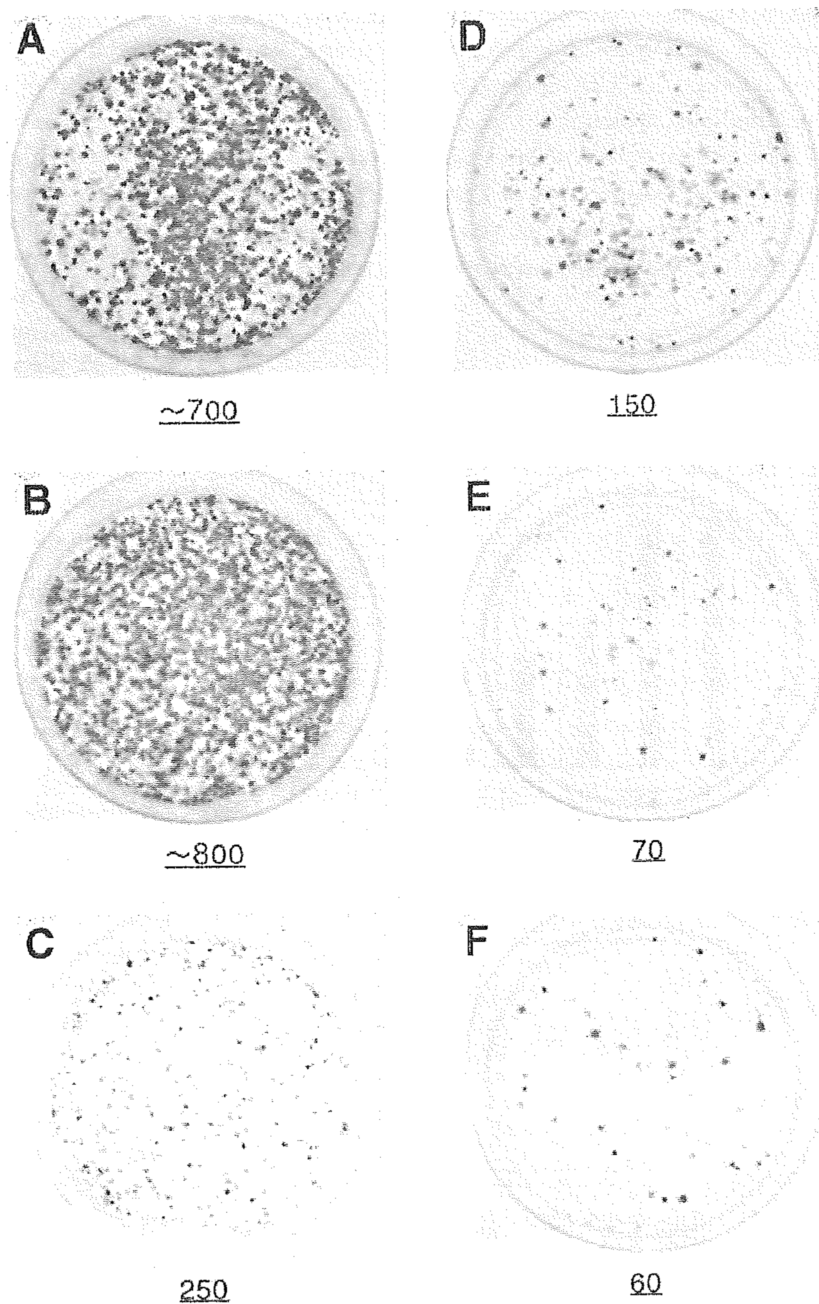
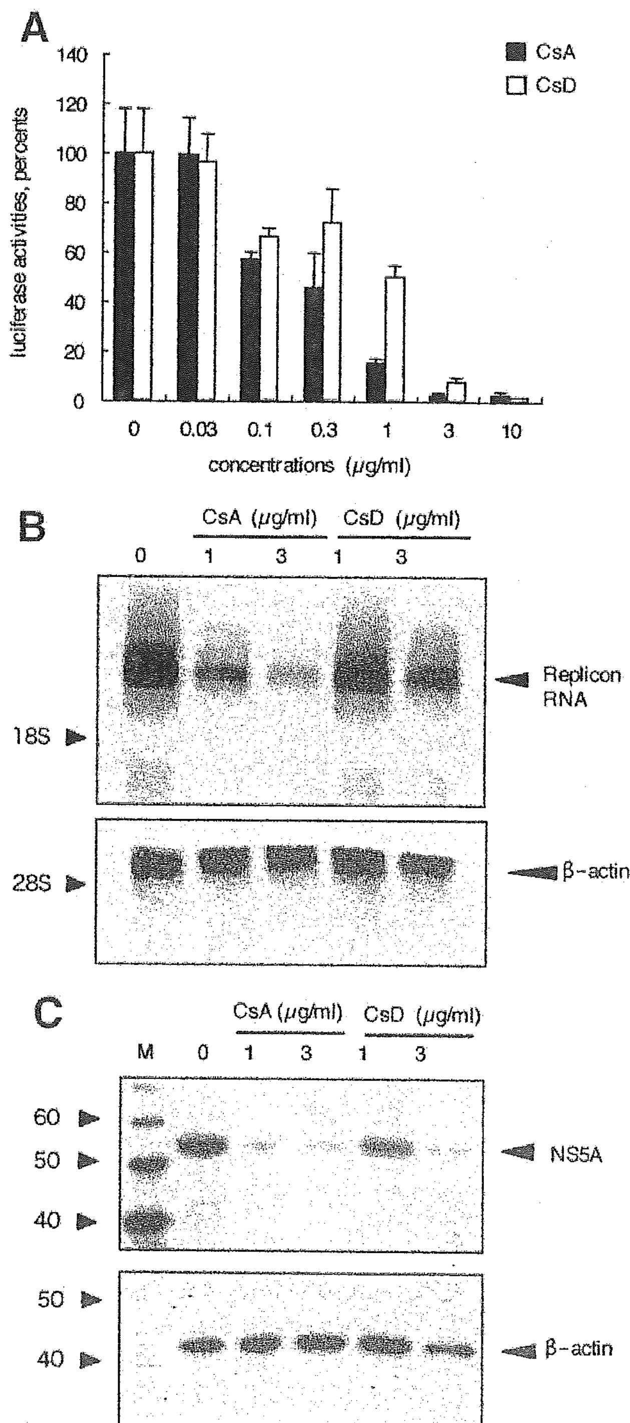


Figure 4. The replicon colony-forming activities of cell lines in which cyclophilins have been stably knocked down by shRNA. Colony-forming activities of HCV replicon were quantified in the cell lines in which cyclophilins were stably knocked down by shRNA-expression vectors. The 6 cyclophilin-directed shRNA-expression vectors were stably transfected into Huh7 cells by retroviral transduction. A replicon, Rep-BSD, was transfected into the cell lines, respectively, and along with cell lines transfected with negative and positive control shRNAs cultured in the presence of BSD. Two weeks after the transfection, the cell colonies were visualized by treating with neutral red solution. Numbers below the plates refer to the approximate numbers of the cell colonies. (*A*) Naive Huh7, (*B*) Huh7/shRNA control, (*C*) Huh7/HCV shRNA#331, (*D*) Huh7/CypA shRNA#3, (*E*) Huh7/CypB shRNA#294, and (*F*) Huh7/CypC shRNA#291.

that CsA has antiviral activity against HCV in vitro.[9,10] Watashi et al reported the in vitro effect of CsA on HCV replication using an HCV replicon system and a cultured cell line that supports HCV replication.[9] However, little is understood about the mechanisms of its action against HCV replication and the mechanism of its clinical efficacy. Our present results show that the action of CsA did not involve the calcineurin/NFAT-mediated pathway that is shared with FK506 (Figure 2*B*), showing that the anti-HCV effect of CsA is not associated with its immunosuppressive activity. On the other hand, knockdown of CypA, CypB, and CypC expression by shRNA suppressed HCV replication substantially (Figures 3 and 4). Furthermore, CsD, an analogue of cyclosporin with weak immunosuppressive activity but that retains the ability to bind cyclophilins, was similarly effective in suppressing HCV replication (Figure 5). Collectively, it was shown that the anti-HCV action of the cyclosporins is through blockade of the activities of cellular cyclophilins.

In our results, both CsA and FK506 suppressed NFAT-mediated luciferase reporter activities in a T lymphocyte–derived cell line, while neither CsA nor FK506 suppressed NFAT activities in hepatoma-derived Huh7 cells (Figure 2*B*). It has been reported that CsA shows divergent effects of NFAT-mediated luciferase activity

among cells of different origins, possibly because NFAT does not participate in autoregulatory activation of its own promoter in CsA-nonresponding cells.[9,30,31] With these findings, although our results may not completely exclude the partial involvement of the NFAT-mediated pathway, the major action of CsA against HCV replication is not through the calcineurin/NFAT pathway but through cyclophilin-mediated mechanisms.



The PPIase activity of cyclophilins has been reported by Takahashi et al and other researchers.[20–22] Through this activity, cyclophilins contribute to the maturation of several proteins, such as carbonic anhydrase[32] and the human immunodeficiency virus *gag* protein.[33,34] Furthermore, the enzymatic activity of cyclophilins may underlie several other functions of these proteins, including cell signaling, mitochondrial function, molecular chaperone activity, RNA splicing, stress response, gene expression, and regulation of kinase activity.[21,35] As for HCV, the viral structural and nonstructural proteins are processed from a single polyprotein of 3000 amino acids by ER membrane-bound signal peptidases and by 2 self-coded serine proteases.[36] It has been reported that folding and assembly of HCV proteins require interaction with ER chaperone proteins such as calreticulin, BiP, and HSP90.[37,38] In this study, the replication levels of the HCV replicon decreased substantially following the knockdown of CypA, CypB, and CypC. Moreover, our preliminary data have shown that overexpression of cyclophilins attenuated the effects of CsA on HCV replication. These results imply that the effects of CsA on HCV replication involve functional blockade of cyclophilins and, more importantly, that constitutive expression of the cytoplasmic cyclophilins such as CypA, CypB, and CypC may be necessary for HCV replication, possibly through assisting functional maturation of the viral proteins as molecular co-chaperone proteins. Although it is still unclear how cyclophilins support the processing of HCV proteins and the replication of the viral genome, these molecules could be potential targets to counteract HCV infection and replication.

One third of nascent proteins are transported to the ER, where they are subjected to posttranslational modifications such as folding, glycosylation, and oligomer-

Figure 5. Suppression of HCV replication by CsD. (*A*) Huh7/Rep-Feo cells were cultured with the concentrations of CsA (*closed boxes*) indicated or an analogue of cyclosporin, CsD (*open boxes*), in the medium. Luciferase assays were performed after 48 hours of culture. Luciferase assays were performed in triplicate. *Error bars* indicate mean ± 2 SD. (*B*) Northern blotting. Huh7/Rep-Neo was cultured with the concentrations of CsA or CsD indicated, and RNA was extracted after 48 hours of culture. Ten micrograms of total cellular RNA was applied to each lane. The upper part of the membrane containing the HCV replicon RNA was hybridized with a digoxigenin-labeled probe specific for the replicon sequence, and the lower part was hybridized with a β-actin probe. *Lane 1,* replicon alone; *lane 2,* 1 μg/mL CsA; *lane 3,* 3 μg/mL CsA; *lane 4,* 1 μg/mL CsD, and *lane 5,* 3 μg/mLCsD. (*C*) Western blotting. Ten micrograms of total cellular protein was separated by polyacrylamide gel electrophoresis and transferred onto the membrane. The membrane was incubated with a monoclonal anti-NS5A antibody or an anti-β-actin antibody. *Lane 1,* protein size markers; *lane 2,* replicon alone; *lane 3,* 1 μg/mL CsA; *lane 4,* 3 μg/mL CsA; *lane 5,* 1 μg/mL CsD; and *lane 6,* 3 μg/mL CsD.

