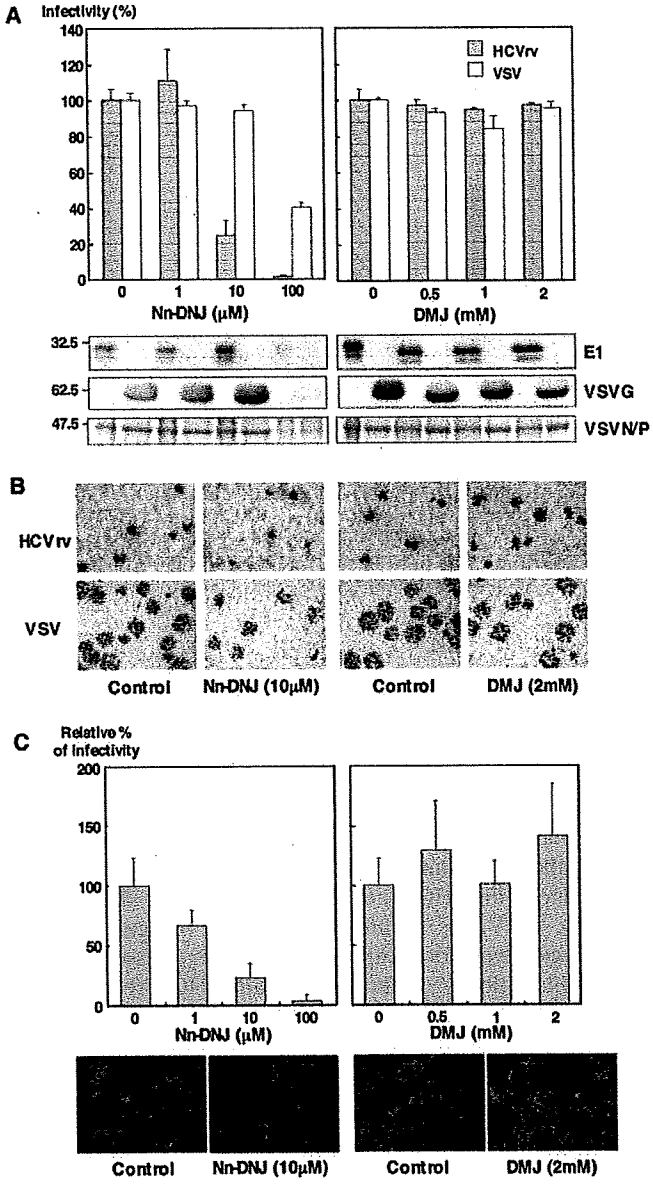

FIG. 8. Effects of ER α-glucosidase inhibitors on the infection with HCVrv and HCVcc. (A) (Top) Production of HCVrv and VSV in the presence of Nn-DNJ (left) or DMJ (right). Huh7 cells infected with HCVrv and VSV at MOIs of 0.1 and 0.01, respectively, were treated with various concentrations of Nn-DNJ or DMJ. Seventy-two hours (HCVrv) or 24 h (VSV) postinfection, culture supernatants were collected and titrated on Huh7 cells by a focus-forming assay. The results shown are from three independent assays, with the error bars representing the standard deviations. (Bottom) Purified viruses generated in Huh7 cells treated with Nn-DNJ or DMJ were analyzed by immunoblotting with anti-E1 (BDI198) and anti-VSVG (ab34774) or Coomassie staining. (B) Focus formation of HCVrv and VSV in the presence of Nn-DNJ or DMJ. Huh7 cells were infected with HCVrv or VSV, treated with Nn-DNJ (10 μM) or DMJ (2 mM) prior to an overlay of culture media containing 0.8% of methylcellulose, and stained with an anti-VSV N antibody after fixation at 72 h (HCVrv) and 24 h (VSV) postinfection. (C) (Top) Production of HCVcc in the presence of Nn-DNJ (left) or DMJ (right). Huh7.5.1 cells infected with HCVcc at an MOI of 0.01 were treated with various concentrations of Nn-DNJ or DMJ. Culture supernatants were collected and titrated by a quantitative core enzyme-linked immunosorbent assay at 96 h postinfection. (Bottom) Immunofluorescence assay of HCVcc infection in the presence of Nn-DNJ or DMJ. Huh7.5.1 cells were infected with HCVcc at an MOI of 0.01, treated with 10 μM of Nn-DNJ or 2 mM of DMJ prior to an overlay of culture media containing 0.8% of methylcellulose, and stained with an anti-NS5A antibody and Alexa 488-conjugated secondary antibody after fixation at 96 h postinfection. Cell nuclei were stained by Hoechst 33258. Pictures were taken using a fluorescence microscope by double exposure of the same fields with filters for Alexa 488 or Hoechst 33258.

the trimming step of N-linked glycosylation, have been shown to prevent the interaction of CNX and/or CRT with the folding glycoproteins, and the production of many enveloped viruses is inhibited by these inhibitors (41). In this study, we found that infection with both HCVrv and HCVcc was inhibited in a dose-dependent manner by treatment with Nn-DNJ, which is an N-alkylated derivative of DNJ exhibiting a stronger effect than DNJ. HCV E1 and E2 proteins were shown to interact with CNX and CRT, and these interactions were inhibited by the treatment with ER α-glucosidase inhibitors (12). One possible function of the HCV p7 protein, the formation of ion channels, has also been shown to be inhibited by the treatment with long-alkyl-chain iminosugar derivatives (50). Recently, it was reported that HCV-LPs produced in the presence of ER α-glucosidase inhibitors incorporated unprocessed, triglucosylated N-glycans and misfolded E1 and E2 proteins and lost their ability to bind hepatoma cell lines (11). Our results demonstrate that the modification of E1 and E2 proteins in the glycosylation steps in the ER is required to confer infectivity to HCVrv and HCVcc. The presence of E1 and E2 proteins on the surrogate viruses and HCVcc possessing high-mannose glycans indicate that these viruses are not released through the *trans*-Golgi network. In the case of West Nile virus, mature particles propagated in mammalian cells possess complex types of carbohydrates, in contrast to those generated in insect cells, which have high-mannose glycans (16). We still do not know the exact nature of modifications of the mature envelope proteins on authentic HCV particles. Further studies of the relationship between the modification of HCV envelope proteins and their infectivity are needed to clarify the life cycle of HCV. The neutralizing activity of antibodies against HCV have been assessed in the past using HCVpv (10, 43), HCVpp (3, 33, 42), and HCVcc (63, 65), as well as by the inhibition of binding of purified E2 protein to hCD81 (24, 53) and of HCV-LP to target cells (59). Sera from patients chronically infected with HCV and experimentally infected chimpanzees were shown to specifically neutralize HCVpp infection (3, 33, 42). In the present study, sera from patients infected with genotype 1b of HCV and anti-E2 monoclonal antibodies exhibited high levels of neutralization activity against infection with both HCVpv and HCVrv bearing HCV envelope proteins of genotypes 1a and 1b. One of the characteristics of HCV infection is the establishment of a persistent infection. Therefore, the high prevalence of neutralizing antibodies to the surrogate viruses and HCVcc suggests that HCV particles exhibiting similar phenotypes to surrogate viruses and HCVcc would be easily eliminated by neutralizing antibodies and thus not be able to participate in the establishment of a persistent infection. Recently, it was reported that HCV escapes from neutralizing antibody and T-cell responses by the continuous generation of escape

variants during chronic infection (51, 62). However, it was demonstrated that viral clearance in acute HCV infection was not correlated with the presence of neutralizing antibodies against HCVpp (33, 42), and 75% of HCVpp bearing HCV envelope proteins of various genotypes are not infectious (29). Therefore, it is reasonable to speculate that HCV particles exhibiting characteristics similar to those of the surrogate viruses are produced in large numbers and act as decoys in HCV patients, eliciting strong neutralizing antibodies against the viruses, and that a small portion of HCV particles exhibiting characteristics different from those of the surrogate viruses may participate in the establishment of persistent infection by escaping from the host immune surveillance system. The authenticity of the surrogate virus systems for the study of HCV infection remains controversial, and further studies are needed to clarify their profiles.

In conclusion, we generated replication-incompetent HCVpv and replication-competent HCVrv possessing HCV envelope proteins as novel surrogate models for the study of HCV. HCVpv and HCVrv were shown to have infection mechanisms similar to those of HCVpp and HCVcc. HCVrv has the following advantages compared to HCVcc: (i) infectious particles bearing HCV envelope proteins of various genotypes are capable of generating in various cell lines or primary cells, in contrast to the strict restriction of generating the infectious HCVcc in the Huh7-derived cell lines; (ii) isolation of escape mutants carrying mutations in the envelope proteins under various pressures may be easily obtained due to the higher replication efficiency than that of HCVcc; and (iii) in vivo investigation of the HCV envelope proteins for entry using humanized mice with human Hc and for immunogenicity for a future vaccine development are possible. Therefore, replication-competent HCVrv established in this study may provide valuable tools not only for understanding the entry mechanisms of HCV in a manner that is cell type and species dependent but also for developing novel therapeutics and vaccines.

