

が、これらをまとめてみると、HCV陽性レシピエントに肝移植後、3～96カ月の間にペグインターフェロンとリバビリンが投与開始されている。男性128例、女性51例で平均年齢54.4歳であった。今まで報告されているペグインターフェロンはすべてa 2bで1.0～1.5 μ g/kg/週で、リバビリンは400～1,000 mgであった。投与期間はI型とIV型は48週間、II型とIII型は24週間投与であった(表2)。

成績全体をまとめると、SVRは179例中46例(26%)であり、いわゆるBiochemical Responderは129例中58例(45%)であった。投与中止例は148例中48例(32%)にみられ、インターフェロンもしくはリバビリンの減量例は75例中46例(61%)に及んでいた。

以前から、言及されていたインターフェロンによる拒絶反応の誘発は記載のある報告だけで見ると75例中16例(13%)に認めたとされている(表3)。

以前のインターフェロンの週3回投与とリバビリン併用療法と比較してみると、なんと大雑把な計算であるが、ペグインターフェロンを用いた最新の報告とペグ化していないインターフェロンとリバビリン併用療法でそのSVR率は26%と全く変わりはないことが判明した。さらに、驚いたことに中止率や脱落率もほとんど変わりのないことが明らかになった。すなわち、インターフェロンでもペグインターフェロン投与でも、リバビリン併用療法による成績は全く変わりのないことが分かった。

従来のインターフェロンよりもペグインターフェロンの方が、血中濃度、AUCさらにはコンプライアンスから通常の慢性肝炎に対してはより普遍化されているが、肝移植医療の領域では必ずしもそうではないようであ

る。すなわち、副作用の発現が認められた際の対処に困難を要する可能性を示すからである。従来型インターフェロンで副作用が出現しても、血中濃度から考慮すると、即座に投与中止で、その濃度は下降し、副作用は改善するだろう。しかし、ペグインターフェロンの場合、その効果を血中濃度を考慮するならば、副作用の継続が、例え投与中止しても改善されない可能性がある。もちろん、中和抗体がない現状では、危険があるといわざるを得ない。したがって、肝移植後のインターフェロン投与に関しては、通常インターフェロンで導入するのが副作用の面からも推奨されるかもしれないが、全くエビデンスのない話で、これらは今後の症例数の増加から結論が得られるのかもしれない。

4 結 論

従来のインターフェロンからペグインターフェロンを新たに用いたりバビリンとの併用療法がさらなる効果と、脱落例や中止例を減少させたか否かが今回の検証時のポイントであった。結論からいうと、ペグインターフェロンを用いても期待される以上の効果と副作用、脱落例と中止例の減少を得ることは困難なようである。通常型のインターフェロンとペグインターフェロンではリバビリン併用療法では全く効果に差異がないことが今このころ判明している。

むしろ、拒絶反応を少し誘発している可能性もある。これら検索には免疫抑制剤の種類による検討はなされていないが、例えばサイクロスポリンにHCVに対する抗ウイルス作用を考慮するならばこの抗ウイルス療法での免疫抑制剤の種類も今後考えなければならぬことかもしれない。

表2 ペグインターフェロンとリバビリン併用療法の背景

報告者(文献)	報告年	症例数	年齢性別	移植後投与開始時期	PEGの種類	PEGの量	RIBの量	投与期間
Didier Samuel (22)	2003	22 (1型17)	58歳男13女9	95.5月	a 2b	1.0 μg/kg/週	7.5mg/kg/日	48 (1型)または24 (1型以外)週
S.Mukherjee (23)	2003	39 (1型31,2型2,3型3,4型3)	50.4歳(19-75)男33女6	22月(2-166)	a 2b	1.5 μg/kg/週	800mg/日	48 (1,4型)または24 (2,3型)週
Hector Rodriguez-Luna (24)	2004	19 (1型12,2型3,3型3,4型1)	53歳男14女5	32月	a 2b	0.5 μg/kg/週最大1.5 μg/kg	400→1,000mg/日	48週
Jerome Dumortier (25)	2004	20 (1型16,2型1,3型2,4型1)	53.8歳男13女7	28月	a 2b	0.5to1.0 μg/kg/週	400→1,200mg/日	48週
M Babatin (26)	2005	13 (1a2,1b6,2型2,3型1,4型1,6型1)	49.4歳(44.3-55.5)男10女3	26.4月	a 2b	平均0.91 μg/kg/週(0.5-1.1)	平均662mg/日(0-1200)	48週,34.5 (2,4-93)
J.M.Moreno Planas (27)	2005	30 (1a3,1b24,3型3)	56歳(40-68)男22女8	52月	a 2b	1.5 μg/kg/週	10.6mg/kg/日	48 (1,4型)またはor24 (2,3型)週
Pierluigi Toniutto (28)	2005	12 (1b)	56.5歳(23-63)男6女6		a 2b	0.5 μg/kg/週	600-800mg/日	48週
Lluis Castells (29)	2005	24 (1b24)	61.4歳男17女7	3.8月	a 2b	1.5 μg/kg/週	600→800mg/日	48週
総計		179例	54.4歳男128例女51例		a 2b	1.0-1.5 μg/kg/週	400mg-1000mg	すべて48週