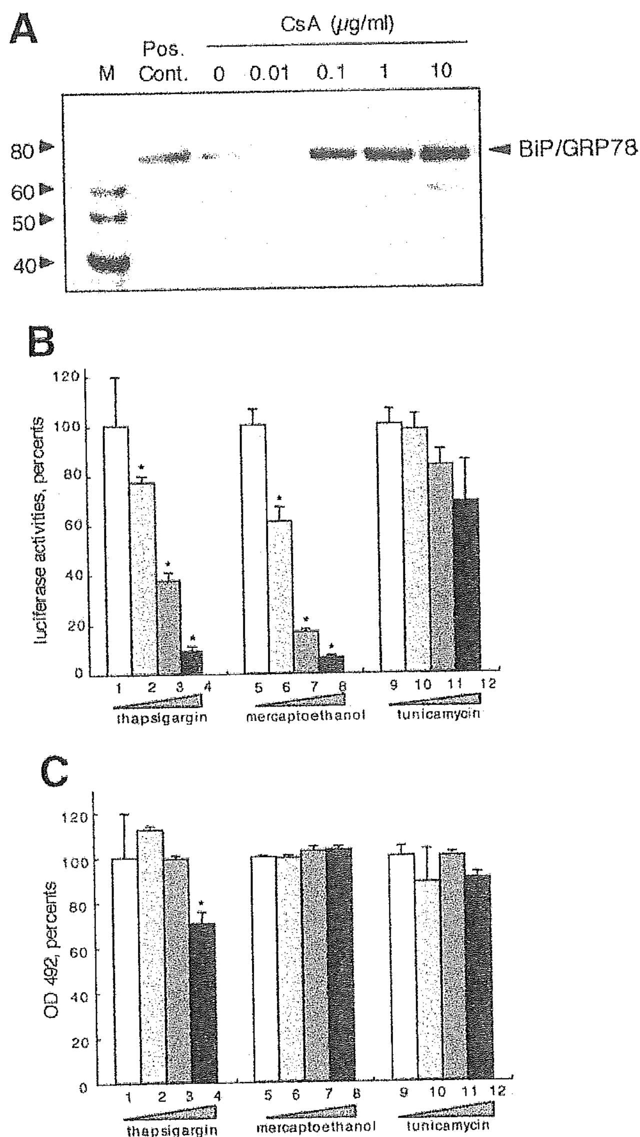


Figure 6. Unfolded protein response following CsA treatment and its effects on HCV replication. (*A*) Huh7 cells were cultured in the presence of CsA at the concentrations indicated, and the cells were harvested at 48 hours after the treatment. Western blotting was performed using anti-Bip/GRP78 antibody. *Lane 1*, protein size markers; *lane 2*, HepG2 lysate as a positive control; *lane 3*, Huh7 alone; *lane 4*, 0.01 μg/mL CsA; *lane 5*, 0.1 μg/mL CsA; *lane 6*, 1 μg/mL CsA; and *lane 7*, 10 μg/mL CsA. (*B*) Huh7/Rep-Feo cells were cultured with various concentrations of thapsigargin, mercaptoethanol, and tunicamycin in the medium. Luciferase assays were performed in triplicate at 48 hours after treatment with each drug. Error bars indicate mean ± 2 SD. Columns 1–4: cells cultured with thapsigargin at concentrations of 0 (column 1), 0.001 (column 2), 0.003 (column 3), and 0.01 μg/mL (column 4). Columns 5–8: cells cultured with mercaptoethanol at concentrations of 0 (column 5), 0.01 (column 6), 0.1 (column 7), and 1 μg/mL (column 8). Columns 9–12: cells cultured with tunicamycin at concentrations of 0 (column 9), 0.01 (column 10), 0.03 (column 11), and 0.1 μg/mL (column 12). *P values of less than .05. (*C*) MTS assay of Huh7/Rep-Feo cells cultured with the various concentrations of ER stress agents. MTS assays at 48 hours after treatment with each drug were performed in triplicate. *Error bars* indicate mean ± 2 SD. Columns 1–12: cells cultured with the drugs that correspond to those in *B*. *P values of less than .05.

ization and are exported to various cellular compartments. Various cellular stresses such as heat shock, ischemia, hypoxia, and viral infection and changes in calcium homeostasis prevent protein folding and maturation in the ER and result in the accumulation of misfolded proteins.[39,40] These defective proteins trigger the unfolded protein response and cause induction of molecular chaperone proteins, suppression of translation, and apoptotic cell death.[27,28,41] Paslaru et al reported that treatment of HeLa cells with CsA induced an unfolded protein response that is characterized by synthesis of a stress protein, BiP/GRP78, located inside the ER.[26] Because PPIases support the correct folding of a sufficient number of proteins, their inhibition ought to lead to the accumulation of denatured proteins in various cell compartments. In this study, treatment with thapsigargin, an inhibitor of the Ca^{2+} adenosine triphosphatase (ATPase) transporter, and mercaptoethanol, which disrupts disulfide bond formation, suppressed HCV replication. These results imply that the effects of CsA on HCV replication may involve induction of an unfolded protein response to a level below the cytotoxic range and that the selective alteration of ER function may abrogate HCV replication.

The expanding applications of CsA to infectious diseases such as HCV may cause substantial problems, particularly undesired immunosuppression and possible interference with the effects of anti-infectious agents such as interferon. One solution to overcome these problems is to use cyclosporin analogues.[42,43] Some types of cyclosporin analogues show attenuated effects in terms of blocking T-cell activation but retain activity against the PPIase activity of cyclophilins. These nonimmunosuppressive cyclosporin analogues have equal or even superior effects against in vitro human immunodeficiency virus replication compared with the immunosuppressive CsA.[44] As we have shown, HCV replication was successfully inhibited by CsD, which has weak immunosuppressive activity.[25,45] The inhibition of T-cell activation by cyclosporin analogues is a function of the binding capacities to cyclophilins, whose binding to the residues of cyclosporine is known to be critical for their immunosuppressive activity in the decreasing order of CsA to CsD.[46] However, in this study, the inhibitory effects of CsA and CsD on HCV replication were found to be similar (Figure 5), showing that the anti-HCV effects did not correlate with immunosuppressive activity. Another cyclosporin analogue, NIM811 ([methyl-Ile4]-cyclosporin), has been reported to be a similar inhibitor of CypA-mediated human immunodeficiency virus 1 replication than the parental CsA.[47] Watashi et al confirmed that NIM811 also is effective against HCV replication in

vitro.[9] The availability of nonimmunosuppressive cyclophilin inhibitors that are less toxic than CsA might hold promise of novel antiviral drugs. These drugs should be reevaluated clinically in the light of the new findings presented here.

Given the current status of limited therapy options against HCV infection and the unsatisfactory outcome of therapy, screening of nonimmunosuppressive cyclosporin analogues or agents targeting cellular cyclophilins may be important to develop novel antiviral therapies. In addition, further investigations of the action of cyclophilins on the expression, processing, or maturation of HCV proteins may elucidate new aspects of the viral infection and replication.

References

1. Alter MJ. Epidemiology of hepatitis C. Hepatology 1997;26:62S–65S.
2. Rosen HR, Gretch DR. Hepatitis C virus: current understanding and prospects for future therapies. Mol Med Today 1999;5:393–399.
3. Hoofnagle JH, Mullen KD, Jones DB, Rustgi V, Di Bisceglie A, Peters M, Waggoner JG, Park Y, Jones EA. Treatment of chronic non-A, non-B hepatitis with recombinant human alpha interferon. A preliminary report. N Engl J Med 1986;315:1575–1578.
4. McHutchison JG, Poynard T. Combination therapy with interferon plus ribavirin for the initial treatment of chronic hepatitis C. Semin Liver Dis 1999;19:57–65.
5. Hadziyannis SJ, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H Jr, Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med 2004;140:346–355.
6. Martinot-Peignoux M, Boyer N, Pouteau M, Castelnau C, Giuily N, Duchatelle V, Auperin A, Degott C, Benhamou JP, Erlinger S, Marcellin P. Predictors of sustained response to alpha interferon therapy in chronic hepatitis C. J Hepatol 1998;29:214–223.
7. Borel JF, Feurer C, Gubler HU, Stahelin H. Biological effects of cyclosporin A: a new antilymphocytic agent. Agents Actions 1976; 6:468–475.
8. Matsuda S, Koyasu S. Mechanisms of action of cyclosporine. Immunopharmacology 2000;47:119–125.
9. Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotohno K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. Hepatology 2003;38:1282–1288.
10. Nakagawa M, Sakamoto N, Enomoto N, Tanabe Y, Kanazawa N, Koyama T, Kurosaki M, Maekawa S, Yamashiro T, Chen CH, Watanabe M. Specific inhibition of hepatitis C virus replication by cyclosporin A. Biochem Biophys Res Commun 2004;313:42–47.
11. Tanabe Y, Sakamoto N, Enomoto N, Kurosaki M, Ueda E, Maekawa S, Yamashiro T, Nakagawa M, Chen CH, Kanazawa N, Watanabe M. Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. J Infect Dis 2004;189:1129–1139.
12. Guo JT, Bichko VV, Seeger C. Effect of alpha interferon on the hepatitis C virus replicon. J Virol 2001;75:8516–8523.
13. Yokota T, Sakamoto N, Enomoto N, Tanabe Y, Miyagishi M, Maekawa S, Yi L, Kurosaki M, Taira K, Watanabe M, Mizusawa H. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep 2003;4: 602–608.
14. Kanazawa N, Kurosaki M, Sakamoto N, Enomoto N, Itsui Y, Yamashiro T, Tanabe Y, Maekawa S, Nakagawa M, Chen CH, Oshima S, Nakamura T, Kato T, Wakita T, Watanabe M. Regulation of hepatitis C virus replication by interferon regulatory factor-1. J Virol 2004;78:9713–9720.
15. Haze K, Yoshida H, Yanagi H, Yura T, Mori K. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell 1999;10:3787–3799.
16. O'Grady JG, Burroughs A, Hardy P, Elbourne D, Truesdale A. Tacrolimus versus microemulsified ciclosporin in liver transplantation: the TMC randomised controlled trial. Lancet 2002;360: 1119–1125.
17. Handschumacher RE, Harding MW, Rice J, Drugge RJ, Speicher DW. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science 1984;226:544–547.
18. Hait WN, Harding MW, Handschumacher RE. Calmodulin, cyclophilin, and cyclosporin A. Science 1986;233:987–989.
19. Warrens AN, Waters JB, Salama AD, Lechler RI. Improving the therapeutic monitoring of cyclosporin A. Clin Transpl 1999;13: 193–200.
20. Fischer G, Wittmann-Liebold B, Lang K, Kiefhaber T, Schmid FX. Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. Nature 1989;337:476–478.
21. Takahashi N, Hayano T, Suzuki M. Peptidyl-prolyl cis-trans isomerase is the cyclosporin A-binding protein cyclophilin. Nature 1989;337:473–475.
22. Ivery MT. Immunophilins: switched on protein binding domains? Med Res Rev 2000;20:452–484.
23. Braaten D, Luban J. Cyclophilin A regulates HIV-1 infectivity, as demonstrated by gene targeting in human T cells. EMBO J 2001; 20:1300–1309.
24. Mottola G, Cardinali G, Ceccacci A, Trozzi C, Bartholomew L, Torrisi MR, Pedrazzini E, Bonatti S, Migliaccio G. Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons. Virology 2002;293:31–43.
25. Sadeg N, Pham-Huy C, Rucay P, Righenzi S, Halle-Pannenko O, Claude JR, Bismuth H, Duc HT. In vitro and in vivo comparative studies on immunosuppressive properties of cyclosporines A, C, D and metabolites M1, M17 and M21. Immunopharmacol Immunotoxicol 1993;15:163–177.
26. Paslaru L, Pinto M, Morange M. GRP78 induction by cyclosporin A in human HeLa cells. FEBS Lett 1994;350:304–308.
27. Munro S, Pelham HR. An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell 1986;46:291–300.
28. Kozutsumi Y, Segal M, Normington K, Gething MJ, Sambrook J. The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. Nature 1988; 332:462–464.
29. Inoue K, Sekiyama K, Yamada M, Watanabe T, Yasuda H, Yoshiba M. Combined interferon alpha2b and cyclosporin A in the treatment of chronic hepatitis C: controlled trial. J Gastroenterol 2003;38:567–572.
30. Duque J, Fresno M, Iniguez MA. Expression and function of the nuclear factor of activated T cells in colon carcinoma cells: involvement in the regulation of cyclooxygenase-2. J Biol Chem 2005;280:8686–8693.
31. Komarova SV, Pereverzev A, Shum JW, Sims SM, Dixon SJ. Convergent signaling by acidosis and receptor activator of NF-kappaB ligand (RANKL) on the calcium/calcineurin/NFAT pathway in osteoclasts. Proc Natl Acad Sci U S A 2005;102:2643–2648.
32. Rycyzyn MA, Reilly SC, O'Malley K, Clevenger CV. Role of cyclophilin B in prolactin signal transduction and nuclear retrotranslocation. Mol Endocrinol 2000;14:1175–1186.