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REFERENCES

1. **Agnello, V., G. Abel, M. Elfahal, G. B. Knight, and Q. X. Zhang.** 1999. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. Proc. Natl. Acad. Sci. USA **96**:12766–12771.
2. **Barth, H., C. Schafer, M. I. Adah, F. Zhang, R. J. Linhardt, H. Toyoda, A. Kinoshita-Toyoda, T. Toida, T. H. Van Kuppevelt, E. Depla, F. Von Weizsacker, H. E. Blum, and T. F. Baumert.** 2003. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J. Biol. Chem. **278**:41003–41012.
3. **Bartosch, B., J. Bukh, J. C. Meunier, C. Granier, R. E. Engle, W. C. Blackwelder, S. U. Emerson, F. L. Cosset, and R. H. Purcell.** 2003. In vitro assay for neutralizing antibody to hepatitis C virus: evidence for broadly conserved neutralization epitopes. Proc. Natl. Acad. Sci. USA **100**:14199–14204.
4. **Bartosch, B., and F. L. Cosset.** 2006. Cell entry of hepatitis C virus. Virology **348**:1–12.
5. **Bartosch, B., J. Dubuisson, and F. L. Cosset.** 2003. Infectious hepatitis C virus pseudo-particles containing functional E1–E2 envelope protein complexes. J. Exp. Med. **197**:633–642.
6. **Bartosch, B., A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, and F. L. Cosset.** 2003. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J. Biol. Chem. **278**:41624–41630.
7. **Baumert, T. F., S. Ito, D. T. Wong, and T. J. Liang.** 1998. Hepatitis C virus structural proteins assemble into viruslike particles in insect cells. J. Virol. **72**:3827–3836.
8. **Blanchard, E., S. Belouzard, L. Goueslain, T. Wakita, J. Dubuisson, C. Wychowski, and Y. Rouille.** 2006. Hepatitis C virus entry depends on clathrin-mediated endocytosis. J. Virol. **80**:6964–6972.
9. **Buonocore, L., K. J. Blight, C. M. Rice, and J. K. Rose.** 2002. Characterization of vesicular stomatitis virus recombinants that express and incorporate high levels of hepatitis C virus glycoproteins. J. Virol. **76**:6865–6872.
10. **Burioni, R., Y. Matsuura, N. Mancini, H. Tani, T. Miyamura, P. E. Varaldo, and M. Clementi.** 2002. Diverging effects of human recombinant anti-hepatitis C virus (HCV) antibody fragments derived from a single patient on the infectivity of a vesicular stomatitis virus/HCV pseudotype. J. Virol. **76**:11775–11779.
11. **Chapel, C., C. Garcia, P. Roingeard, N. Zitzmann, J. Dubuisson, R. A. Dwek, C. Trepo, F. Zoulim, and D. Durantel.** 2006. Antiviral effect of alpha-glucosidase inhibitors on viral morphogenesis and binding properties of hepatitis C virus-like particles. J. Gen. Virol. **87**:861–871.
12. **Choukhi, A., S. Ung, C. Wychowski, and J. Dubuisson.** 1998. Involvement of endoplasmic reticulum chaperones in the folding of hepatitis C virus glycoproteins. J. Virol. **72**:3851–3858.
13. **Clayton, R. F., A. Owsianka, J. Aitken, S. Graham, D. Bhella, and A. H. Patel.** 2002. Analysis of antigenicity and topology of E2 glycoprotein present on recombinant hepatitis C virus-like particles. J. Virol. **76**:7672–7682.
14. **Cormier, E. G., F. Tsamis, F. Kajumo, R. J. Durso, J. P. Gardner, and T. Dragic.** 2004. CD81 is an entry coreceptor for hepatitis C virus. Proc. Natl. Acad. Sci. USA **101**:7270–7274.
15. **Courageot, M. P., M. P. Frenkiel, C. D. Dos Santos, V. Deubel, and P. Despres.** 2000. Alpha-glucosidase inhibitors reduce dengue virus production by affecting the initial steps of virion morphogenesis in the endoplasmic reticulum. J. Virol. **74**:564–572.
16. **Davis, C. W., H. Y. Nguyen, S. L. Hanna, M. D. Sanchez, R. W. Doms, and T. C. Pierson.** 2006. West Nile virus discriminates between DC-SIGN and DC-SIGNR for cellular attachment and infection. J. Virol. **80**:1290–1301.
17. **Dubuisson, J.** 2000. Folding, assembly and subcellular localization of hepatitis C virus glycoproteins. Curr. Top. Microbiol. Immunol. **242**:135–148.
18. **Evans, M. J., T. von Hahn, D. M. Tscherne, A. J. Syder, M. Panis, B. Wolk, T. Hatziioannou, J. A. McKeating, P. D. Bieniasz, and C. M. Rice.** 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature **446**:801–805.
19. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83**:8122–8126.
20. **Garbutt, M., R. Liebscher, V. Wahl-Jensen, S. Jones, P. Moller, R. Wagner, V. Volchkov, H. D. Klenk, H. Feldmann, and U. Stroher.** 2004. Properties of replication-competent vesicular stomatitis virus vectors expressing glycoproteins of filoviruses and arenaviruses. J. Virol. **78**:5458–5465.
21. **Gardner, J. P., R. J. Durso, R. R. Arrigale, G. P. Donovan, P. J. Maddon, T. Dragic, and W. C. Olson.** 2003. L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. Proc. Natl. Acad. Sci. USA **100**:4498–4503.
22. **Hamamoto, I., Y. Nishimura, T. Okamoto, H. Aizaki, M. Liu, Y. Mori, T. Abe, T. Suzuki, M. M. Lai, T. Miyamura, K. Moriishi, and Y. Matsuura.** 2005. Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. J. Virol. **79**:13473–13482.
23. **Hsu, M., J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc. Natl. Acad. Sci. USA **100**:7271–7276.
24. **Ishii, K., D. Rosa, Y. Watanabe, T. Katayama, H. Harada, C. Wyatt, K. Kiyosawa, H. Aizaki, Y. Matsuura, M. Houghton, S. Abrignani, and T. Miyamura.** 1998. High titers of antibodies inhibiting the binding of envelope to human cells correlate with natural resolution of chronic hepatitis C. Hepatology **28**:1117–1120.
25. **Jeetendra, E., K. Ghosh, D. Odell, J. Li, H. P. Ghosh, and M. A. Whitt.** 2003. The membrane-proximal region of vesicular stomatitis virus glycoprotein G ectodomain is critical for fusion and virus infectivity. J. Virol. **77**:12807–12818.
26. **Kanda, T., A. Basu, R. Steele, T. Wakita, J. S. Ryerse, R. Ray, and R. B. Ray.** 2006. Generation of infectious hepatitis C virus in immortalized human hepatocytes. J. Virol. **80**:4633–4639.
27. **Lagging, L. M., K. Meyer, R. J. Owens, and R. Ray.** 1998. Functional role of hepatitis C virus chimeric glycoproteins in the infectivity of pseudotyped virus. J. Virol. **72**:3539–3546.
28. **Lavillette, D., Y. Morice, G. Germanidis, P. Donot, A. Soulier, E. Pagkalos, G. Sakellariou, L. Intrator, B. Bartosch, J. M. Pawlotsky, and F. L. Cosset.**

2005. Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection. J. Virol. **79**:6023–6034.
29. **Lavillette, D., A. W. Tarr, C. Voisset, P. Donot, B. Bartosch, C. Bain, A. H. Patel, J. Dubuisson, J. K. Ball, and F. L. Cosset.** 2005. Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus. Hepatology **41**:265–274.
30. **Lawson, N. D., E. A. Stillman, M. A. Whitt, and J. K. Rose.** 1995. Recombinant vesicular stomatitis viruses from DNA. Proc. Natl. Acad. Sci. USA **92**:4477–4481.
31. **Lichty, B. D., A. T. Power, D. F. Stojdl, and J. C. Bell.** 2004. Vesicular stomatitis virus: re-inventing the bullet. Trends Mol. Med. **10**:210–216.
32. **Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice.** 2005. Complete replication of hepatitis C virus in cell culture. Science **309**:623–626.
33. **Logvinoff, C., M. E. Major, D. Oldach, S. Heyward, A. Talal, P. Balfe, S. M. Feinstone, H. Alter, C. M. Rice, and J. A. McKeating.** 2004. Neutralizing antibody response during acute and chronic hepatitis C virus infection. Proc. Natl. Acad. Sci. USA **101**:10149–10154.
34. **Lozach, P. Y., H. Lortat-Jacob, A. de Lacroix de Lavalette, I. Staropoli, S. Foung, A. Amara, C. Houles, F. Fieschi, O. Schwartz, J. L. Virelizier, F. Arenzana-Seisdedos, and R. Altmeyer.** 2003. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. J. Biol. Chem. **278**:20358–20366.
35. **Majid, A. M., H. Ezelle, S. Shah, and G. N. Barber.** 2006. Evaluating replication-defective vesicular stomatitis virus as a vaccine vehicle. J. Virol. **80**: 6993–7008.
36. **Major, M. E., B. Rehermann, and S. M. Feinstone.** 2001. Hepatitis C viruses, p. 1127–1162. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
37. **Matsuo, E., H. Tani, C. K. Lim, Y. Komoda, T. Okamoto, H. Miyamoto, K. Moriishi, S. Yagi, A. H. Patel, T. Miyamura, and Y. Matsuura.** 2006. Characterization of HCV-like particles produced in a human hepatoma cell line by a recombinant baculovirus. Biochem. Biophys. Res. Commun. **340**:200–208.
38. **Matsuura, Y., T. Suzuki, R. Suzuki, M. Sato, H. Aizaki, I. Saito, and T. Miyamura.** 1994. Processing of E1 and E2 glycoproteins of hepatitis C virus expressed in mammalian and insect cells. Virology **205**:141–150.
39. **Matsuura, Y., H. Tani, K. Suzuki, T. Kimura-Someya, R. Suzuki, H. Aizaki, K. Ishii, K. Moriishi, C. S. Robison, M. A. Whitt, and T. Miyamura.** 2001. Characterization of pseudotype VSV possessing HCV envelope proteins. Virology **286**:263–275.
40. **Meertens, L., C. Bertaux, and T. Dragic.** 2006. Hepatitis C virus entry requires a critical postinternalization step and delivery to early endosomes via clathrin-coated vesicles. J. Virol. **80**:11571–11578.
41. **Mehta, A., N. Zitzmann, P. M. Rudd, T. M. Block, and R. A. Dwek.** 1998. Alpha-glucosidase inhibitors as potential broad based anti-viral agents. FEBS Lett. **430**:17–22.
42. **Meunier, J. C., R. E. Engle, K. Faulk, M. Zhao, B. Bartosch, H. Alter, S. U. Emerson, F. L. Cosset, R. H. Purcell, and J. Bukh.** 2005. Evidence for cross-genotype neutralization of hepatitis C virus pseudo-particles and enhancement of infectivity by apolipoprotein C1. Proc. Natl. Acad. Sci. USA **102**:4560–4565.
43. **Meyer, K., A. Basu, C. T. Przysiecki, L. M. Lagging, A. M. Di Bisceglie, A. J. Conley, and R. Ray.** 2002. Complement-mediated enhancement of antibody function for neutralization of pseudotype virus containing hepatitis C virus E2 chimeric glycoprotein. J. Virol. **76**:2150–2158.
44. **Moriishi, K., and Y. Matsuura.** 2003. Mechanisms of hepatitis C virus infection. Antivir. Chem. Chemother. **14**:285–297.
45. **Niwa, H., K. Yamamura, and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108**:193–199.
46. **Ogino, M., H. Ebihara, B. H. Lee, K. Araki, A. Lundkvist, Y. Kawaoka, K. Yoshimatsu, and J. Arikawa.** 2003. Use of vesicular stomatitis virus pseudotypes bearing hantaan or seoul virus envelope proteins in a rapid and safe neutralization test. Clin. Diagn. Lab. Immunol. **10**:154–160.
47. **Okamoto, T., Y. Nishimura, T. Ichimura, K. Suzuki, T. Miyamura, T. Suzuki, K. Moriishi, and Y. Matsuura.** 2006. Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. EMBO J. **25**:5015–5025.
48. **Op De Beeck, A., C. Voisset, B. Bartosch, Y. Ciczora, L. Cocquerel, Z. Keck, S. Foung, F. L. Cosset, and J. Dubuisson.** 2004. Characterization of functional hepatitis C virus envelope glycoproteins. J. Virol. **78**:2994–3002.
49. **Owsianka, A., A. W. Tarr, V. S. Juttla, D. Lavillette, B. Bartosch, F. L. Cosset, J. K. Ball, and A. H. Patel.** 2005. Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. J. Virol. **79**:11095–11104.
50. **Pavlovic, D., D. C. Neville, O. Argaud, B. Blumberg, R. A. Dwek, W. B. Fischer, and N. Zitzmann.** 2003. The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. Proc. Natl. Acad. Sci. USA **100**:6104–6108.
51. **Pestka, J. M., M. B. Zeisel, E. Blaser, P. Schurmann, B. Bartosch, F. L. Cosset, A. H. Patel, H. Meisel, J. Baumert, S. Viazov, K. Rispeter, H. E. Blum, M. Roggendorf, and T. F. Baumert.** 2007. Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. Proc. Natl. Acad. Sci. USA **104**:6025–6030.
52. **Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, and S. Abrignani.** 1998. Binding of hepatitis C virus to CD81. Science **282**:938–941.
53. **Rosa, D., S. Campagnoli, C. Moretto, E. Guenzi, L. Cousens, M. Chin, C. Dong, A. J. Weiner, J. Y. Lau, Q. L. Choo, D. Chien, P. Pileri, M. Houghton, and S. Abrignani.** 1996. A quantitative test to estimate neutralizing antibodies to the hepatitis C virus: cytofluorimetric assessment of envelope glycoprotein 2 binding to target cells. Proc. Natl. Acad. Sci. USA **93**:1759–1763.
54. **Rose, J. K., L. Buonocore, and M. A. Whitt.** 1991. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. BioTechniques **10**:520–525.
55. **Sandrin, V., P. Boulanger, F. Penin, C. Granier, F. L. Cosset, and B. Bartosch.** 2005. Assembly of functional hepatitis C virus glycoproteins on infectious pseudoparticles occurs intracellularly and requires concomitant incorporation of E1 and E2 glycoproteins. J. Gen. Virol. **86**:3189–3199.
56. **Sandrin, V., D. Muriaux, J. L. Darlix, and F. L. Cosset.** 2004. Intracellular trafficking of Gag and Env proteins and their interactions modulate pseudotyping of retroviruses. J. Virol. **78**:7153–7164.
57. **Scarselli, E., H. Ansuini, R. Cerino, R. M. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, and A. Vitelli.** 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J. **21**:5017–5025.
58. **Schneider-Schaulies, J.** 2000. Cellular receptors for viruses: links to tropism and pathogenesis. J. Gen. Virol. **81**:1413–1429.
59. **Steinmann, D., H. Barth, B. Gissler, P. Schurmann, M. I. Adah, J. T. Gerlach, G. R. Pape, E. Depla, D. Jacobs, G. Maertens, A. H. Patel, G. Inchauspe, T. J. Liang, H. E. Blum, and T. F. Baumert.** 2004. Inhibition of hepatitis C virus-like particle binding to target cells by antiviral antibodies in acute and chronic hepatitis C. J. Virol. **78**:9030–9040.
60. **Tamura, K., A. Oue, A. Tanaka, N. Shimizu, H. Takagi, N. Kato, A. Morikawa, and H. Hoshino.** 2005. Efficient formation of vesicular stomatitis virus pseudotypes bearing the native forms of hepatitis C virus envelope proteins detected after sonication. Microbes Infect. **7**:29–40.
61. **Tscherne, D. M., C. T. Jones, M. J. Evans, B. D. Lindenbach, J. A. McKeating, and C. M. Rice.** 2006. Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. J. Virol. **80**:1734–1741.
62. **von Hahn, T., J. C. Yoon, H. Alter, C. M. Rice, B. Rehermann, P. Balfe, and J. A. McKeating.** 2007. Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. Gastroenterology **132**:667–678.
63. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11**:791–796.
64. **Wu, S. F., C. J. Lee, C. L. Liao, R. A. Dwek, N. Zitzmann, and Y. L. Lin.** 2002. Antiviral effects of an iminosugar derivative on flavivirus infections. J. Virol. **76**:3596–3604.
65. **Yi, M., R. A. Villanueva, D. L. Thomas, T. Wakita, and S. M. Lemon.** 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc. Natl. Acad. Sci. USA **103**:2310–2315.
66. **Yu, M. Y., B. Bartosch, P. Zhang, Z. P. Guo, P. M. Renzi, L. M. Shen, C. Granier, S. M. Feinstone, F. L. Cosset, and R. H. Purcell.** 2004. Neutralizing antibodies to hepatitis C virus (HCV) in immune globulins derived from anti-HCV-positive plasma. Proc. Natl. Acad. Sci. USA **101**:7705–7710.
67. **Zhang, J., G. Randall, A. Higginbottom, P. Monk, C. M. Rice, and J. A. McKeating.** 2004. CD81 is required for hepatitis C virus glycoprotein-mediated viral infection. J. Virol. **78**:1448–1455.
68. **Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari.** 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. USA **102**:9294–9299.
69. **Zhong, J., P. Gastaminza, J. Chung, Z. Stamataki, M. Isogawa, G. Cheng, J. A. McKeating, and F. V. Chisari.** 2006. Persistent hepatitis C virus infection in vitro: coevolution of virus and host. J. Virol. **80**:11082–11093.

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Involvement of Dendritic Cell Frequency and Function in Virological Relapse in Pegylated Interferon-α and Ribavirin Therapy for Chronic Hepatitis C Patients

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A combination of pegylated interferon α (PEG-IFNα) and ribavirin has been used widely. Enhancement of immune response against hepatitis C virus (HCV) is known to be involved in the efficacy of the combination therapy. The aim of the study was to elucidate whether the frequency or function of immunocompetent blood cells is related to the outcome of the therapy. Twenty-five chronic hepatitis C patients with high viral load of HCV genotype 1 who underwent 48 weeks of PEG-IFNα2b and ribavirin therapy were examined. During the treatment, frequencies of dendritic cell subsets, helper T cell subsets, and NK cells were phenotypically determined. In some patients, the ability of dendritic cells to stimulate allogeneic $CD4^+$T cells was examined at the end and after the therapy. Among the 25 patients, 11 showed a sustained virological response, 11 a transient response, and 3 no response. In comparison with sustained virological responders, non-sustained virological responders showed impaired dendritic cell function at the end and after the treatment. The transient responders showed a decline of plasmacytoid dendritic cell frequency from Weeks 1–12 and impaired dendritic cell function as well. Even in patients who attained negative serum HCV RNA at Week 12, the transient responders showed a significant decrease of plasmacytoid dendritic cell frequency and impaired dendritic cell function. In conclusion, in PEG-IFNα and ribavirin combination therapy for chronic hepatitis C patients, the early-phase plasmacytoid dendritic cell frequency and/or end-of-treatment dendritic cell function are related to the virological outcome of the therapy.
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KEY WORDS: chronic hepatitis C; PEG interferon; ribavirin; dendritic cell

INTRODUCTION

Hepatitis C virus (HCV) infection causes various types of liver diseases including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [Seeff, 2002]. The most effective way to prevent the progression of disease is to eradicate HCV from the infected hosts [Alter et al., 1989]. At present, combination therapy with pegylated interferon alpha (PEG-IFNα) and ribavirin is considered as the standard treatment for chronic HCV infection. The rate of the sustained virological response achieved by the combination therapy has been up to 50% in patients with HCV genotype 1 and a high HCV RNA titer; however, half of the patients do not attain sustained virological response [Manns et al., 2001; Fried et al., 2002]. In addition to HCV genotype and HCV quantity, several factors have been reported as

Abbreviations: HCV, hepatitis C virus; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cells; NK, natural killer; MLR, mixed leukocyte reaction

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therapeutic determinants in PEG-IFNα and ribavirin combination therapy, such as liver fibrosis, age, gender, and ethnicity [Manesis et al., 1997; Poynard et al., 1998; Jacobson et al., 2005]. It is accepted that initial changes of serum HCV RNA titer from the beginning of the therapy, i.e., HCV dynamics, correlates well with the clinical outcomes of the treated patients [Davis et al., 2003; Hayashi and Takehara, 2006]. In PEG-IFNα and ribavirin therapy, an early virological response is defined as a reduction in serum HCV RNA quantity by at least 2 $\log_{10}$ units or to an undetectable level by a sensitive qualitative PCR after the first 12 weeks of the treatment or negative serum HCV RNA at Week 24 of the therapy [Davis et al., 2003]. It has been reported that the patients who fail to attain early virological response at Week 12 or 24 are not likely to gain sustained virological response after 48 weeks of the combination therapy, suggesting that early virological response can serve as a negative predictor of sustained virological response [Ferenci, 2004; Ferenci et al., 2005]. Prolongation of the duration of PEG-IFNα and ribavirin combination therapy from 48–72 weeks is likely to improve sustained virological response rate by decreasing relapsers [Berg et al., 2006]. Therefore, identifying potential relapsers during therapy and providing additional weeks of treatment may be clinically important, since it can offer them a better chance of attaining sustained virological response. However, no reliable marker is currently available for predicting virological relapse in PEG-IFNα and ribavirin therapy.

In chronic hepatitis C, multifaceted immune dysfunction may be implicated in the persistence of HCV including dendritic cells, NK cells, and T cells [Kanto et al., 1999; Auffermann-Gretzinger et al., 2001; Rosen et al., 2002; Nattermann et al., 2006]. It is reported that sustained viral responders maintained vigorous and multispecific HCV-specific $CD4^+$ Th1 responses, suggesting that the restoration of $CD4^+$ T cell responses may be related to successful HCV eradication [Kamal et al., 2002]. However, it is not known whether the frequency or the function of other immune cells during the combination therapy has any relationship to the therapy outcome.

In the present study, in order to determine immunological markers correlated with the efficacy of the treatment, the frequency of peripheral blood cell subsets and their dynamics were studied during and after the combination therapy. The function of dendritic cells from the patients was examined to clarify whether it was correlated with the therapeutic efficacy. This study supports the view that the reactivity of the immune system to the combination therapy is involved critically in the outcome of the treatment.