33. Taylor P, Husi H, Kontopidis G, Walkinshaw MD. Structures of cyclophilin-ligand complexes. Prog Biophys Mol Biol 1997;67: 155–181.
34. Streblow DN, Kitabwalla M, Malkovsky M, Pauza CD. Cyclophilin A modulates processing of human immunodeficiency virus type 1 p55Gag: mechanism for antiviral effects of cyclosporin A. Virology 1998;245:197–202.
35. Gothel SF, Marahiel MA. Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. Cell Mol Life Sci 1999;55:423–436.
36. Bartenschlager R, Lohmann V. Replication of hepatitis C virus. J Gen Virol 2000;81:1631–1648.
37. Choukhi A, Ung S, Wychowski C, Dubuisson J. Involvement of endoplasmic reticulum chaperones in the folding of hepatitis C virus glycoproteins. J Virol 1998;72:3851–3858.
38. Waxman L, Whitney M, Pollok BA, Kuo LC, Darke PL. Host cell factor requirement for hepatitis C virus enzyme maturation. Proc Natl Acad Sci U S A 2001;98:13931–13935.
39. Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev 1999;13:1211–1233.
40. Pahl HL. Signal transduction from the endoplasmic reticulum to the cell nucleus. Physiol Rev 1999;79:683–701.
41. Mori K. Tripartite management of unfolded proteins in the endoplasmic reticulum. Cell 2000;101:451–454.
42. Bierer BE, Somers PK, Wandless TJ, Burakoff SJ, Schreiber SL. Probing immunosuppressant action with a nonnatural immunophilin ligand. Science 1990;250:556–559.
43. Sigal NH, Dumont F, Durette P, Siekierka JJ, Peterson L, Rich DH, Dunlap BE, Staruch MJ, Melino MR, Koprak SL, et al. Is cyclophilin involved in the immunosuppressive and nephrotoxic mechanism of action of cyclosporin A? J Exp Med 1991;173: 619–628.
44. Billich A, Hammerschmid F, Peichl P, Wenger R, Zenke G, Quesniaux V, Rosenwirth B. Mode of action of SDZ NIM 811, a nonimmunosuppressive cyclosporin A analog with activity against human immunodeficiency virus (HIV) type 1: interference with HIV protein-cyclophilin A interactions. J Virol 1995;69:2451–2461.
45. Mizuno K, Furuhashi Y, Misawa T, Iwata M, Kawai M, Kikkawa F, Kano T, Tomoda Y. Modulation of multidrug resistance by immunosuppressive agents: cyclosporin analogues, FK506 and mizoribine. Anticancer Res 1992;12:21–25.
46. Quesniaux VF, Schreier MH, Wenger RM, Hiestand PC, Harding MW, Van Regenmortel MH. Cyclophilin binds to the region of cyclosporine involved in its immunosuppressive activity. Eur J Immunol 1987;17:1359–1365.
47. Thali M, Bukovsky A, Kondo E, Rosenwirth B, Walsh CT, Sodroski J, Gottlinger HG. Functional association of cyclophilin A with HIV-1 virions. Nature 1994;372:363–365.

Received September 10, 2004. Accepted June 9, 2005.

Address requests for reprints to: Naoya Sakamoto, MD, PhD, Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. e-mail: nsakamoto.gast@tmd.ac.jp; fax: (81) 3-5803-0268.

M.N. and N.S. contributed equally to this work.

Supported by grants 15590629 and 16590580 from the Japan Society for the Promotion of Science and in part by a grant from the Viral Hepatitis Research Foundation of Japan.

Journal of Gastroenterology and Hepatology (2005) **20**, 1401–1409 DOI: 10.1111/j.1440-1746.2005.04024.x

HEPATOLOGY

Mutations in the NS5B region of the hepatitis C virus genome correlate with clinical outcomes of interferon-alpha plus ribavirin combination therapy

KOSEI HAMANO,* NAOYA SAKAMOTO,* NOBUYUKI ENOMOTO,*,† NAMIKI IZUMI,‡ YASUHIRO ASAHINA,‡ MASAYUKI KUROSAKI,*,‡ ERI UEDA,* YOKO TANABE,* SHINYA MAEKAWA,* JUN ITAKURA,* HIDEKI WATANABE,* SEI KAKINUMA* AND MAMORU WATANABE*

**Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, †First Department of Medicine, Yamanashi University, Yamanashi, and ‡Devision of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan*

Abstract

Background and Aim: Combination treatments of interferon-alpha (IFN) and ribavirin (RBV) are more effective than those of IFN alone in hepatitis C virus (HCV) infection. However, mechanisms of the action of the combination regimen are not well understood. To elucidate the viral genetic basis of IFN plus RBV combination therapy, genetic variabilities of HCV-1b were analyzed.

Methods: We performed pair-wise comparisons of full-length HCV genomic sequences in three patients' sera before and after initiation of IFN plus RBV treatment. Subsequently, we analyzed amino acid sequences of the NS5B region, which codes for the viral RNA-dependent RNA polymerase, and compared these with the outcomes of the therapy in 81 patients.

Results: Analysis of the entire HCV sequence in patients who received IFN plus RBV therapy did not show consistent amino acid changes between before and after the initiation of the therapy. NS5B sequence analyses revealed that mutations at positions 300–358 of NS5B, including polymerase motif B to E, occurred more frequently in a group of patients exhibiting a sustained viral response (SVR) or an end-of-treatment response (ETR) compared with a group of patients exhibiting a non-response (NR). Closer examination revealed that mutations at aa 309, 333, 338 and 355 of NS5B occurred significantly more frequently in the SVR plus ETR group than in the NR group ($P = 0.0004$). Multivariate analysis showed that the number of mutations at these four sites was an independent predictor of SVR plus ETR *versus* NR.

Conclusions: Particular amino acid changes in the NS5B region of HCV may correlate with outcomes of IFN plus RBV combination therapy.

© 2005 Blackwell Publishing Asia Pty Ltd

Key words: amino acid sequence, error catastrophe, RNA-dependent RNA polymerase, transition.

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis, which can lead to liver cirrhosis and hepatocellular malignancy.[1,2] Interferon (IFN) is the agent of choice for treating HCV infection. However, IFN monotherapy produces sustained virological responses in only 15–20% of patients treated, most of whom relapse after completion of the therapy.[3,4] Several recent studies of combination therapy with IFN alpha 2b and ribavirin (RBV) have shown that the regimen induces higher sustained virological responses than IFN monotherapy. Unfortunately, 50–60% of patients still do not respond to the combination therapy.[5–8]

RBV is a synthetic guanosine analog with broad antiviral actions *in vitro* against various DNA and RNA

Correspondence: Naoya Sakamoto, Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8519, Japan. Email: nsakamoto.gast@tmd.ac.jp
Accepted for publication 8 September 2004.

viruses.[9,10] At present, four mechanisms of action have been postulated: (i) immune modulatory effects by a switching of T-cell phenotype from Th2 to Th1 that induces type 1 cytokine responses;[11–13] (ii) inhibition of inosine monophosphate dehydrogenase (IMPDH) leading to depletion of cellular GMP pool;[14] (iii) mutagenic activity against RNA viruses that induces misincorporation of RBV triphosphate into viral RNA leading to error prone replication of viral genome;[15–18] and (iv) inhibition of the activity of HCV NS5B RNA-dependent RNA polymerase (RdRp).[19,20] However, it has not been fully understood which mechanisms of actions of RBV are effective against HCV infection.

Certain genetic structures of viruses may affect the sensitivity to their therapeutic drugs. Nucleoside analogs are widely used against viruses such as human immunodeficiency virus type 1 (HIV) and hepatitis B virus (HBV).[21,22] The antiviral effect of those reagents arises from the inhibition of viral DNA/RNA polymerase activity. However, single or multiple mutation(s) in the viral polymerase confer drug resistance and help the drug resistant strains emerge.[22–30] Also in HCV infection, the INF sensitivity determining region (ISDR) of HCV genome, which we have previously identified, critically determines the virological response to IFN and the treatment outcomes.[31,32] As to RBV, one study of five HCV genotype 1a patients who had undergone RBV monotherapy has reported one mutation in NS5B that may correlate with RBV sensitivity.[33] These findings make us speculate that genetic variability of HCV NS5B region, which codes for RdRp, may correlate with sensitivity to RBV and may influence the outcomes of IFN plus RBV combination therapy.

In the present study, we first analyzed effects of RBV on HCV genomic structure and the viral genetic basis of RBV resistance by performing pair-wise comparisons of full-length HCV genomic sequences in patient sera before and after initiation of IFN plus RBV treatment. Subsequently, we have investigated a hypothesis that genomic variability of HCV RdRp may confer resistance or susceptibility to RBV and may correlate with the outcomes of IFN plus RBV combination therapy. Thus, we analyzed amino acid sequences of the NS5B region and the outcomes of IFN plus RBV combination therapy in 81 patients, and found that certain amino acid variations in the NS5B region may associate with the treatment outcomes.

METHODS

Patients of interferon plus ribavirin non-responders

Three patients infected with HCV, genotype 1b, were studied. All patients were non-responders to combination therapy with IFN alfa-2b (Intron A, Schering Plough, Kenilworth, NJ, USA), 6 million units three times per week plus RBV (Rebetoron, Schering Plough), 800 mg/day (> 12.1 mg/kgBW) for 24 weeks. Serum samples were obtained before treatment and at 12 weeks after initiation of the treatment, and pair-wise comparisons of the consensus sequences of full-length HCV genomes were performed. As controls for the IFN plus RBV therapy data, we analyzed our previously published HCV sequence data for three non-responders of IFN monotherapy[32] (deposited with the DDBJ/GenBank/EMBL data libraries under accession number D50483, D50480, D50485, D50481, D50484 and D50482).

RNA extraction, reverse transcription-polymerase chain reaction and direct sequencing

RNA was extracted from patient sera by the modified acid guanidinium thiocyanate-phenol-chloroform (AGPC) method,[34] using ISOGEN reagent (Wako Pure Chemical Industries, Osaka, Japan), and reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described.[32] Full-length HCV genomes were amplified by nested PCR with 21 partially overlapping sets of primers, as previously reported.[32] M13-forward and M13-reverse sequencing primer sequences were attached to the 5′-termini of sense and antisense nested PCR primers. Each PCR product was purified by a spin filtration column (Suprec-02; Takara). Both strands of the PCR products were cycle sequenced with the PRISM dye termination kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions, and consensus nucleotide sequences were determined by an automated DNA sequencer model 373 A (Applied Biosystems).

Sequence analyses

Nucleotide sequencing analysis was performed with a software program (MEGA version 2.1) to calculate values for d_N (non-synonymous substitution), d_S (synonymous substitution), d_N/d_S ratios, and the number of point mutations.

Clinical outcome of combination therapy

Patients were placed into one of three outcome groups.

- Sustained virologic response (SVR): HCV-RNA was not detectable by RT-PCR for 6 months following completion of the therapy.
- End-of-treatment response (ETR): HCV-RNA was not detected at the end of the treatment, but reappeared within 6 months thereafter.
- Non-response (NR): HCV-RNA did not disappear during the treatment.

Nucleoside sequencing analyses of the NS5b region

Amino acid mutations in the conserved motifs (motif A, B, C, D, E, F)[35–38] in NS5B RdRp were retrospectively analyzed in 81 HCV genotype 1b patients who were

treated with IFN alfa-2b, 6 million units three times per week plus RBV, 800 mg/day (> 12.1 mg/kgBW) for 24 weeks. All patients had biopsy-proven chronic hepatitis with positive serum HCV antibodies and serum HCV-RNA. RNA was extracted from sera of the patients before treatment. NS5B region, including motifs A to F, was amplified by RT-PCR and sequences corresponding to nucleotides 7730–8874 of HCV-J were determined.[32] The deduced amino acid sequences of all patients were aligned and compared with consensus sequences for mutations and analyzed for correlation between amino acid mutations of NS5B and the clinical outcome of the combination therapy.

Statistical analyses

Comparisons of differences in categorical data between groups were performed using the χ^2 test and Fishers exact test. Distributions of continuous variables were analyzed by the Mann–Whitney *U*-test for two groups and by the Kruskal–Wallis test or Scheffé method for three groups. Multivariate analysis was carried out by multiple logistic regression analysis. *P*-values of less than 0.05 were defined as statistically significant.

RESULTS

Pair-wise comparisons of the full-length HCV genome in three patients before and after initiation of IFN/RBV treatment

HCV genomes from the three study patients comprised 9423 nucleotides and contained an open reading frame of 3010 amino acids. In patient one, 31 amino acid changes were found in the HCV genome. These amino acid changes were clustered in the E2-hypervariable regions (8 of 31) and the NS5A regions (11 of 31). Before treatment, the INF-sensitivity determining lesion (ISDR)[31,32] were 'mutant' type with five amino acid changes compared with consensus sequence, which changed to 'intermediate' type with two amino acid changes after the initiation of treatment. In patient two, 37 amino acid changes were found in the entire HCV genome. The changes were exclusively found in the E2-hypervariable region (16 out of 37 amino acids), while there was no change in the ISDR. In patient three, 56 amino acid changes were found. The changes were exclusively found in the E2 region (24 out of 56 amino acids). Distribution of amino acid changes during the therapy in the three patients treated with combination therapy and three non-responders to IFN monotherapy are illustrated in Figure 1. The numbers of nucleotide changes for the three study patients were 88, 130 and 272, respectively. The d_N/d_S ratios were 0.195, 0.148 and 0.099, respectively. Among the three control subjects who received IFN monotherapy, the numbers of nucleotide changes were 138, 160 and 175, respectively. The d_N/d_S ratios were 0.158, 0.061 and 0.089, respectively. As shown in Figure 2, d_N/d_S ratios tended to be higher in the E2 region than in the other regions during

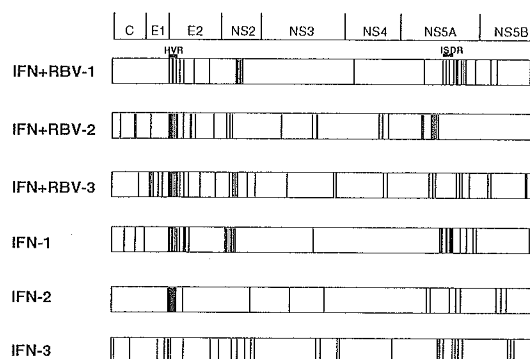


Figure 1 Schematic representation of the distribution of mutations in amino acid residues during the combination therapy and interferon (IFN) monotherapy. Distributions of amino acid changes in the entire hepatitis C virus (HCV) genome in patient serum before treatment and 12 weeks after initiation of treatment are shown. The upper three data are from patients treated with IFN/ribavirin (RBV) combination therapy (IFN + RBV 1–3), and the lower three data are those treated with IFN monotherapy (IFN 1–3). Vertical lines in each HCV polyproteins show position of amino acid differences during the therapy.

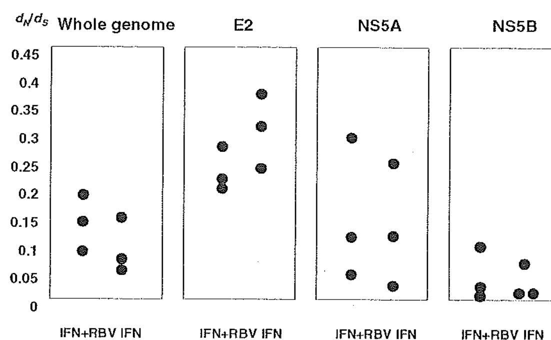


Figure 2 Ratio of non-synonymous to synonymous distances for the E2, NS5A, NS5B and whole hepatitis C virus (HCV) genome. The d_N/d_S ratio in E2 region tended to be higher than other regions during interferon (IFN) monotherapy and during combination therapy. All pairwise d_N/d_S ratios were calculated using MEGA version 2.1 for each subject.

both IFN monotherapy and combination therapy. The numbers of transitional mutations in patients who received the combination therapy had 71 (80.2% of total mutations), 104 (80.0%) and 218 (80.1%) transitional mutations, respectively, and in patients who received IFN monotherapy these were 108 (78.3%), 131 (81.9%) and 130 (75.4%), respectively. The proportion of transitions among IFN monotherapy patients did not differ from the proportion among combination therapy patients.

Two studies have observed two key transitions, C-to-U and G-to-A, in genomic sequences of RBV-treated RNA viruses.[17,18] In the present study, C-to-U and G-to-A mutations comprised 35.5%, 40.6% and 58% of

total mutations, respectively, in the three patients treated with IFN monotherapy, and 43.2%, 38.3% and 37.8%, respectively, in those treated with combination therapy. These results showed no obvious increase in key mutations of C-to-U and G-to-A associated with the combination therapy (Table 1).

Sequence analyses of NS5b region in 81 patients treated with IFN and RBV therapy

To study the correlation between the genetic structures of NS5B and the outcome of IFN plus RBV combination therapy, amino acid sequences of HCV NS5B (aa. 61–407), including motif A-F, were analyzed in 81 patients treated with IFN plus RBV combination therapy. The clinical characteristics of the patients are shown in Table 2. Nineteen (23.5%) patients were SVR, 40 (49.4%) were ETR, and 22 (27.2%) were NR. Clinical variables were analyzed according to the results of the combination therapy. Univariate analysis identified fibrosis stage as significantly lower in the SVR patients than in the other patients. No other clinical parameters were significantly correlated with the responses.

The amino acid sequences of the essential motif B to E of NS5B in these 81 patients are aligned with consensus sequences in Figure 3. Comparison of the NS5B sequences between patients with SVR and patients with non-SVR (ETR and NR) showed no obvious differences. Instead, when we compared the sequences of a patient group of SVR plus ETR with those of patients with NR, the mutations at position NS5B 300–358, including motif B to E between, were more frequent in the SVR plus ETR group than in the NR group. When we analyzed mutations of individual amino acid positions, the frequencies of mutations at aa 309, 333, 338 and 355 of NS5B (the four sites) were found to be more frequent in patients with SVR or ETR than those with NR (Fig. 4). The total number of amino acid changes at these four sites was significantly higher in patients with SVR or ETR than those with NR (0.93 ± 0.89 *vs* 0.27 ± 0.70, $P = 0.0004$). In 19 SVR patients, five patients had no mutations, 10 patients had one mutation, and four patients had two or more mutations at the four sites. In the 40 ETR patients, 18 patients had no mutations, 13 patients had one mutation, and nine patients had two or more mutations at the four sites. In 22 NR patients, 19 patients had no mutations, two patients had one mutation, and one patient had three mutations at the four sites (Fig. 5a). The SVR rates were 11.9% (5 of 42) and 35.9% (14 of 39) in patients who had none and one or more mutations at the four sites, respectively (Fig. 5b). Patients with increased mutations at the four sites tended to be in the SVR or ETR groups. We subsequently analyzed various clinical factors by multivariate analysis among the three response groups to determine the independent predictors for SVR and NR (Table 3). Among these clinical factors, the NS5B mutation described above was independently associated with NR ($P = 0.0185$).

Mutations of the NS5B region, which codes for the viral RdRp, may alter its enzymatic activities which may influence serum virus load of each patient. In our results, however, there was no obvious correlation between the number of NS5B mutations and serum viral loads in each patient, nor was there a difference in the serum virus loads between the patient groups categorized by the numbers of mutations at aa 309, 333, 338 and 355 of NS5B.

Table 1 Sequence analysis of full genome of hepatitis C virus (HCV) RNA treated with interferon (IFN) plus ribavirin

	G-to-A and C-to-U	Other transition (A-to-G and U-to-C)
IFN plus ribavirin	58.3	72.5
No ribavirin (IFN monotherapy)	60.8	70.4

Mutations per 10 000 nucleotides. A total of 56 538 nucleotides were sequenced.

DISCUSSION

In the present study, we have demonstrated that particular amino acid changes in the NS5B region of HCV

Table 2 Baseline characteristics of the group of 81 patients, segregated according to the clinical outcome of interferon (IFN) plus ribavirin combination therapy

	SVR	ETR	NR	*P*-value
Number of patients	19	40	22	
Age (years)	49.5 ± 12.2	55.9 ± 8.1	57.2 ± 10.6	NS
Sex (male/female)	15/4	27/13	11/11	NS
Baseline ALT (IU/L)	122.2 ± 88.0	80.2 ± 43.2	107.4 ± 73.7	NS
Platelet count ($10^3/mm^3$)	16.0 ± 5.5	16.3 ± 5.5	14.7 ± 4.5	NS
Fibrosis stage (SD)	1.41 ± 0.71	1.92 ± 0.94	2.10 ± 0.72	0.012†
Serum HCV RNA at baseline (KIU/mL)	480.5 ± 295.7	594.6 ± 239.3	599.9 ± 271.3	NS
Number of ISDR mutations	1.73 ± 2.92	0.80 ± 1.22	1.00 ± 1.80	NS

†Significant differences between SVR and others. Values are expressed as mean ± SD, except where noted. ALT, alanine aminotransferase; ETR, end-of-treatment responder; NR,-non-responder; NS, not significant; SR, sustained responder.

```
              B                          C                              D                      E
                              300                                                   355
Consensus  RRCRASGVLTTSCGNTLTCYLKASAAC RAAKL QDCTMLVNGDDLVVICES AGTQEDAAS LRVFTEAMTRYSAPPGD PPQPEYDLE LITSCSSNVSVAHDA
Hcv-J      ---------------------T--- ----- ------------------ --------A --A-------------- --------- ---------------
```

Non responders (n=22)

End of treatment responders (n=40)

Sustained virologic responders (n=19)

309 333 338 355

Figure 3 Amino acid sequence alignments of the NS5B motif B-E in 81 patients with chronic hepatitis C virus (HCV)-1b infection and treated with interferon (IFN) plus ribavirin for 24 weeks. Amino acid residues are indicated by the standard single-letter codes, and dashes indicate the identical amino acid residues with consensus sequence and HCV-J shown at the top. At four amino acid positions (NS5B 309, 333, 338 and 355), usages of amino acid residues differ between non-responders (NR) and others. Changes of this part are indicated by bold letter. Outcome of combination therapy is shown on the left side.

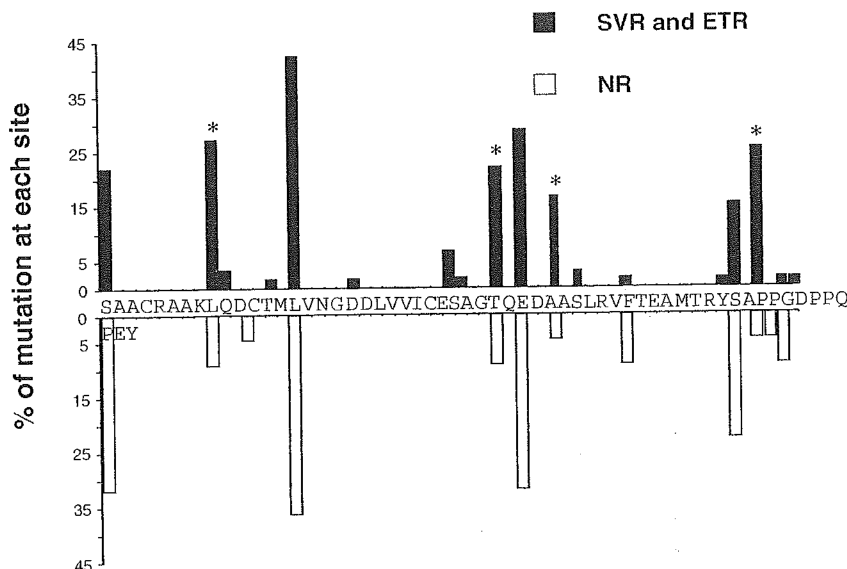


Figure 4 Relationship between frequency of mutations at each site in NS5B 300–358 and the efficacy of interferon (IFN) plus ribavirin treatment. Amino acid residues are indicated by the standard single-letter codes. Among these 59 sites, mutations of aa NS5B 309, 333, 338 and 355 (identified by *) are frequent in sustained virologic response (SVR) and end-of-treatment response (ETR) patients. NR, non-response.