MATERIALS AND METHODS

Patients

Among chronic hepatitis C patients who had been followed at Osaka University Hospital, Osaka Koseinenkin Hospital, and Osaka National Hospital, 32 patients who received PEG-IFNα2b and ribavirin combination therapy for 48 weeks were enrolled in the present study. The study was approved by the ethical committee of the Osaka University Graduate School of Medicine. Written informed consent was obtained from all patients. At enrollment, the patients were confirmed to be positive for both serum anti-HCV antibody and HCV RNA, but were negative for other viral infections, including hepatitis B virus and human immunodeficiency virus. All the patients were infected with HCV genotype 1b with a serum HCV RNA quantity of more than 100 kilocopies/ml, as determined by methods described elsewhere [Pawlotsky et al., 2000]. All patients had shown persistent or fluctuating serum alanine aminotransferase abnormalities at enrollment. The presence of other causes of liver disease, such as autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, alcohol abuse, and metabolic disorders was excluded by laboratory and imaging analyses. With all patients, a combination of biochemical markers and ultrasonography or computed tomography scan analyses ruled out the presence of cirrhosis and tumors in the liver. Histological analyses of liver disease were performed with liver tissue obtained by ultrasonography-guided biopsy. The activity and stage of the disease were assessed by two independent pathologists according to the classification proposed by Desmet [Desmet et al., 1994].

Study Design

All patients were treated with PEG-IFNα2b subcutaneously at a dose of 75 μg/week (body weight > 40 kg and ≤ 60 kg) or 105 μg/week (body weight > 60 kg and ≤ 80 kg) or 135 μg/week (body weight > 80 kg and ≤ 100 kg) and oral ribavirin at a dose of 600 mg/day (body weight > 40 kg and ≤ 60 kg) or 800 mg/day (body weight > 60 kg and ≤ 80 kg) or 1000 mg/day (body weight > 80 kg and ≤ 100 kg). Ribavirin was administered divided into two doses per day. All patients were treated for 48 weeks and followed for 24 weeks after the cessation of therapy. The early responders were defined as those who showed a reduction in serum HCV RNA quantity to an undetectable level by qualitative PCR at Week 12 of the therapy. Virological response was estimated at 24 weeks after cessation of the treatment. Sustained virological response was defined as the maintenance of negative serum HCV RNA by PCR for more than 6 months after completion of the therapy. Transient response was defined as the reappearance of serum HCV RNA within 6 months after cessation of therapy in patients who had achieved negative serum HCV RNA at the end of the treatment. No response meant that there was persistently positive serum HCV RNA throughout the therapy period. Non-sustained virological response group is comprised of transient responders and no responders.

Analysis of Dendritic Cell Subsets, Helper T Cells, and NK Cells

For the numerical analyses of blood dendritic cells, helper T cells, and NK cells, venous blood was drawn

from patients before treatment and at Weeks 1, 4, 8, 12, 24, and 48 during the therapy. Peripheral blood mononuclear cells (PBMCs) were collected by density-gradient centrifugation on a Ficoll–Hypaque cushion. After viable PBMCs had been counted, the cells were stained with combinations of various antibodies for phenotypic markers.

The following monoclonal antibodies were purchased from BD Biosciences (San Jose, CA): anti-lineagemarker (Lin; CD3 (clone SK7), CD14 (clone MφP9), CD16 (clone 3G8), CD19 (clone SJ25C1), CD20 (clone L27), and CD56 (clone NCAM16.2)), anti-CD4 (clone RPA-T4), anti-CD11c (clone B-ly6), anti-CD123 (clone 7G3), anti-CD3 (clone UCHT1), anti-CD45RO (clone UCHL1), anti-CD56 (clone B159), anti-HLA-DR (clone L243), anti-CCR4 (clone 1G1). Anti-CXCR3 (clone 49801) monoclonal antibody was purchased from R&D Systems (Minneapolis, MN). Staining was performed with FITC, PE, PerCP, and APC conjugated antibodies as described previously. The acquisitions and analyses of data were performed with FACSCalibur (BD Biosciences) and CellQuest software.

Blood dendritic cells were defined as Lin^- and HLA-DR^+ cells. Myeloid dendritic cells are Lin^-, HLA-DR^+, $CD11c^+$, $CD123^{low}$ cells, and plasmacytoid dendritic cells are Lin^-, HLA-DR^+, $CD11c^-$, and $CD123^{high}$ cells, respectively. Helper T cell subpopulations were defined by the pattern of CXCR3 and CCR4; Th1 cells are $CD4^+$, $CD45RO^+$, $CXCR3^+$, and Th2 cells are $CD4^+$, $CD45RO^+$, and $CCR4^+$, respectively. NK cells were defined as $CD3^-$, $CD56^+$ cells. The percentages of dendritic cell subsets and NK cells in PBMCs or Th1 and Th2 cells in $CD4^+$ T cells were determined by FACS. In order to examine the dynamics of dendritic cell subsets after initiation of the treatment, we used the ratio of frequencies at each time point to those before the therapy.

Allogeneic Mixed Leukocyte Reaction With Dendritic Cells

In some patients, we examined whether the allostimulatory ability of dendritic cells was related to the clinical outcomes. At the end of treatment and at Week 4 after completion of the treatment, monocyte-derived dendritic cells were generated from PBMC obtained from the patients according to methods reported previously [Romani et al., 1994]. As controls, monocyte-derived dendritic cells were generated simultaneously from healthy donors. As responder cells in mixed leukocyte reaction (MLR), naive $CD4^+$ T cells were isolated from PBMC of irrelevant healthy donors by using a naive $CD4^+$ T cell enrichment kit (Stemcell Technologies, Vancouver, BC). Allogeneic MLR with monocyte-derived dendritic cells was performed as reported previously [Kanto et al., 1999]. In order to compare the ability of monocyte-derived dendritic cells among patients, we determined the MLR ratio between patients and controls as counts per minute (cpm) of ^3H-thymidine incorporated into $CD4^+$ T cells at the T cell/dendritic cell ratio of 10/1.

Statistical Analyses

For statistical analysis, the non-parametric Mann–Whitney *U*-test was used between the groups. To analyze paired data, we used Wilcoxon's signed rank test. Differences of continuous variables between groups were compared by two-way ANOVA. *P*-values of less than 0.05 were considered to be statistically significant. These statistical analyses were performed with StatView software (Cary, NC).

RESULTS

Outcome of the PEG-IFNα and Ribavirin Therapy

Among the 32 patients who received PEG-IFNα2b and ribavirin combination therapy, 25 completed the therapy while 7 patients dropped out due to various adverse effects. Among the 25 patients who completed the therapy, 11 (44%) achieved sustained virological response, 11 (44%) showed transient response, and 3 (12%) showed no response (Table I). There was no difference in the baseline clinical parameters among these groups (Table I). With regard to HCV RNA at Week 12 in patients who completed the therapy, 11 were negative for HCV RNA (early responders), while the remaining 14 were not. Among 11 patients with early response, 7 were sustained virological responders and 4 were transient responders. Among 14 patients who were positive for serum HCV RNA at Week 12, 4 patients achieved sustained virological response, 7 showed transient response, and 3 showed no response. Details of the therapeutic response in the current study are shown in Figure 1.

TABLE I. Baseline Clinical Characteristics of the Patients

	All patients	SVR	TR	NR
Age[a]	50.0 ± 10.9	46.7 ± 12.4	54.1 ± 8.9	46.7 ± 9.3
Sex (M/F)	20/5	9/2	8/3	3/0
ALT (IU/l)[a]	99.3 ± 47.8	97.5 ± 50.9	103 ± 51.3	94.0 ± 34.6
HCV RNA (kilo copies/ml)[a]	3146 ± 2675	3685 ± 3023	2743 ± 2338	2647 ± 3163
Activity (minimal/mild/moderate)	7/7/11	5/3/3	1/4/6	1/0/2
Fibrosis (mild/moderate/severe)	11/12/2	6/5/0	3/7/1	2/0/1

ALT, alanine aminotransferase.
Histological activity and fibrosis were assessed according to the classification proposed by Desmet.
[a]Mean ± SD.

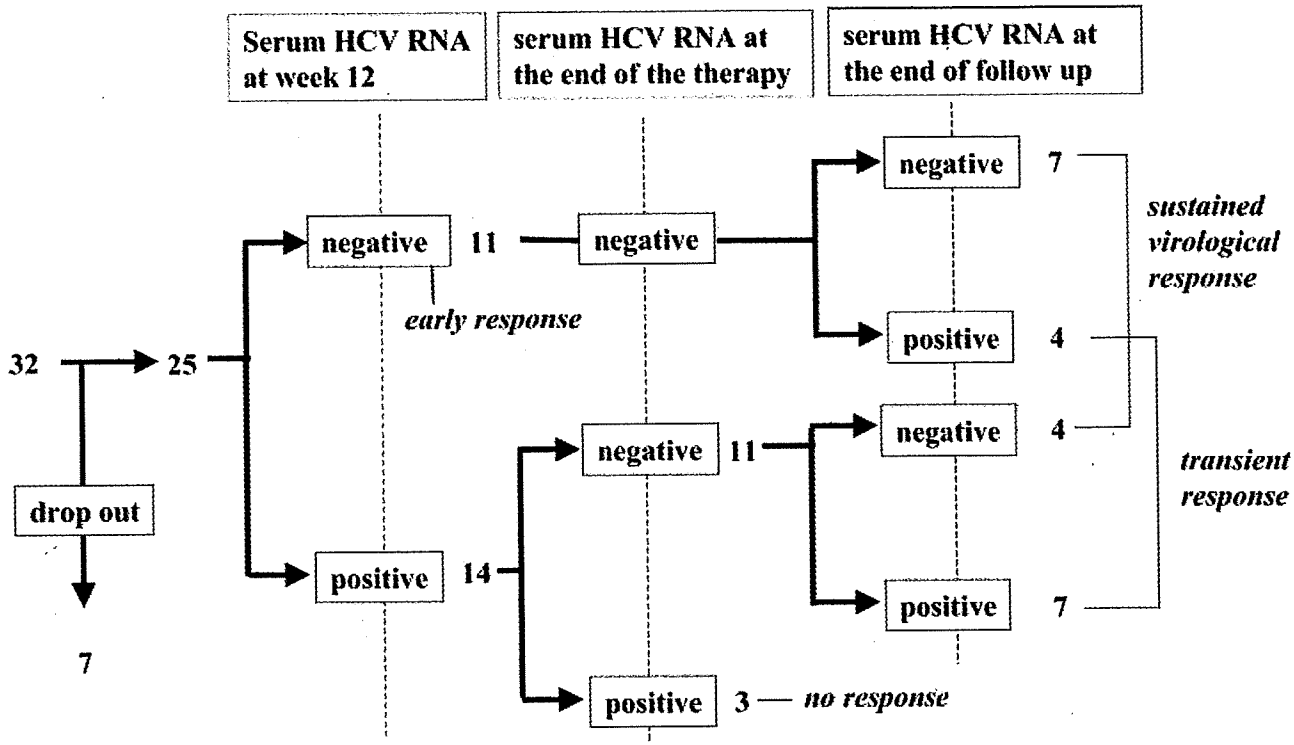

Fig. 1. Detailed outcomes of chronic hepatitis C patients treated with 48-week PEG-IFNα2b and ribavirin combination therapy. Thirty-two patients received the therapy, but seven dropped out due to various adverse effects. Among the 25 who completed the therapy, 11 achieved sustained virological response, 11 were transient responders, and 3 were non-responders. The early responders were defined as those who showed a reduction in HCV RNA quantity to an undetectable level by qualitative PCR at Week 12 of the therapy. According to this criterion, 11 patients were early responders and were further categorized into 7 sustained virological response (sustained virological responders with early response) and 4 transient response (transient responders with early response). Of the other 14 patients who were not early responders, 4 were sustained virological responders, 7 were transient responders, and 3 were non-responders.

Non-Sustained Virological Responders Had a Lower MLR Ratio Than Sustained Virological Responders

In order to clarify whether the frequency and function of immune cells are involved in the outcomes of the combination therapy, these parameters were compared between sustained virological responders and non-sustained virological responders, including transient responders and no responders. The pretreatment percentages of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1, and Th2 were not different between the sustained virological responders and non-sustained virological responders (Fig. 2A). As for the changes of dendritic cell subsets during the therapy, frequencies of both plasmacytoid dendritic cells and myeloid dendritic cells at each time point did not differ between sustained virological responders and non-sustained virological responders (Fig. 2B,C). The percentages of NK cells in non-sustained virological responders tended to be higher than those in sustained virological responders from Weeks 4–48, which did not reach statistical significance ($P = 0.0533$ ANOVA) (Fig. 2F). The frequencies of Th1 and Th2 did not differ between these two groups (Fig. 2G,H). As for dendritic cell function, dendritic cells from the non-sustained virological responders showed a lower MLR ratio than those from the sustained virological responders at the end ($P < 0.01$) and at 4 weeks after the completion of therapy ($P < 0.005$) (Fig. 3). These results show that lesser ability of dendritic cells at the end of treatment may be related to non-sustained virological response.

Transient Responders Had a Lower MLR Ratio in Dendritic Cell Function Than Sustained Virological Responders in the Course of Combination Therapy

In order to elucidate if the above-mentioned immunological markers are related to virological relapse, a

Fig. 2. Pretreatment frequency of blood cells and its changes during 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and non-sustained virological responders. Frequencies of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1 cells, and Th2 cells in the patients before the treatment (**A**), during the combination therapy (**B**, **C**, **F–H**) and the ratios of myeloid dendritic cell or plasmacytoid dendritic cell frequency (**D**, **E**) were determined as described in Materials and Methods, which were compared between sustained virological responders and non-sustained virological responders. Black bars (A) or closed triangles (B–H) depict sustained virological responders and white bars (A) or closed circles (B–H) depict non-sustained virological responders. The results are expressed as the mean ± SEM of 11 sustained virological responders and 14 non-sustained virological responders. PBMC, peripheral blood mononuclear cells; NK, natural killer.

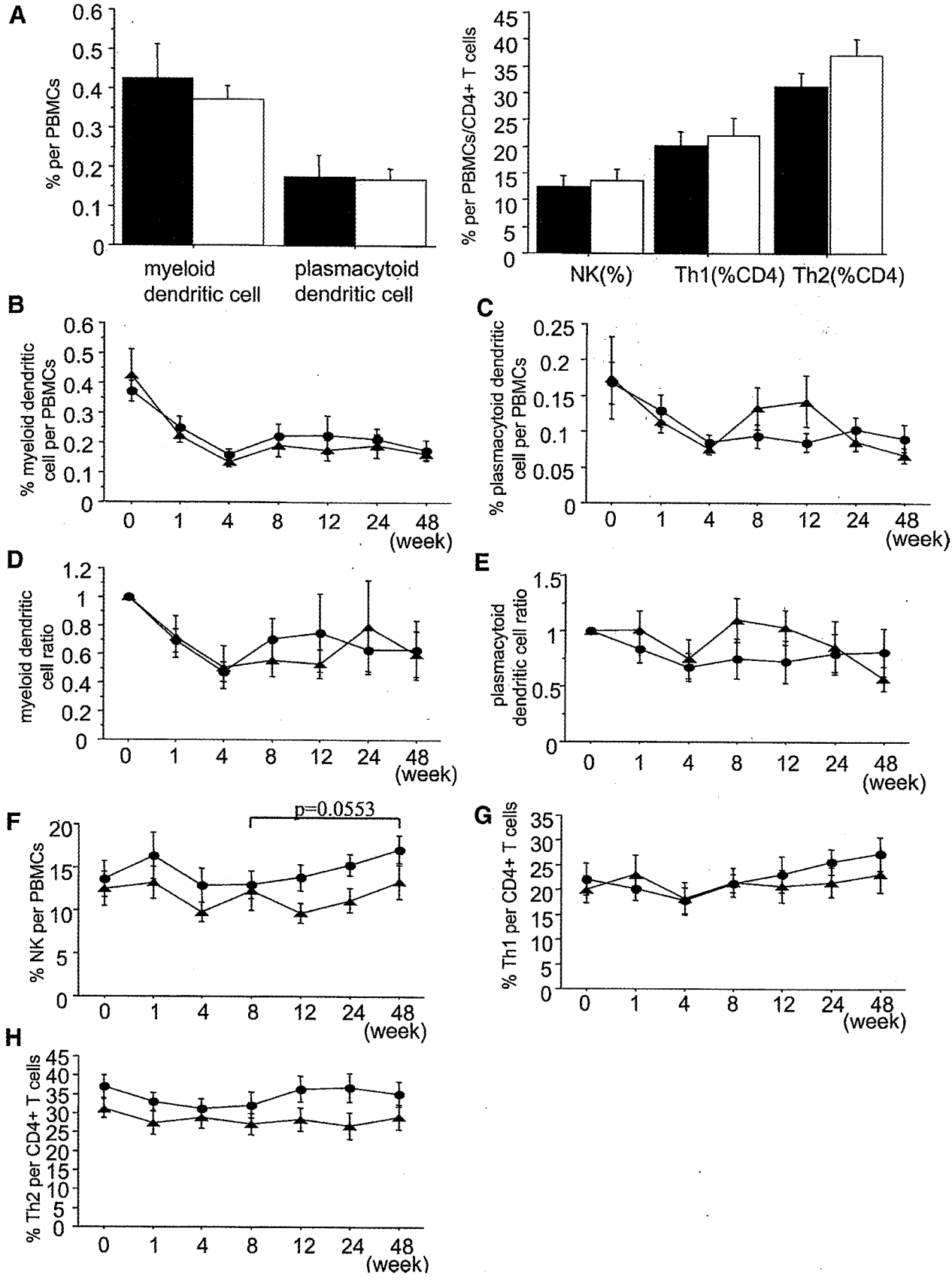
A
% per PBMCs
myeloid dendritic cell
plasmacytoid dendritic cell
% per PBMCs/CD4+ T cells
NK(%)
Th1(%CD4)
Th2(%CD4)
B
% myeloid dendritic cell per PBMCs
C
% plasmacytoid dendritic cell per PBMCs
D
myeloid dendritic cell ratio
E
plasmacytoid dendritic cell ratio
F
p=0.0553
% NK per PBMCs
G
% Th1 per CD4+ T cells
H
% Th2 per CD4+ T cells
0 1 4 8 12 24 48
(week)

Fig. 2.

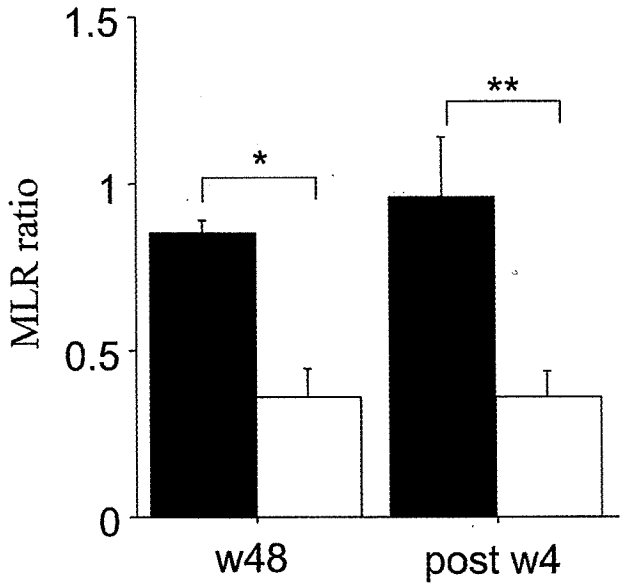

Fig. 3. Allostimulatory activity of dendritic cells in patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and non-sustained virological responders. At the end of treatment (Week 48) and at Week 4 after completion of the treatment, monocyte-derived dendritic cells were generated from the patients or healthy donors and their allostimulatory capacity was evaluated as described in Materials and Methods. The MLR ratio between patients and controls was determined from the counts per minute (cpm) of ^{3}H-thymidine incorporated into CD4$^+$ T cells at T cell/dendritic cell ratio of 10/1. The results are expressed as the mean ± SEM of 11 sustained virological responders and 14 non-sustained virological responders. Black bars indicate sustained virological responders and white bars indicate non-sustained virological responders. *$P < 0.01$, **$P < 0.005$.

comparison was undertaken between sustained virological responders and transient responders. The pretreatment percentages of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1, and Th2 were not different between the sustained virological responders and transient responders (Fig. 4A).

The percentages of myeloid dendritic cells and plasmacytoid dendritic cells were not different between the sustained virological responders and transient responders at each time point (Fig. 4B,C). The transient responders tended to show a lower plasmacytoid dendritic cell ratio than sustained virological responders from Weeks 1–12 ($P = 0.0553$, ANOVA) (Fig. 4E), suggesting that plasmacytoid dendritic cell is likely to decrease in the early phase in transient responders whereas those in sustained virological responders tend to be maintained. By contrast, no difference was observed in the myeloid dendritic cell ratio between the groups (Fig. 4D). The percentages of NK cells in transient responders were significantly higher than those in sustained virological responders from Weeks 8–48 ($P < 0.05$) (Fig. 4F). The frequencies of Th1 or Th2 at each point during therapy did not differ between the sustained virological responders and transient responders (Fig. 4G,H).