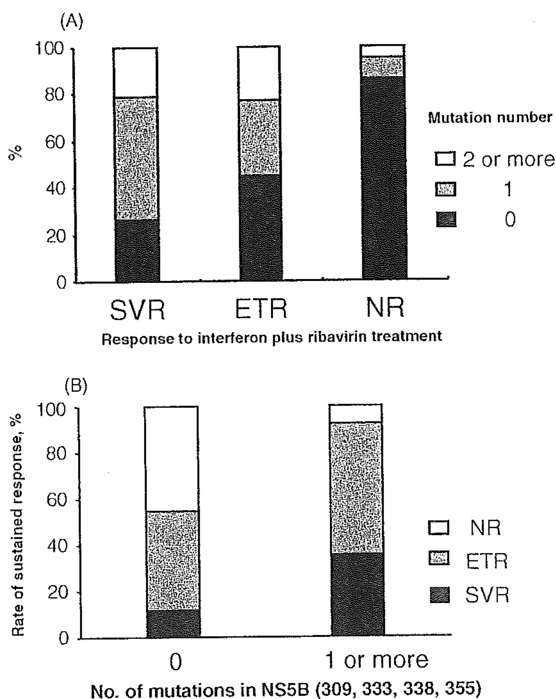


Figure 5 Relationship between number of mutations in NS5B 309, 333, 338, 355 and the outcome of interferon (IFN) plus ribavirin treatment. (a) Distribution of total numbers of mutations at aa. 309, 333, 338 and 355 of NS5B according to sustained virologic response (SVR), end-of-treatment response (ETR) and non-response (NR) patients. (b) Proportion of SVR, ETR and NR patients between groups with or without mutations at aa. 309, 333, 338 and 355 of NS5B.

Table 3 Multivariate analysis for the clinical and virological factors affecting virological responses (SVR and NR) to interferon (IFN) plus ribavirin combination therapy in the group of 81 patients

	Patient with SVR P-value	Patient with NR P-value
Age (years)	0.572	0.598
Sex (male/female)	0.814	0.158
Baseline ALT (IU/L)	0.022	0.981
Platelet count (10^3/mm^3)	0.749	0.627
Mean fibrosis stage (SD)	0.037	0.330
Serum HCV RNA at baseline	0.227	0.890
No. of ISDR mutations	0.491	0.754
No. of NS5B mutations (309,333,338,355)	0.057	0.019

ALT, alanine aminotransferase; ETR, end-of-treatment response; ISDR, interferon sensitivity determining region; NR, non-response; SR, sustained response.

correlate with the clinical outcome of combination therapy. Pair-wise comparisons of the full-length HCV genome in three patient sera obtained before and 12 weeks after the start of IFN plus RBV therapy did not show consistent amino acid changes. The results suggest negative evidence against the presence of treatment-resistant viral sub-populations. On the contrary, subsequent analyses of mutation patterns in the NS5B region in 81 patients showed a significant correlation between particular amino acid mutations of NS5B and the outcome of the combination therapy. Mutations of aa. 309, 333, 338 and 355 of the NS5B were significantly more frequent in SVR and ETR patients, in which the virus has been persistently or at least tempo-

rarily eliminated. Total numbers of mutations at the four amino acid positions were significantly more in SVR and ETR patients compared to NR patients (0.93 ± 0.89 *vs* 0.27 ± 0.70; $P = 0.0004$). These data suggest that particular amino acid mutations of NS5B-RdRp protein may confer sensitivity to combination therapy.

Recently, several studies on mutational analyses of HCV NS5B have identified several key residues responsible for its RdRp activity. Lohmann *et al.* noted that one single amino acid substitution in NS5B increased the efficacy of colony formation by 500-fold in HCV subgenomic replicon.[39] Cheney *et al.* noted that several amino acid substitutions (K155A, R168A, D225N and R386Q) were detrimental to both *in vitro* polymerase activity and replicon RNA replication in Huh-7 cells.[40] Recently, Young *et al.* suggested that NS5B F415Y mutation in HCV-1a was a key resistant variant for RBV monotherapy.[33] However, Y415 is the consensus residue for all genotypes except for 1a and 6a. In the present study of three non-responders, there was no difference at NS5B Y415 between sera collected before treatment and sera collected 12 weeks after the start of treatment with combination therapy.

The locations of the four mutations within the calculated tertiary structure of NS5B RdRp are illustrated in Figure 6a,b. The mutations in NS5B, which were more frequently found in the SVR and the ETR patients, were clustered in motif B to E of RdRp. The amino acid 309 and 355 are both located on the enzyme surface of the substrate entry site. NS5B 333 and 338 are adjacent to the NTP tunnel (Fig. 6b). Because mutations found in HBV and HIV DNA polymerase/reverse transcriptase are known to be located on the surface of the catalytic domain, the mutations in HCV RdRp that were found in the present study may considerably affect their enzymatic activity. Our preliminary data have shown that the HCV subgenomic replicon carrying point mutations in aa. 141 in NS5B less efficiently than the original sequences. Further studies are needed to clarify the role of these point mutations in NS5B in determining the activity of RdRp.

A recent study by Crotty *et al.* has shown that direct antireplicative effects of RBV on viruses include 'error catastrophe' theory in which misincorporations of RBV triphosphate into the viral genome lead to accumulation of mutations in the viral genome and yield defective virus genome. Characteristic pattern of nucleotide mutations by RBV are an increase of G-to-A and C-to-U transition mutations.[17,18] In our present study, although the majority of the mutations were transitions, there was no significant difference in the ratios of the G-to-A and C-to-U mutations between IFN monotherapy and combination therapy (Table 1). One explanation for the discrepancy is that the concentration of RBV in clinical use is too low to act as a mutagen. The clinically achievable blood concentration of RBV is 10–30 μM.[41] On the contrary, an *in vitro* study of polio virus has shown that RBV concentration of 100 μM is required to increase the mutation frequency by at least 1.2-fold.[17] Highly mutated HCV can be excluded or escape detection by RT-PCR and minor clone of HCV quasi-species are excluded by direct sequence of nested PCR products. Therefore, although it is not clear whether RBV is a mutagen against viral genome, our results suggest other mechanisms of RBV contribute to suppress HCV replication, such as inhibition of enzymatic activities of viral RNA polymerase.

Many studies have endeavored to identify factors predictive of the outcome of IFN plus RBV combination therapy. Factors that have been examined include pre-treatment clinical parameters such as baseline viral load, degree of fibrosis, and gender.[42] One study has

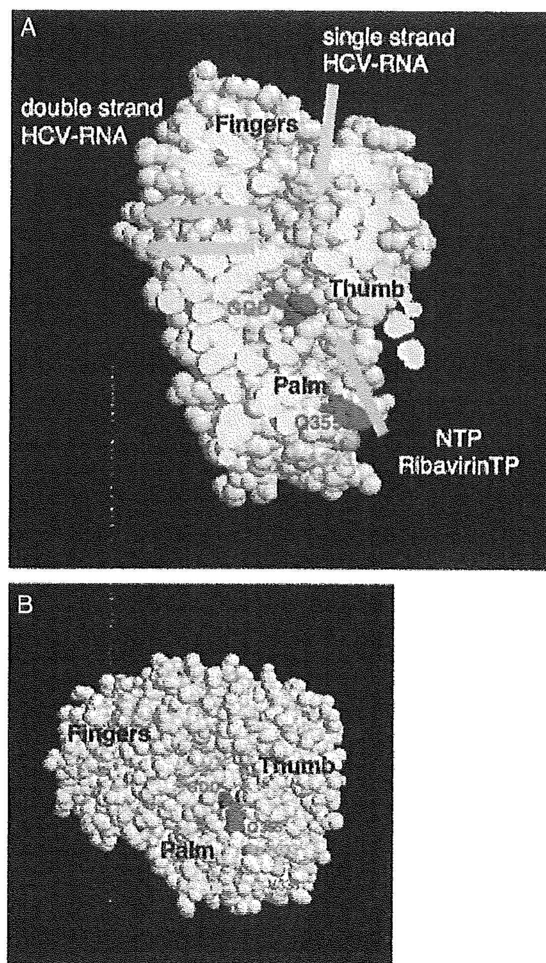


Figure 6 Crystal structure of the hepatitis C virus (HCV) NS5B-RNA dependent RNA polymerase (RdRp). The molecular model of NS5B was constructed using 1QUV from Protein Data Bank (PDB). A space-filling representation of each atom is shown. Graphics were generated using Rasmol 2.7.2.1. (a) Cross-section of the RdRp at level of nucleotide tunnels. The single stranded HCV RNA enters the enzyme through a groove at the top of the finger domain, and the NTP or ribavirin enters the enzyme through the right lower dNTP tunnel (between β fingers and thumb). The essential GDD motif is shown in pink. NS5B 309, 333, 338 and 355 are shown in yellow, orange, green and red, respectively. (b) View from the dNTP entry site.

found early viral response (two-log decline of HCV RNA) to be predictive of SVR.[43] Another study showed that ISDR mutations were correlated with the SVR in chronic HCV 1b infection in Taiwan.[44] In the present study, multivariate analysis identified baseline ALT and the degree of fibrosis as independent factors for SVR. Further multivariate analysis showed that the number of mutations at positions NS5B 309, 333, 338 and 355 were independently associated with NR ($P = 0.0185$). The possible implications of our results are that the number of the above-described NS5B mutations is an independent predictive factor and that the parameter predicts NR patients exclusively from SVR or ETR patients. Our results which may enable prediction of NR before initiation of therapy might be of value when we consider indication for IFN plus RBV antiviral therapy or when making a decision about early cessation of the therapy, which may avoid possible side-effects and therapy costs. Although further studies of a larger population of patients are needed, the mutation number might be used to tailor therapy and is a useful factor for clinicians in making a clinical decision to stop treating HCV infection with combination therapy.

Given the absence of proven anti-HCV agents other than IFN and RBV, these combinations will continue to dominate therapy against HCV. Our present results provide evidence of a significant correlation between the response to IFN plus RBV combination therapy in patients with chronic HCV-1b infection and the amino acid changes that were present before therapy in conserved regions of NS5B. Certain amino acid changes in the HCV NS5B-RdRp domain may correlate with the clinical outcome of combination therapy and could thus be an initial predictor for response to IFN plus RBV combination therapy.

REFERENCES

1 Seeff LB, Hoofnagle JH. Appendix: The National Institutes of Health Consensus Development Conference Management of Hepatitis C 2002. *Clin. Liver Dis.* 2003; 7: 261–87.

2 Hoofnagle JH. Course and outcome of hepatitis C. *Hepatology* 2002; **36**: S21–9.

3 Hoofnagle JH, di Bisceglie AM. The treatment of chronic viral hepatitis. *N. Engl. J. Med.* 1997; **336**: 347–56.

4 Poynard T, Bedossa P, Chevallier M *et al.* A comparison of three interferon alfa-2b regimens for the long-term treatment of chronic non-A, non-B hepatitis. Multicenter Study Group. *N. Engl. J. Med.* 1995; **332**: 1457–62.

5 Poynard T, Marcellin P, Lee SS *et al.* 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* 1998; **352**: 1426–32.

6 Reichard O, Norkrans G, Fryden A, Braconier JH, Sonnerborg A, Weiland O. Randomised, double-blind, placebo-controlled trial of interferon alpha-2b with and without ribavirin for chronic hepatitis C. The Swedish Study Group. *Lancet* 1998; **351**: 83–7.