With regard to the dendritic cell function, the transient responders showed a lower MLR ratio than the sustained virological responders from Weeks 4–48 after the end of the therapy ($P < 0.05$) (Fig. 5). These results suggest that sustained impairment of dendritic cell function at the end and after the treatment may be related to the virological relapse after cessation of the therapy.

Early-Phase Decline of Plasmacytoid Dendritic Cell Frequency and Sustained Impairment of Dendritic Cell Ability Are Related to Transient Response in the Combination Therapy Even in Patients Who Lost Serum HCV RNA at Week 12 of the Treatment

In order to estimate more precisely the involvement of immunological markers in the outcomes of the combination therapy, we examined the above-mentioned parameters in patients who attained negative serum HCV RNA at Week 12 (early response group), as they were considered to be comparable with respect to the virological response to the therapy. Among 11 patients who were clear of serum HCV at Week 12, 7 were categorized into sustained virological response (sustained virological responders with early response) and the remaining 4 into transient response (transient responders with early response) (Fig. 1). Among patients with early response, the pretreatment percentages of myeloid dendritic cells, plasmacytoid dendritic cells, Th1, Th2, and NK cells (Fig. 6A) and those at any points during the therapy did not differ between sustained virological responders and transient responders (Fig. 6B,C,F–H). The plasmacytoid dendritic cell ratios in transient responders were lower than those in sustained virological responders from Weeks 1–12 ($P < 0.05$, ANOVA) (Fig. 6E), whereas the myeloid dendritic cell ratio did not differ between the groups (Fig. 6D).

As for MLR, dendritic cells from the transient responders showed a lower MLR ratio than those from the sustained virological responders at the end and at 4 weeks after the completion of therapy (Fig. 7) ($P < 0.001$).

DISCUSSION

In the PEG-IFNα and ribavirin therapy for chronic hepatitis C, viral and host factors are critically involved in the efficacy of treatment. As for viral factors, HCV

Fig. 4. Pretreatment frequency of blood cells and its changes during 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and transient responders. Frequencies of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1 cells, and Th2 cells in the patients before the treatment (**A**), during the combination therapy (**B, C, F–H**), and the ratios of myeloid dendritic cell or plasmacytoid dendritic cell frequency (**D, E**) were determined as described in Materials and Methods, which were compared between sustained virological responders and transient responders ones. Black bars (A) or closed triangles (B–H) depict sustained virological responders and white bars (A) or closed circles (B–H) depict transient responders. The results are expressed as the mean ± SEM of 11 sustained virological responders and 11 transient responders. PBMC, NK are shown in Figure 2. *$P < 0.05$ (sustained virological responders vs. transient responders).

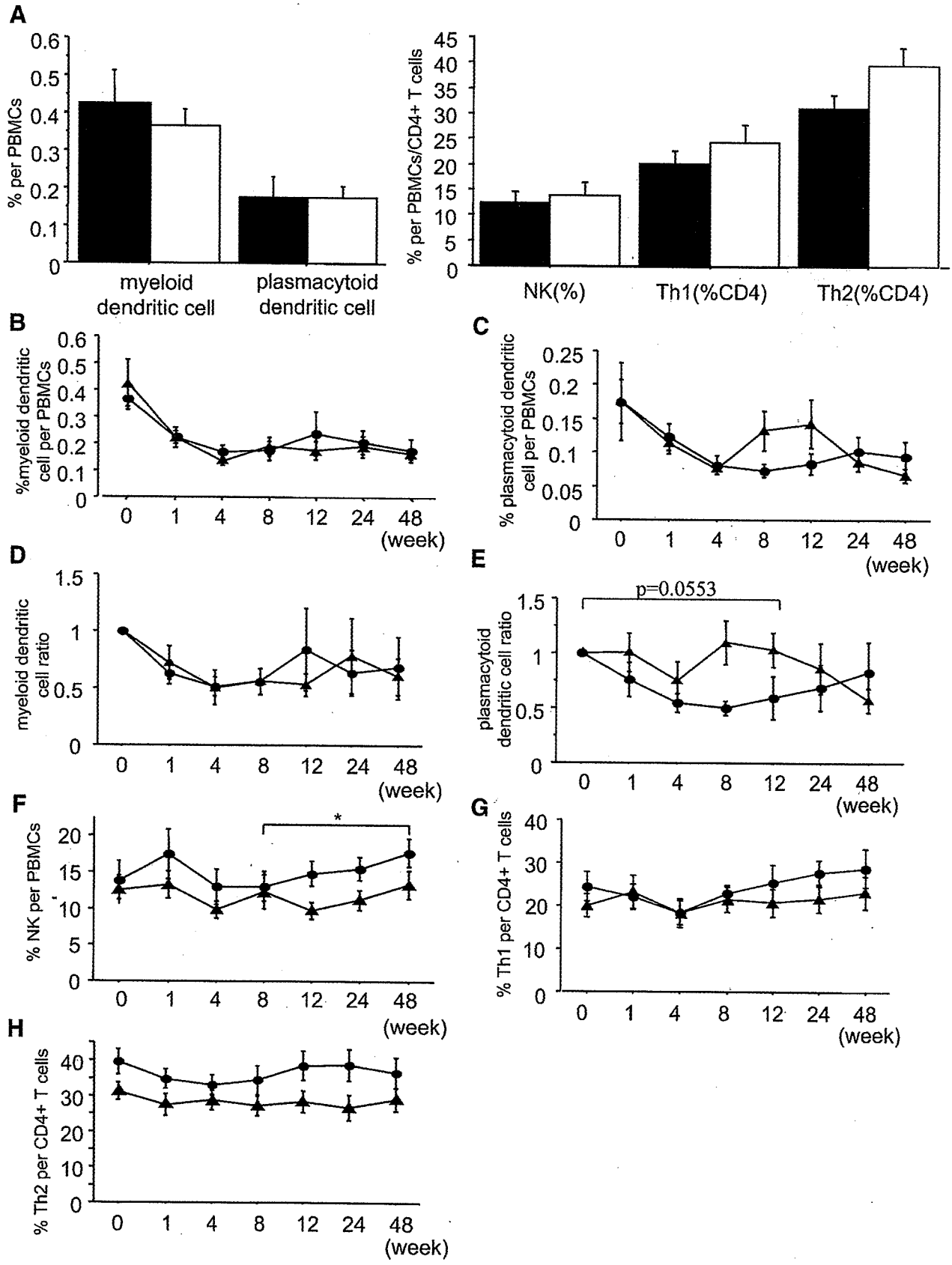
A
% per PBMCs
myeloid dendritic cell
plasmacytoid dendritic cell
% per PBMCs/CD4+ T cells
NK(%)
Th1(%CD4)
Th2(%CD4)
B
%myeloid dendritic cell per PBMCs
C
% plasmacytoid dendritic cell per PBMCs
D
myeloid dendritic cell ratio
E
p=0.0553
plasmacytoid dendritic cell ratio
F
% NK per PBMCs
*
G
% Th1 per CD4+ T cells
H
% Th2 per CD4+ T cells
0 1 4 8 12 24 48
(week)

Fig. 4.

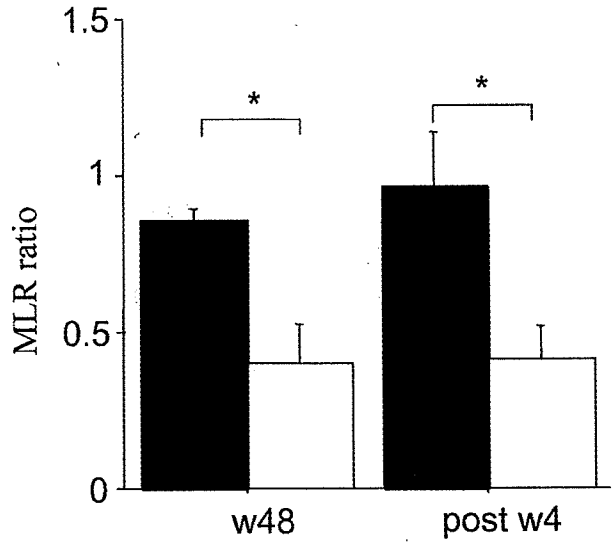

Fig. 5. Allostimulatory activity of dendritic cells in patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in sustained virological responders and transient responders. At the end of treatment (Week 48) and at Week 4 after completion of the treatment, monocyte-derived dendritic cells were generated from the patients or healthy donors and their allostimulatory capacity was evaluated as described in Materials and Methods. The MLR ratio between patients and controls was determined as the same as Figure 3. The results are expressed as the mean ± SEM of 11 sustained virological responders and 11 transient responders. Black bars indicate sustained virological responders and white bars indicate transient responders. *$P < 0.05$.

genotypes and baseline HCV RNA titers are major determinants dictating therapeutic outcomes. In addition, failure of rapid decline in serum HCV RNA from the beginning of the treatment, i.e., non-early virological response, has been used as a negative predictor for sustained virological response. Alternatively, the enhancement of immunity has been implicated to play a key role in the successful responses in PEG-IFNα and ribavirin therapy. However, it is yet to be determined which parameters are practically feasible for the assessment of treatment-induced immune responses correlating with therapeutic efficacy.

In the present study, it was determined whether the frequencies of dendritic cells, NK cells, Th1 and Th2 cells, as well as dendritic cell function in patients are related to the outcome of the PEG-IFNα and ribavirin therapy. By comparing these markers in the course of the treatment between sustained virological responders and non-sustained virological responders, it was demonstrated that non-sustained virological responders showed impaired dendritic cell function in MLR than sustained virological responders. When the analyses were extended to comparison between sustained virological responders and transient responders, transient responders exhibited (1) lower plasmacytoid dendritic cell ratio, (2) higher NK cell frequency, and (3) impaired dendritic cell function than sustained virological responders. Of particular interest were the findings of a lower plasmacytoid dendritic cell ratio as well as lower MLR even in transient responders with early response compared to sustained virological responders with early response. Since patients with early response are defined as those who showed negative serum HCV RNA at Week 12, they are considered to be similar in virological response to the combination therapy. Thus, such parameters could serve as immunological markers for virological relapse, presumably being independent of the early virological response.

In general, homeostasis of blood cell number is regulated by their life span and their recruitment from the bone marrow to circulating blood. A reduction of blood cell numbers is frequently observed in patients who are treated with PEG-IFNα and ribavirin combination therapy, which may be due to bone marrow suppression, enhancement of cellular apoptosis, or alteration of localization. However, the dynamics of dendritic cell subsets or NK cells under combination therapy is yet to be clarified. Some investigators have reported that the frequency or the absolute number of blood dendritic cell is dynamically changed by various stresses, such as infection [Hotchkiss et al., 2002] or surgery [Ho et al., 2001]. The present study showed that reduction of plasmacytoid dendritic cells after the introduction of combination therapy is much greater in the transient responders than in the sustained virological responders. IFNα is reported to act as a regulatory factor on $CD11c^-$ dendritic cells to sustain their viability and to inhibit gaining the ability to stimulate Th2 development [Ito et al., 2001]. Thus, patients who respond well to IFNα, as demonstrated by better plasmacytoid dendritic cell survival during the treatment, are likely to have better chances to eradicate HCV. Limited information is available about the factors influencing the number of NK cells. In chronic HCV infection, it has been reported that the progression of liver disease is associated with a decrease of peripheral as well as liver-residing NK cells [Kawarabayashi et al., 2000]. It is plausible that the lower frequency of peripheral NK cells in the sustained virological responders compared to the transient responders, as shown in this study, may be related to the accumulation of NK cells in the liver, where they presumably produce IFNγ to suppress HCV replication. Further study is needed to disclose the reasons for the dynamics of these cells being related to the virological response in the combination therapy.

Fig. 6. Pretreatment frequency of blood cells and changes during 48-week PEG-IFNα2b and ribavirin therapy in patients who showed negative serum HCV RNA at Week 12 of the therapy. Frequencies of myeloid dendritic cells, plasmacytoid dendritic cells, NK cells, Th1 cells, and Th2 cells in the patients before the treatment (**A**), during the combination therapy (**B**, **C**, **F–H**) and the ratios of myeloid dendritic cell or plasmacytoid dendritic cell frequency (**D**, **E**) were determined as described in Materials and Methods, which were compared between sustained virological responders and transient responders ones. Black bars (A) or closed triangles (B–H) depict sustained virological responders and white bars (A) or closed circles (B–H) depict transient responders. The results are expressed as the mean±SEM of seven sustained virological responders with early response and four transient responders with early response. PBMC, NK are shown in Figure 2. *$P < 0.05$ (sustained virological responders vs. transient responders).

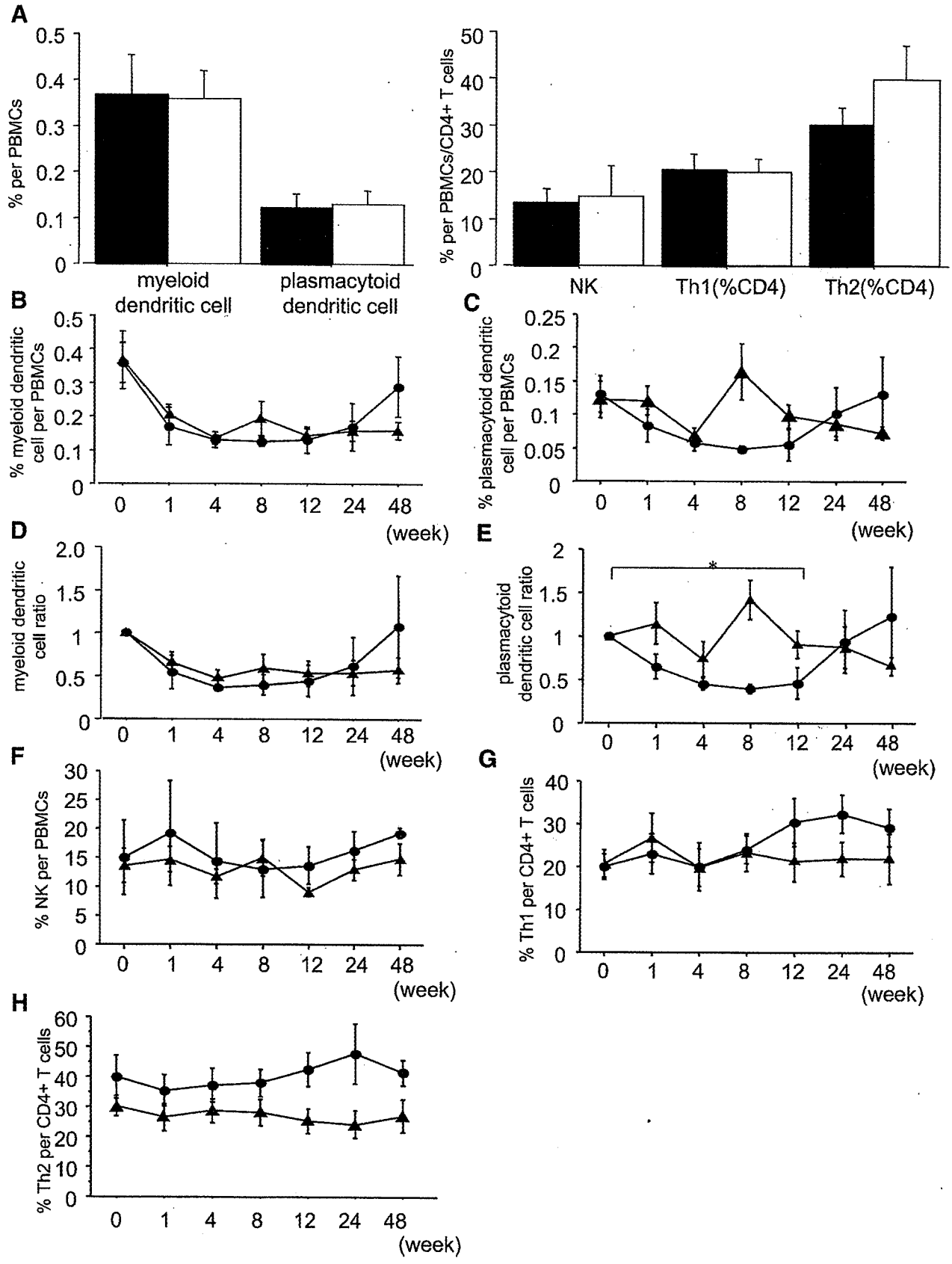
A
% per PBMCs
myeloid dendritic cell
plasmacytoid dendritic cell
% per PBMCs/CD4+ T cells
NK
Th1(%CD4)
Th2(%CD4)
B
% myeloid dendritic cell per PBMCs
C
% plasmacytoid dendritic cell per PBMCs
D
myeloid dendritic cell ratio
E
plasmacytoid dendritic cell ratio
F
% NK per PBMCs
G
% Th1 per CD4+ T cells
H
% Th2 per CD4+ T cells
0 1 4 8 12 24 48
(week)

Fig. 6.

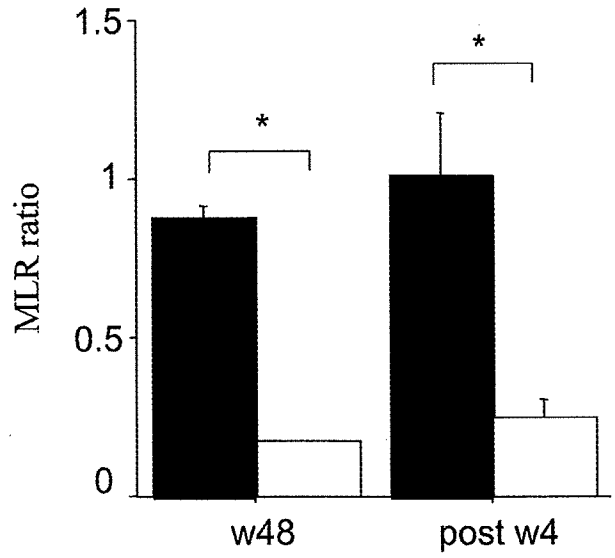

Fig. 7. Allostimulatory activity of dendritic cells in the patients who underwent 48-week PEG-IFNα2b and ribavirin therapy in patients who showed negative serum HCV RNA at Week 12 of the therapy. At the end of treatment (Week 48) and at Week 4 after the completion of the treatment, monocyte-derived dendritic cells were generated from the patients or healthy donors and their allostimulatory capacity was evaluated as described in Materials and Methods. The MLR ratio between patients and controls was determined as the same as Figure 3. The results are expressed as the mean ± SEM of seven sustained virological responders with early response and four transient responders with early response. Black bars indicate sustained virological responders and white bars indicate transient responders, respectively. $^*P < 0.05$.

In the present study, non-sustained virological responders or transient responders showed a lesser capacity for dendritic cell function than sustained virological responders at the end and after cessation of the therapy. Even in the patients who lost serum HCV RNA at Week 12, the dendritic cell function was lower in transient responders than sustained virological responders. One of the mechanisms of impaired dendritic cell function in non-sustained virological responders or transient responders may be residual HCV both in serum and in cells. It is reported that the relapse rate was higher in the patients who were positive for HCV RNA by sensitive transcription-mediated amplification (TMA) at the end of combination therapy than those who were negative for it, even when they were negative for HCV RNA by conventional PCR [Gerotto et al., 2006]. Other investigators have shown that residual HCV is detectable by means of sensitive PCR in blood cells from patients who cleared HCV from the serum by IFNα and ribavirin combination therapy [Pham et al., 2004], supporting the possibility that blood cells are reservoirs of HCV replication. Taking these findings into consideration, it is conceivable that a small quantity of HCV might exist in the blood cells in some transient responders. Since direct HCV infection of monocytes or blood dendritic cells is considered to be one of the mechanisms of the functional impairment of dendritic cell [Navas et al., 2002; Goutagny et al., 2003; Ducoulombier et al., 2004], persistent HCV may delay the restoration of dendritic cell function in non-sustained virological responders or transient responders compared to sustained virological responders.

In summary, it was shown that the frequencies of plasmacytoid dendritic cells or NK cells and dendritic cell function might be related to the outcomes of the combination therapy. Since the present study was performed with a relatively small number of patients, a greater number of patients should be examined in order to validate the feasibility of using these as immunological markers of relapse. The prediction of virological non-response or relapse during therapy can help improve the clinical outcomes of treated patients, as prolongation of combination therapy offers potential relapsers a better chance of sustained virological response by suppressing HCV reappearance.

REFERENCES

Alter HJ, Purcell RH, Shih JW, Melpolder JC, Houghton M, Choo QL, Kuo G. 1989. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. N Engl J Med 321:1494–1500.