7 McHutchison JG, Gordon SC, Schiff ER *et al.* Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N. Engl. J. Med.* 1998; **339**: 1485–92.

8 Davis GL, Esteban-Mur R, Rustgi V *et al.* Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. Interventional Hepatitis Interventional Therapy Group. *N. Engl. J. Med.* 1998; **339**: 1493–9.

9 Witkowski JT, Robins RK, Sidwell RW, Simon LN. Design, synthesis, and broad spectrum antiviral activity of 1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide and related nucleosides. *J. Med. Chem.* 1972; **15**: 1150–4.

10 Sidwell RW, Huffman JH, Khare GP, Allen LB, Witkowski JT, Robins RK. Broad-spectrum antiviral activity of Virazole: 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. *Science* 1972; **177**: 705–6.

11 Cramp ME, Rossol S, Chokshi S, Carucci P, Williams R, Naoumov NV. Hepatitis C virus-specific T-cell reactivity during interferon and ribavirin treatment in chronic hepatitis C. *Gastroenterology* 2000; **118**: 346–55.

12 Tam RC, Lim C, Bard J, Pai B. Contact hypersensitivity responses following ribavirin treatment in vivo are influenced by type 1 cytokine polarization, regulation of IL-10 expression, and costimulatory signaling. *J. Immunol.* 1999; **163**: 3709–17.

13 Ning Q, Brown D, Parodo J *et al.* Ribavirin inhibits viral-induced macrophage production of TNF, IL-1, the procoagulant fgl2 prothrombinase and preserves Th1 cytokine production but inhibits Th2 cytokine response. *J. Immunol.* 1998; **160**: 3487–93.

14 Streeter DG, Witkowski JT, Khare GP *et al.* Mechanism of action of 1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole), a new broad-spectrum antiviral agent. *Proc. Natl. Acad. Sci. USA* 1973; **70**: 1174–8.

15 Severson WE, Schmaljohn CS, Javadian A, Jonsson CB. Ribavirin causes error catastrophe during Hantaan virus replication. *J. Virol.* 2003; **77**: 481–8.

16 Contreras AM, Hiasa Y, He W, Terella A, Schmidt EV, Chung RT. Viral RNA mutations are region specific and increased by ribavirin in a full-length hepatitis C virus replication system. *J. Virol.* 2002; **76**: 8505–17.

17 Crotty S, Cameron CE, Andino R. RNA virus error catastrophe: direct molecular test by using ribavirin. *Proc. Natl. Acad. Sci. USA* 2001; **98**: 6895–900.

18 Crotty S, Maag D, Arnold JJ *et al.* The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. *Nat. Med.* 2000; **6**: 1375–9.

19 Tam RC, Lau JY, Hong Z. Mechanisms of action of ribavirin in antiviral therapies. *Antivir. Chem. Chemother.* 2001; **12**: 261–72.

20 Maag D, Castro C, Hong Z, Cameron CE. Hepatitis C virus RNA-dependent RNA polymerase (NS5B) as a mediator of the antiviral activity of ribavirin. *J. Biol. Chem.* 2001; **276**: 46094–8.

21 Hirsch MS, D'Aquila RT. Therapy for human immunodeficiency virus infection. *N. Engl. J. Med.* 1993; **328**: 1686–95.

22 Dienstag JL, Schiff ER, Wright TL *et al.* Lamivudine as initial treatment for chronic hepatitis B in the United States. *N. Engl. J. Med.* 1999; **341**: 1256–63.

23 Arts EJ, Quinones-Mateu ME, Albright JL *et al.* 3′-Azido-3′-deoxythymidine (AZT) mediates cross-resistance to

nucleoside analogs in the case of AZT-resistant human immunodeficiency virus type 1 variants. *J. Virol.* 1998; **72**: 4858–65.

24 Benhamou Y, Bochet M, Thibault V *et al.* Long-term incidence of hepatitis B virus resistance to lamivudine in human immunodeficiency virus-infected patients. *Hepatology* 1999; **30**: 1302–6.

25 de Jong MD, Veenstra J, Stilianakis NI *et al.* Host-parasite dynamics and outgrowth of virus containing a single K70R amino acid change in reverse transcriptase are responsible for the loss of human immunodeficiency virus type 1 RNA load suppression by zidovudine. *Proc. Natl. Acad. Sci. USA* 1996; **93**: 5501–6.

26 Gauthier J, Bourne EJ, Lutz MW *et al.* Quantitation of hepatitis B viremia and emergence of YMDD variants in patients with chronic hepatitis B treated with lamivudine. *J. Infect. Dis.* 1999; **180**: 1757–62.

27 Imamichi T, Berg SC, Imamichi H *et al.* Relative replication fitness of a high-level 3′-azido-3′-deoxythymidine-resistant variant of human immunodeficiency virus type 1 possessing an amino acid deletion at codon 67 and a novel substitution (Thr-->Gly) at codon 69. *J. Virol.* 2000; **74**: 10958–64.

28 Miller V, Ait-Khaled M, Stone C *et al.* HIV-1 reverse transcriptase (RT) genotype and susceptibility to RT inhibitors during abacavir monotherapy and combination therapy. *Aids* 2000; **14**: 163–71.

29 Shah FS, Curr. KA, Hamburgh ME *et al.* Differential influence of nucleoside analog-resistance mutations K65R and L74V on the overall mutation rate and error specificity of human immunodeficiency virus type 1 reverse transcriptase. *J. Biol. Chem.* 2000; **275**: 27037–44.

30 Winters MA, Shafer RW, Jellinger RA, Mamtora G, Gingeras T, Merigan TC. Human immunodeficiency virus type 1 reverse transcriptase genotype and drug susceptibility changes in infected individuals receiving dideoxyinosine monotherapy for 1–2 years. *Antimicrob. Agents Chemother.* 1997; **41**: 757–62.

31 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N. Engl. J. Med.* 1996; **334**: 77–81.

32 Enomoto N, Sakuma I, Asahina Y *et al.* Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. *J. Clin. Invest.* 1995; **96**: 224–30.

33 Young KC, Lindsay KL, Lee KJ *et al.* Identification of a ribavirin-resistant NS5B mutation of hepatitis C virus during ribavirin monotherapy. *Hepatology* 2003; **38**: 869–78.

34 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 1987; **162**: 156–9.

35 Poch O, Sauvaget I, Delarue M, Tordo N. Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J.* 1989; **8**: 3867–74.

36 Lesburg CA, Cable MB, Ferrari E, Hong Z, Mannarino AF, Weber PC. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nat. Struct. Biol.* 1999; **6**: 937–43.

37 Lohmann V, Korner F, Herian U, Bartenschlager R. Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. *J. Virol.* 1997; **71**: 8416–28.

38 Lohmann V, Roos A, Korner F, Koch JO, Bartenschlager R. Biochemical and structural analysis of the NS5B RNA-dependent RNA polymerase of the hepatitis C virus. *J. Viral Hepat.* 2000; **7**: 167–74.

39 Lohmann V, Korner F, Dobierzewska A, Bartenschlager R. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J. Virol.* 2001; **75**: 1437–49.

40 Cheney IW, Naim S, Lai VC *et al.* Mutations in NS5B polymerase of hepatitis C virus: impacts on in vitro enzymatic activity and viral RNA replication in the subgenomic replicon cell culture. *Virology* 2002; **297**: 298–306.

41 Larrat S, Stanke-Labesque F, Plages A, Zarski JP, Bessard G, Souvignet C. Ribavirin quantification in combination treatment of chronic hepatitis C. *Antimicrob. Agents Chemother.* 2003; **47**: 124–9.

42 Poynard T, McHutchison J, Goodman Z, Ling MH, Albrecht J. Is an 'a la carte' combination interferon alfa-2b plus ribavirin regimen possible for the first line treatment in patients with chronic hepatitis C? The ALGOVIRC Project Group. *Hepatology* 2000; **31**: 211–18.

43 Seeff LB, Hoofnagle JH. National Institutes of Health Consensus Development Conference: management of hepatitis C 2002. *Hepatology* 2002; **36**: S1–2.

44 Hung CH, Lee CM, Lu SN *et al.* Mutations in the NS5A and E2-PePHD region of hepatitis C virus type 1b and correlation with the response to combination therapy with interferon and ribavirin. *J. Viral Hepat.* 2003; **10**: 87–94.

SPECIAL ARTICLE

Consensus Proposals for a Unified System of Nomenclature of Hepatitis C Virus Genotypes

Peter Simmonds,[1] Jens Bukh,[2] Christophe Combet,[3] Gilbert Deléage,[3] Nobuyuki Enomoto,[4] Stephen Feinstone,[5] Phillippe Halfon,[6] Geneviève Inchauspé,[7] Carla Kuiken,[8] Geert Maertens,[9] Masashi Mizokami,[10] Donald G. Murphy,[11] Hiroaki Okamoto,[12] Jean-Michel Pawlotsky,[13] François Penin,[3] Erwin Sablon,[9] Tadasu Shin-I,[10] Lieven J. Stuyver,[14] Heinz-Jürgen Thiel,[15] Sergei Viazov,[16] Amy J. Weiner,[17] and Anders Widell[18]

International standardization and coordination of the nomenclature of variants of hepatitis C virus (HCV) is increasingly needed as more is discovered about the scale of HCV-related liver disease and important biological and antigenic differences that exist between variants. A group of scientists expert in the field of HCV genetic variability, and those involved in development of HCV sequence databases, the Hepatitis Virus Database (Japan), euHCVdb (France), and Los Alamos (United States), met to re-examine the status of HCV genotype nomenclature, resolve conflicting genotype or subtype names among described variants of HCV, and draw up revised criteria for the assignment of new genotypes as they are discovered in the future. A comprehensive listing of all currently classified variants of HCV incorporates a number of agreed genotype and subtype name reassignments to create consistency in nomenclature. The paper also contains consensus proposals for the classification of new variants into genotypes and subtypes, which recognizes and incorporates new knowledge of HCV genetic diversity and epidemiology. A proposal was made that HCV variants be classified into 6 genotypes (representing the 6 genetic groups defined by phylogenetic analysis). Subtype name assignment will be either confirmed or provisional, depending on the availability of complete or partial nucleotide sequence data, or remain unassigned where fewer than 3 examples of a new subtype have been described. In conclusion, these proposals provide the framework by which the HCV databases store and provide access to data on HCV, which will internationally coordinate the assignment of new genotypes and subtypes in the future. (HEPATOLOGY 2005;42:962-973.)

Abbreviations: HCV, hepatitis C virus; IDU, injection drug user; RF, recombinant form; ICTV, International Committee for the Taxonomy of Viruses.

From the [1]Centre for Infectious Diseases, University of Edinburgh, Summerhall, Edinburgh, United Kingdom; [2]Hepatitis Viruses Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD; [3]Institut de Biologie et Chimie des Protéines, Pôle BIoinformatique de Lyon, BioSciences Lyon-Gerland, Lyon, France; [4]First Department of Internal Medicine, University of Yamanashi, Tamaho, Yamanashi, Japan; [5]Laboratory of Hepatitis Viruses, Division of Viral Products, Food and Drug Administration, Bethesda, MD; [6]Laboratoire Alphabio, Marseille, France; [7]CNRS-BioMérieux, Immunothérapie des Maladies Infectieuses Chroniques, Lyon, France; [8]Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM; [9]Innogenetics n.v., Ghent, Belgium; [10]Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya, Japan; [11]Laboratoire de Santé Publique du Québec, Institut National de Santé Publique du Québec, Québec, Canada; [12]Division of Virology, Department of Infection and Immunity, Jichi Medical School, Tochigi, Japan; [13]Department of Virology, INSERM U635, Henri Mondor Hospital, University of Paris, Créteil, France; [14]Virco BVBA, Generaal De Wittelaan, Mechelen, Belgium; [15]Institut für Virologie, Justus-Liebig-Universität, Giessen, Germany; [16]Institute of Virology, Essen University Hospital, Essen, Germany; [17]Chiron Corporation, Emeryville, CA; and the [18]Department of Medical Microbiology, Malmö University Hospital, Lund University, Malmö, Sweden.

Received April 21, 2005; accepted June 2, 2005.