Auffermann-Gretzinger S, Keeffe EB, Levy S. 2001. Impaired dendritic cell maturation in patients with chronic, but not resolved, hepatitis C virus infection. Blood 97:3171–3176.

Berg T, von Wagner M, Nasser S, Sarrazin C, Heintges T, Gerlach T, Buggisch P, Goeser T, Rasenack J, Pape GR, Schmidt WE, Kallinowski B, Klinker H, Spengler U, Martus P, Alshuth U, Zeuzem S. 2006. Extended treatment duration for hepatitis C virus type 1: Comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. Gastroenterology 130:1086–1097.

Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. 2003. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology 38:645–652.

Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. 1994. Classification of chronic hepatitis: Diagnosis, grading and staging. Hepatology 19:1513–1520.

Ducoulombier D, Roque-Afonso AM, Di Liberto G, Penin F, Kara R, Richard Y, Dussaix E, Feray C. 2004. Frequent compartmentalization of hepatitis C virus variants in circulating B cells and monocytes. Hepatology 39:817–825.

Ferenci P. 2004. Predicting the therapeutic response in patients with chronic hepatitis C: The role of viral kinetic studies. J Antimicrob Chemother 53:15–18.

Ferenci P, Fried MW, Shiffman ML, Smith CI, Marinos G, Goncales FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Chaneac M, Reddy KR. 2005. Predicting sustained virological responses in chronic hepatitis C patients treated with peginterferon alfa-2a (40 KD)/ribavirin. J Hepatol 43:425–433.

Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347:975–982.

Gerotto M, Dal Pero F, Bortoletto G, Ferrari A, Pistis R, Sebastiani G, Fagiuoli S, Realdon S, Alberti A. 2006. Hepatitis C minimal residual viremia (MRV) detected by TMA at the end of Peg-IFN plus ribavirin therapy predicts post-treatment relapse. J Hepatol 44: 83–87.

Goutagny N, Fatmi A, De Ledinghen V, Penin F, Couzigou P, Inchauspe G, Bain C. 2003. Evidence of viral replication in circulating dendritic cells during hepatitis C virus infection. J Infect Dis 187: 1951–1958.

Hayashi N, Takehara T. 2006. Antiviral therapy for chronic hepatitis C: Past, present, and future. J Gastroenterol 41:17–27.

Ho CS, Lopez JA, Vuckovic S, Pyke CM, Hockey RL, Hart DN. 2001. Surgical and physical stress increases circulating blood dendritic cell counts independently of monocyte counts. Blood 98:140–145.

Hotchkiss RS, Tinsley KW, Swanson PE, Grayson MH, Osborne DF, Wagner TH, Cobb JP, Coopersmith C, Karl IE. 2002. Depletion of

dendritic cells, but not macrophages, in patients with sepsis. J Immunol 168:2493–2500.

Ito T, Amakawa R, Inaba M, Ikehara S, Inaba K, Fukuhara S. 2001. Differential regulation of human blood dendritic cell subsets by IFNs. J Immunol 166:2961–2969.

Jacobson IM, Gonzalez SA, Ahmed F, Lebovics E, Min AD, Bodenheimer HC Jr, Esposito SP, Brown RS Jr, Brau N, Klion FM, Tobias H, Bini EJ, Brodsky N, Cerulli MA, Aytaman A, Gardner PW, Geders JM, Spivack JE, Rahmin MG, Berman DH, Ehrlich J, Russo MW, Chait M, Rovner D, Edlin BR. 2005. A randomized trial of pegylated interferon alpha-2b plus ribavirin in the retreatment of chronic hepatitis C. Am J Gastroenterol 100:2453–2462.

Kamal SM, Fehr J, Roesler B, Peters T, Rasenack JW. 2002. Peginterferon alone or with ribavirin enhances HCV-specific CD4 T-helper 1 responses in patients with chronic hepatitis C. Gastroenterology 123:1070–1083.

Kanto T, Hayashi N, Takehara T, Tatsumi T, Kuzushita N, Ito A, Sasaki Y, Kasahara A, Hori M. 1999. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. J Immunol 162:5584–5591.

Kawarabayashi N, Seki S, Hatsuse K, Ohkawa T, Koike Y, Aihara T, Habu Y, Nakagawa R, Ami K, Hiraide H, Mochizuki H. 2000. Decrease of CD56(+)T cells and natural killer cells in cirrhotic livers with hepatitis C may be involved in their susceptibility to hepatocellular carcinoma. Hepatology 32:962–969.

Manesis EK, Papaioannou C, Gioustozi A, Kafiri G, Koskinas J, Hadziyannis SJ. 1997. Biochemical and virological outcome of patients with chronic hepatitis C treated with interferon alfa-2b for 6 or 12 months: A 4-year follow-up of 211 patients. Hepatology 26:734–739.

Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: A randomised trial. Lancet 358:958–965.

Nattermann J, Feldmann G, Ahlenstiel G, Langhans B, Sauerbruch T, Spengler U. 2006. Surface expression and cytolytic function of natural killer cell receptors is altered in chronic hepatitis C. Gut 55:869–877.

Navas MC, Fuchs A, Schvoerer E, Bohbot A, Aubertin AM, Stoll-Keller F. 2002. Dendritic cell susceptibility to hepatitis C virus genotype 1 infection. J Med Virol 67:152–161.

Pawlotsky JM, Bouvier-Alias M, Hezode C, Darthuy F, Remire J, Dhumeaux D. 2000. Standardization of hepatitis C virus RNA quantification. Hepatology 32:654–659.

Pham TN, MacParland SA, Mulrooney PM, Cooksley H, Naoumov NV, Michalak TI. 2004. Hepatitis C virus persistence after spontaneous or treatment-induced resolution of hepatitis C. J Virol 78:5867–5874.

Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, Bain V, Heathcote J, Zeuzem S, Trepo C, Albrecht J. 1998. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). Lancet 352: 1426–1432.

Romani N, Gruner S, Brang D, Kampgen E, Lenz A, Trockenbacher B, Konwalinka G, Fritsch PO, Steinman RM, Schuler G. 1994. Proliferating dendritic cell progenitors in human blood. J Exp Med 180:83–93.

Rosen HR, Miner C, Sasaki AW, Lewinsohn DM, Conrad AJ, Bakke A, Bouwer HG, Hinrichs DJ. 2002. Frequencies of HCV-specific effector CD4+ T cells by flow cytometry: Correlation with clinical disease stages. Hepatology 35:190–198.

Seeff LB. 2002. Natural history of chronic hepatitis C. Hepatology 36: S35–S46.

Immunity
Article

Alveolar Macrophages Are the Primary Interferon-α Producer in Pulmonary Infection with RNA Viruses

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SUMMARY

Type I interferons (IFNs) are critical for antiviral responses. Here we generated a knockin mouse in which green fluorescence protein (GFP) was expressed under the control of the *Ifna6* promoter. Virus-induced expression of GFP recapitulated various IFN-α subtypes. Systemic infection of the mice with Newcastle disease virus (NDV) increased GFP$^+$ plasmacytoid dendritic cells (pDCs) via the Toll-like receptor system, and GFP$^+$ conventional dendritic cells (cDCs) and macrophages via the RIG-I-like helicase system. By contrast, lung infection with NDV led to IFN-α production in alveolar macrophages (AMs) and cDCs, but not in pDCs. Specific depletion of AMs caused a marked defect in the initial viral elimination in the lung. pDCs produced IFN-α in the absence of AM-mediated viral recognition, suggesting that pDCs function when the first defense line is broken. Thus, AMs act as a type I IFN producer that is important for the initial responses to viral infection in the lung.

INTRODUCTION

The innate immune system senses viral invasion and evokes quick responses by producing various cytokines. Among them, type I interferons (IFNs) are pleiotropic cytokines essential for antiviral immune responses. They are comprised of multiple IFN-αs and single IFN-β, and other members such as IFN-ω, -ε, and -κ (Honda et al., 2006). Humans and mice have more than 13 IFN-α family members. Type I IFNs induce apoptosis of virus-infected cells and cellular resistance to viral infection, and also activate natural killer (NK) and T cells (Stetson and Medzhitov, 2006). Thus, type I IFNs have an important role not only in the innate antiviral responses, but also in the activation of the adaptive immune system.

Two innate immune receptor families, Toll-like receptors (TLRs) and RIG-I-like helicases (RLHs), have been shown to recognize viral components and induce type I IFNs (Akira et al., 2006). The TLR system senses various viral components, including double-stranded RNA (dsRNA) single-stranded RNA (ssRNA), and unmethylated DNA with CpG motifs via TLR3, TLR7, and TLR9, respectively. TLR3 triggers signaling cascades via an adaptor protein, the Toll-IL-1 receptor (TIR) domain containing adaptor-inducing IFN-β (TRIF), which activates two IκB kinase (IKK)-related kinases, TANK-binding kinase 1 (TBK1) and inducible IKK (IKK-*i*). These kinases are known to directly phosphorylate transcription factors of IFN regulatory factor 3 (IRF-3) and IRF-7 (Kawai and Akira, 2006). These transcription factors then form a dimer, translocate to the nucleus, and activate the transcription of type I IFNs and IFN-inducible genes. On the other hand, TLR7 and TLR9 activate IRF-7 via an adaptor, MyD88 (Honda et al., 2004; Kawai et al., 2004), IL-1R-associated kinase 1 (IRAK1) (Uematsu et al., 2005), and IKK-α (Hoshino et al., 2006), but not TBK1 or IKK-*i*.

The RLH family is comprised of the retinoic acid-inducible gene I (RIG-I), the melanoma differentiation-associated gene 5 (MDA5), and Lgp2 (Akira et al., 2006). RIG-I and MDA5, but not Lgp2, contain caspase-recruit domains (CARDs) in addition to a RNA helicase domain. RIG-I is responsible for detection of various RNA viruses (Kato et al., 2005; Yoneyama et al., 2004), in vitro transcribed dsRNA (Kato et al., 2006), and 5′-triphosphate RNA (Hornung et al., 2006; Pichlmair et al., 2006), whereas MDA5 recognizes picornaviruses and polyinosinic polycytidylic acid [poly (I:C)] (Kato et al., 2006). RIG-I and MDA5 activate TBK1 and IKK-*i* via a CARD domain containing IFN-β promoter stimulator-1 (IPS-1), an adaptor also known as MAVS, CARDIF, or VISA (Kawai et al., 2005; Kumar et al., 2006; Meylan et al., 2005; Seth et al., 2005; Sun et al., 2006; Xu et al., 2005).

Although various cells are reported to have the potential to produce type I IFNs when exposed to viruses in vitro,