The European HCV database is funded by HepCVax Cluster (EU FP5 grant QLK2-2002-01329) and VIRGIL Network of Excellence (EU FP6 grant LSHM-CT-2004-503359).

Address reprint requests to: Peter Simmonds, Centre for Infectious Diseases, University of Edinburgh, Summerhall, Edinburgh, EH9 1QH, United Kingdom. E-mail: Peter.Simmonds@ed.ac.uk; fax: (44) 131 650 6511.

Copyright © 2005 by the American Association for the Study of Liver Diseases.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.20819

Potential conflict of interest: Nothing to report.

The VIIIth Report of the International Committee for the Taxonomy of Viruses (ICTV) currently classifies hepatitis C virus (HCV) and GB virus B as members of the *Hepacivirus* genus in the virus family, *Flaviviridae*.[1] Although the report recognizes the existence of 6 main genetic groups of HCV and designates them as "clades," it is beyond the remit of the ICTV to extend classification proposals below the level of species. Thus, separate arrangements are required for the standardization of genotype and subtype assignments of genetic variants of HCV.

A meeting was convened at the 11th International Symposium on HCV and Related Viruses, Heidelberg, Germany, October 2004. This was a successor to the first HCV classification meeting in Santa Fe, New Mexico, in 1997, with a similar membership of scientists from North America, Europe, and Japan working in the field of HCV sequence variation.[2] The purpose of the meeting was to analyze the current description, assignment, and nomenclature of HCV genetic variants and to review new developments in studies of HCV genetic variability and epidemiology. A new aim was to formally link genotype nomenclature proposals with the organization and sequences retrieval systems available on three HCV sequence databases that provide a resource to study genetic variability of HCV and its clinical, epidemiological, and therapeutic manifestations. The first database was created in Japan by Prof. Masashi Mizokami and co-workers (http://s2as02.genes.nig.ac.jp/), the second in the European Union by Prof. Gilbert Deleage et al.[3] (http://euhcvdb.ibcp.fr/), and the third in the United States by Dr. Carla Kuiken et al.[4] (http://hcv.lanl.gov/ or http://hcv-db.org). The accessibility of these databases and the provision for users to download and analyze annotated sequences make them ideal vehicles for reinforcing a standardized nomenclature system, and their support is an integral part of the outlined proposals. This support entails assisting users to avoid naming conflicts, providing advice and analysis support, ensuring that the nomenclature used in the 3 databases is standardized and follows the guidelines in this paper, and trying to increase awareness of these guidelines in the HCV research community and among journal reviewers and editors.

The meeting was convened with the following broad aims:

1. Standardize nomenclature for existing variants of HCV:
   - Develop consistent nomenclature for variants within each clade
   - Resolve conflicting subtype and genotype designations
   - Publish a complete list of currently classified recognized genotypes and subtypes, with acknowledgment of originating authors
2. Formulate agreed criteria for the designation of new HCV variants:
   - New genetic groups/clades/genotypes
   - Subtypes, recognizing that designation of subtypes may only be epidemiologically relevant in certain cases
   - Recombinant forms of HCV
3. Provide a classification scheme for HCV for research and database use:
   - Standardize nomenclature to provide a common interface for sequence retrieval from HCV databases
   - Provide a relevant classification for investigation of clinical and biological differences between HCV variants

Background

A standard system for HCV classification is of importance in studies of the epidemiology, evolution, and pathogenesis of HCV. Of particular clinical importance is the need to understand genotype-specific differences in response to interferon-α–based treatments. A classification system has to be robust, based on objective criteria, and able to accommodate new genetic variants and recombinant forms that are discovered in the future. To achieve this, the classification of HCV should be based, as with other biological systems, on its evolutionary history (as far as it is currently understood). The following section reviews current thoughts on the origins and epidemiology underlying the observed genetic diversity of HCV, and how these aspects may be incorporated into the proposed classification scheme.

HCV Sequence Variability. When the extent of the genetic heterogeneity of HCV was discovered in the early 1990s, a number of different methods were used for classifying variants.[5-12] These differed from each other in the methods used to delineate different genotypes (by pairwise distance measurements or by phylogeny), whether they incorporated the two levels of sequence variability in the nomenclature system, and finally, in the letters or numbers assigned to each recognized genetic group. Progress toward resolving these uncertainties in HCV classification was made by publication of a consensus paper in 1994,[13] proposing the classification of HCV by phylogenetic methods into 6 genotypes (updated phylogenetic tree shown in Fig. 1). These approximately equidistant genetic groups each contain a variable number of more closely related, genetically (and epidemiologically) distinct "subtypes." Genotypes differ from each other by

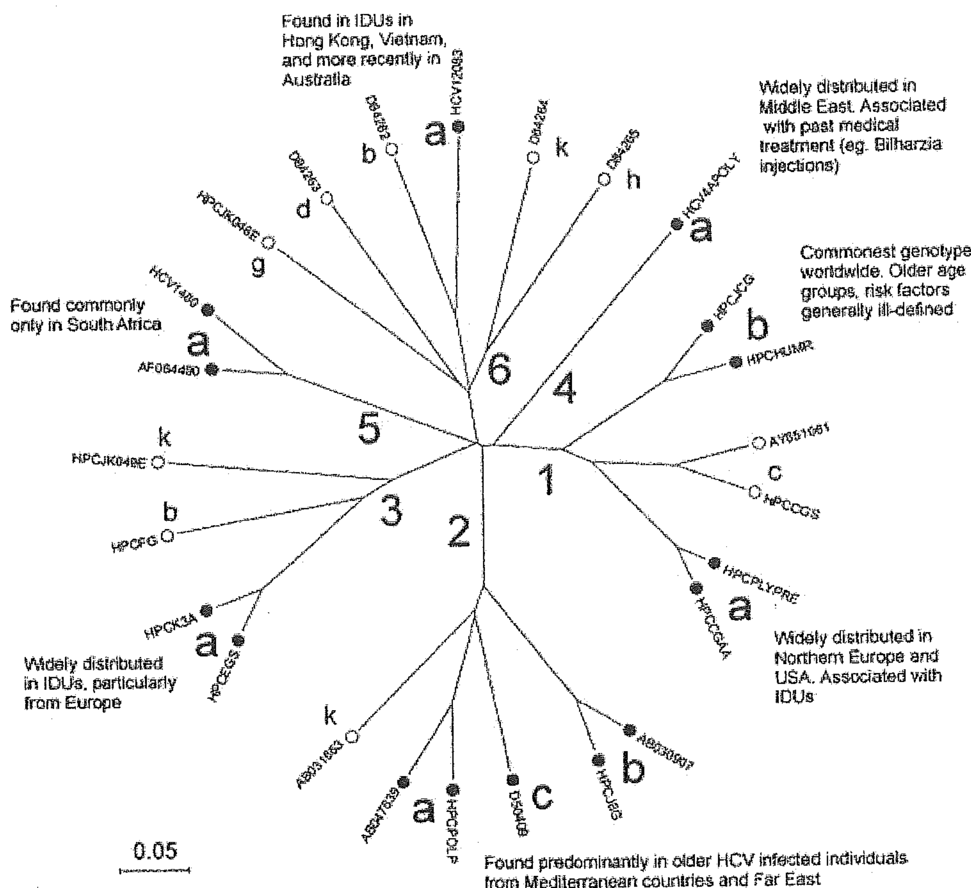


Fig. 1. Evolutionary tree of available complete open-reading frame sequences for each HCV genotype. Phylogenetic analysis was carried out on complete coding sequences of genotypes of HCV (maximum 2 where multiple sequences available; prioritized as in Table 1). The main identified risk groups for each genotype (IDUs, recipients of unscreened blood or blood products, other parenteral exposures) has been indicated where information is available (filled circles and accompanying text). These represent the main variants believed to have become prevalent in industrialized countries over the course of the 20th century. HCV genotypes 3k, 6d, 6g, 6h, and 6k are the re-assigned names of the previously described genotypes "10a," "7b," "11a," "9a," and "8b," respectively (Table 2). The tree was constructed by neighbor-joining as implemented in the MEGA package,[97] using Jukes-Cantor corrected distances.

31% to 33% at the nucleotide level, compared with 20% to 25% between subtypes. Despite the sequence diversity of HCV, all genotypes share an identical complement of co-linear genes of similar or identical size in the large open reading frame, and the genetic inter-relationships of HCV variants are remarkably consistent throughout the genome.[2] This has enabled many of the currently recognized variants of HCV to be provisionally classified, based on partial sequences from subgenomic regions such as core/E1 or NS5B.[14] The most conserved regions of the HCV genome are the 5′ untranslated region, and the terminal 99 bases of the 3′untranslated region. The inferred amino acid sequence of the core gene is also relatively invariant between genotypes. The most variable region of the HCV genome is the hypervariable region of E2.[15,16] Here, the large number of likely immune-selected amino acid changes[17-22] distorts the underlying phylogeny of HCV apparent from comparison of other genomic regions.

Each genetic group of HCV comprises varying numbers of more closely related variants, typically different from each other at 20% to 25% of nucleotides, compared with more than 30% between genotypes (Fig. 1). The most common variants found in Western countries have previously been classified with subtype labels, such as 1a and 1b in genotype 1; and 2a, 2b, and 2c in genotype 2. These variants have become very widely distributed over the past 50 to 70 years as a result of transmission through blood transfusion and various other invasive medical and surgical procedures, and by needle sharing between injection drug users (IDUs). They now represent the vast majority of infections in Western countries encountered clinically, and for which most information has been collected on disease progression and response to α-interferon–based treatment.

Since the original classification of HCV, further molecular epidemiology studies have revealed the existence of much greater diversity in certain regions of sub-Saharan Africa and in South and Southeast Asia (Fig. 2). Most new variants originate from specific geographical regions; for example, infections in Western Africa are predominantly by genotype 2,[23-27] whereas those in Central Africa, such as the Democratic Republic of Congo and Gabon, are by genotypes 1 and 4.[12,24,28-32] Taking this geographical mapping further, genotypes 3 and 6 show similar genetic diversity in South and Eastern Asia.[24,33-35]

These observations indicate the likely long-term presence in human populations in parts of Africa and Asia, distinct from HCV transmission patterns in Western and other non-tropical countries. The relatively recent ap-

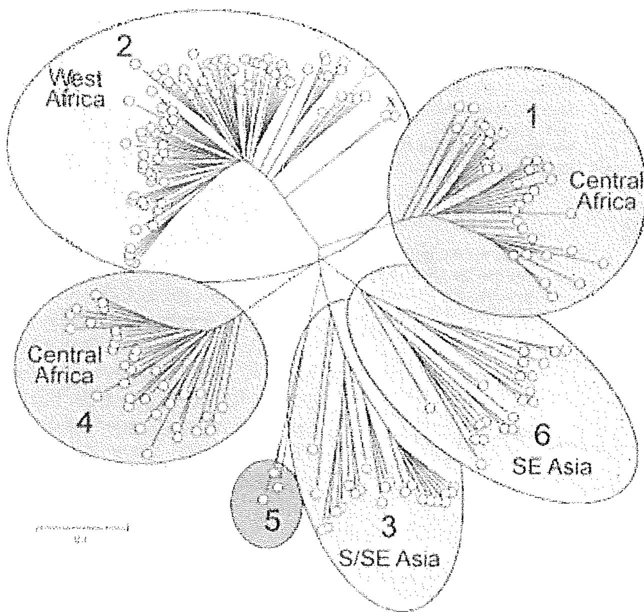


Fig. 2. Evolutionary tree of all available NS5B sequences of HCV. This phylogenetic analysis of the NS5B region of all publicly available nucleotide sequences in the region from 8276 to 8615 (numbered as in the H77 reference sequences, AF009606[63]) demonstrates that HCV variants still fall into 6 distinct genotypes but each contains numerous novel variants discovered in high-diversity areas in sub-Saharan Africa and Southeast Asia. The tree was constructed by neighbor-joining as implemented in the MEGA package,[97] using Jukes-Cantor corrected distances. More divergent members of genotype 2 are indicated with an "x."

pearance of new risk groups and routes of spread,[32,36] such as blood transfusion since the 1940s, the medical use of unsterilized needles for injections and vaccinations, and most specifically to industrialized countries, injecting drug use and the sharing of injection equipment,[32,37-39] has allowed the rapid spread and amplification of "founder" viruses. What we now call subtypes 1a, 1b, 2a, 2b, 3a, and 4a are likely to be the descendants of HCV variants that "seeded" these new, rapidly expanding transmission networks. As discussed later, HCV classification should both recognize the epidemiological associations of these "founder" viruses and incorporate their subtype names into the genotype nomenclature, while acknowledging that such labels are of little or no value in the description of HCV variants in high-diversity areas in sub-Saharan Africa and Southeast Asia.

Recombination. A recent discovery with implications for HCV classification is recombination between genotypes of HCV.[40,41] Homologous recombination in HCV could clearly be facilitated by the overlap in genotype distributions in many parts of the world. It also may be favored by the nature of HCV risk behavior, in which there may be frequent exposures around the time of primary infection (*e.g.*, repeated needle-sharing between several IDUs), and lack of protective immunity from reinfection during chronic HCV infection. Recently, a viable and rapidly spreading recombinant containing structural genes from genotype 2k and non-structural genes from genotype 1b was found in IDUs in St. Petersburg, Russia.[40,41] Inter-subtype (or intra-genotype) recombinants have also been described, such as a 1a/1b recombinant in Peru.[42] The true frequency of recombination may be underestimated because it would be difficult to detect if it occurred between variants of the same subtype. Similarly, it would be difficult to document intersubtype recombinants where HCV is highly diverse, such as within genotype 2 in West Africa. Finally, although there is a comparative wealth of complete genome sequences of common HCV genotypes, such as 1b, most studies of HCV variability in high diversity areas are based on analysis of single sub-genomic regions, such as NS5B or core/E1, making detection of potential recombination events unlikely.

HCV Genotype Identification. Genotype identification is clinically important because genotypes 1 and 4 are more resistant than genotypes 2 and 3 to the current standard of care, pegylated interferon-α and ribavirin combination therapy.[43] Indeed, most treatment protocols require genotype information to tailor dose and duration of treatment. Genotyping assays are usually based on sequence analysis of an amplified segment of the genome, commonly the 5′untranslated region, because this region is targeted by most diagnostic assays for HCV RNA. Although this region is highly conserved, a well-characterized set of polymorphisms predict genotype and can be conveniently detected by probe hybridization,[44,45] changes in restriction sites[46,47] or by direct sequencing.[48]

For the purposes for which they are normally used (prediction of treatment response and dose scheduling),[43] currently used 5′UTR-based assays are acceptably accurate, with more than 95% concordance with genotypes identified by nucleotide sequencing in NS5B or other coding regions of the genome.[49-55] Several factors, however, preclude their use for definitive genotype identification, for identification of subtypes, and more generally, as an HCV classification tool:

- Although several genotype-specific nucleotide changes in the 5′UTR usually allow each of the 6 main genotypes to be differentiated from each other, there are exceptions. Some genotype 6 variants found in Southeast Asia have 5′UTR sequences identical to those of genotype 1a or 1b.[34-36,56] This illustrates a more general point that, even for genotype identification, the performance of genotyping assays is very much a property of the range of HCV variants tested. The currently used 5′UTR-based assays are unlikely to operate to the published level of accuracy (>95%; see above) in high-diversity areas.

• Even for well-characterized variants of HCV, such as those circulating in Western countries, sequence differences between subtypes may be variable or non-existent in the 5′UTR. For example, a sequence polymorphism at position 243 (numbered as in the H77 reference sequence), frequently used to differentiate subtypes 1a and 1b, is unreliable. In one of the original surveys, 6 (7.5%) of 80 subtype 1a sequences and 1 of 79 1b sequences would have been incorrectly identified on the basis of this polymorphism.[57] A related problem is that although some subtypes may be separately identifiable in the 5′UTR (such as 2a and 2b), others, such as 2c, may not, even though all 3 subtypes are approximately equally divergent from each other elsewhere in the genome (Fig. 1).

• Even relatively short coding regions of the HCV genome provide more definitive information on the genotype or subtype of an HCV variant than the 5′UTR. Although not necessarily required clinically, the nucleotide sequence of a sub-genomic region (including the conserved core gene) allows definitive identification of genotype and generally of the subtype, as well as being able to predict the existence of HCV variants not yet classified.

• For all genotyping assays, whether based on the 5′UTR or elsewhere in the genome, there is an intrinsic assumption that the genotype inferred from 1 region reflects that of the genome as a whole. Although few recombinant forms have been described, the spread of HCV variants such as the 2k/1b recombinant and the generation of further hybrid viruses in multiply exposed individuals would increasingly limit the accuracy of genotyping assays, and importantly for their clinical use, attenuate their predictive value for treatment response.

As should be evident from these points, HCV identification is an activity distinct from HCV classification. Classification provides the framework on which the specificity and accuracy of genotyping assays can be assessed, and for this purpose an agreed and consistent set of classification criteria, and a system of assigning genotype names is required. The following section discusses the issues in HCV classification in which consensus is required, and is followed by a series of classification and nomenclature proposals designed to maintain clarity in this field.

Current Issues to Resolve in HCV Classification

Several problems and uncertainties with current classification schemes for HCV have been identified and cause both inconsistencies with the nomenclature of HCV variants in published papers and difficulties for the organization and retrieval of HCV sequences from the 3 databases. These can be summarized as follows:

Diversity Within Genetic Groups. Although the primary division of HCV variants into 6 genetic groups is evident from phylogenetic analysis (Figs. 1 and 2), it has been increasingly recognized that there is considerably more genetic diversity within groups 2, 3, and 6 than found between the originally classified subtypes 1a and 1b, and 2a, 2b, and 2c.[34] In the past, it had been additionally proposed that more divergent variants within groups 3 and 6 qualify as separate major genotypes of HCV. At the HCV Classification meeting in Santa Fe, genetic group 6 was proposed to be re-designated as "clade 6," its variants retain their proposed genotype designations as genotypes 6, 7, 8, 9, and 11; similarly, "clade 3" should contain variants classified as genotypes 3 and 10.[2] In this scheme, the one-to-one correspondence between genetic group and genotype is lost.

The imposition of an additional tier of variability, however, leads to largely arbitrary classification decisions that compromised the simplicity of the original primary assignment of HCV genetic groups as genotypes. For example, both subtype 3b and the proposed new genotype 10a are both in genetic group 3 but are both highly divergent in sequence from subtype 3a, much more so than other subtypes of genotype 3 (Fig. 1). The decision to classify 10a as a genotype and 3b as a subtype was based on a difference in nucleotide sequence divergence in the coding region of only 3% (23% between 3a and 3b, 26% between 3a and 10a). This is much lower than the 31% to 34% divergence between variants in different genetic groups (such as between 1a and 2a). Divergence between the various proposed genotypes in group 6 is similarly consistently lower (mean, 27%; range, 21%-29%) than between the originally classified genotypes. Genetic group 2 may similarly contain more divergent sequences than the norm for subtypes (marked as "x" in Fig. 2). This might lead to the addition of further, equally arbitrary, genotype designations in a geographical region where otherwise genotype 2 variants are predominant in the population.

Apart from the difficulty in placing this further dividing line between genotype and clade, the resulting classification in a subtype/genotype/clade hierarchy is geographically inconsistent. To many, the scheme has been confusing, because in some cases, a clade contains only 1 genotype and the terms are interchangeable (*e.g.*, genotype 1/clade 1); in others a clade may contain 5 or more genotypes (*e.g.*, clade 6, genotypes 6, 7, 8, 9, and 11). This confusion and lack of consensus has led to continuing nomenclature differences between publications

whenever variants from Southeast Asia and elsewhere are described.

Conflicting Subtype Designations. There are many examples of conflicting nomenclature within currently classified HCV variants. Most of these inconsistencies comprise 2 different subtypes being referred to by the same name, such subtypes "4a" found in Egypt[7] and Zaire.[12] Conversely, the same variant may be described with different subtype designation, such as VAT96, designated as "2k,"[58] and RU169 designated as "2j."[59] These occurrences will have to be resolved in an agreed catalogue of HCV variants, and for retrieval of sequences from the HCV databases.

Recombination. Currently no method exists for classifying recombinant forms of HCV. For database retrieval and for cataloguing the occurrence of recombinant viruses, a nomenclature system that recorded its genotype composition and provided unique identifiers for pattern of breakpoints would be of value. This system is in place for HIV-1 and might be used as a model for HCV.[60] Here, designation of inter-subtype recombinant viruses as (circulating) recombinant forms (RFs) requires detection and complete genome sequences of a recombinant virus from 3 or more independently infected individuals. Each new recombinant should have breakpoints in the same positions in each sequence. Each is then numbered sequentially in order of discovery, with subtype identification letters listed alphabetically to approximately indicate their composition. The HCV recombinant in St. Petersburg[40,41] would therefore be designated as RF 01_1b2k.

Table 1. Confirmed HCV Genotypes/Subtypes

Genotype*	Locus/Isolate(s)†	Accession number(s)	Reference(s)
Genotype 1			
1a	HPCPLYPRE, HPCCGAA	M62321, M67463	67, 68
1b	HPCJCG, HPCHUMR	D90208, M58335	69, 70
1c	HPCCGS, AY051292	D14853, AY051292	71
Genotype 2			
2a	HPCPOLP, JFH-1	D00944, AB047639	72, 73
2b	HPCJ8G, JPUT971017	D10988, AB030907	9, 74
2c	BEBE1	D50409	75
2k	VAT96	AB031663	58
Genotype 3			
3a	HPCEGS, HPCK3A	D17763, D28917	76, 77
3b	HPCFG	D49374	78
3k	HPCJK049E1	D63821	59
Genotype 4			
4a	HCV4APOLY	Y11604	79
Genotype 5			
5a	EUH1480, SA13‡	Y13184, AF064490	80, 81
Genotype 6			
6a	HCV12083, 6a33	Y12083, AY858526	82
6b	Th580	D84262	83
6d	VN235	D84263	83
6g	HPCJK046E2	D63822	59
6h	VN004	D84265	83
6k	VN405	D84264	83

NOTE. Tables 1, 2, and 3 were compiled by a working group of Donald Murphy, Erwin Sablon, and Phillipe Halfon.

*Consensus proposed genotype/subtype names. For instances in which multiple sequences of a HCV genotype are available, two sequences have been listed, prioritized by (1) publication date, or (2) submission date when unpublished.

†Locus (or isolate name, if locus is the same as the accession number).

‡Sequence obtained from acute phase plasma of a chimpanzee experimentally infected with (human-derived) isolate SA13.

Consensus Classification Proposals

Each of these issues in HCV classification was discussed, and the following consensus decisions were made. These are proposals for standardizing the nomenclature of currently described variants of HCV, and the future designation of new subtypes and genotypes as they are discovered.

Division of HCV Into Clades/Genotypes. The primary division of HCV variants remains the 6 genetic groups, irrespective of the hugely increased numbers of subtypes or variants since found within these groups. The consensus acknowledges that different levels of within-group diversity are found between genotypes, and different relationships within them. Nevertheless, varying degrees of diversity are becoming apparent in other genotypes (*e.g.,* among the genotype 2 variants from West Africa), and it is difficult and arbitrary to specify a degree of sequence divergence below which a subtype designation is made, and above which a new genotype is assigned. This difficulty is epitomized by the problems with the classifications of 3b and 10a within genotype 3 (see above).

The following points summarize the recommendations concerning the designation of HCV genotypes:

1. The primary division of HCV will henceforth be based on the 6 genetic groups apparent from Figs. 1, 2, and other published sequence analyses of HCV. Division of HCV variants into the 6 genetic groups of HCV is supported by each of the principal methods of phylogenetic analysis of the core/E1, NS5B, and complete genome sequences (Table 1). These comprise tree-building by: (i) neighbor-joining and unweighted pair group method with arithmetic mean from pairwise distances computed with a variety of substitution models, (ii) parsimony, and (iii) maximum likelihood. For distance-based methods, greater than 70% of trees (actually invariably greater than 90%) support the primary division of HCV variants into the 6 genetic groups, with no consistent support for any higher-level grouping. Consistency between phylogenetic methods is required for the assignment of new genotypes (see specific proposals below).