表3 ペグインターフェロンとリバビリン併用療法の成績

報告者(文献)	報告年	SVR率	BR率	NC率	中止率	減量率	拒絶反応
Didier Samuel (22)	2003	4/22 (18%)	11/22 (50%)		14/22 (64%)		
S.Mukherjee (23)	2003	12/39 (31%)	12/39 (31%)	8/39 (21%)	17/39 (44%)		0
Hector Rodriguez-Luna (24)	2004	5/19 (26%)			7/12 (58%)		1/19 (5%)
Jerome Dumortier (25)	2004	9/20 (45%)	15/20 (75%)		4/20 (20%)	6 (IFN)13 (RIB)/20 (95%)	5/20 (25%)
M Babatin (26)	2005	4/13 (31%)	9/13 (69%)		4/13 (31%)	6 (ribavirin)/13 (47%)	3/13 (23%)
J.M. Moreno Planas (27)	2005	14/30 (47%)			11/30 (37%)	12/30 (40%)	0
Pierluigi Toniutto (28)	2005	1/12 (8%)	3/12 (25%)		7/12 (58%)	11/12 (92%)	0
Lluis Castells (29)	2005	8/23 (35%)	8/23 (35%)				1/23 (4%)
総計(平均)		46/178 (26%)	58/129 (45%)	8/39 (21%)	48/148 (32%)	46/75 (61%)	10/75 (13%)

5 今後の課題

予後が不良とされてきたHCV陽性レシピエントに対して、今、考えられているのは、以下の点である。①肝移植後は3～6カ月以内にインターフェロンとリバビリン併用療法を速やかに開始することである。②可能な限りステロイドは離脱状況が望ましい。③そして、副作用の点からインターフェロン用量、投与間隔、リバビリン用量は適宜漸減して、少なくとも半年、可能であれば48週間継続投与が望ましい。④脱落例に対しては、通常慢性肝炎治療に即してトランスアミナーゼ値を正常化させる目的の抗炎症療法の継続を選択せざるを得ないと思われる。適切な薬剤として強力ネオミノファーゲンCとUDCA辺りが妥当であろうか。その場合、線維化の進展抑制が目標となるであろうが、臨床的事実は一つもないのが現状である。

特に、肝移植後、いつ抗ウイルス療法を開始するかが、一つの問題点である。肝移植後のプロトコル肝生検の妥当性は別として、術後3～6カ月、1年、1.5年後に肝生検を施行して、F1/A1以上の変化がみられた段階で抗ウイルス療法(ペグインターフェロンとリバビリン)を開始するのが世界の趨勢になりつつあるようであるが、preemptive treatmentの成績も良好なので、今後の症例の積み重ねが必要となってくるであろう。

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成人生体肝移植におけるネオーラル投与の実際

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Optimal application of Neoral in adult living donor liver transplantation

key words : 成人生体肝移植, ネオーラル, AUC

近年, 生体肝移植において, 免疫抑制剤の血中濃度が脳死肝移植に比して相対的に高値を示すこと¹⁾, タクロリムス(FK506)の至適投与量がグラフト重量に規定されること²⁾などが報告され, 免疫抑制剤の薬物動態が生体肝移植と脳死肝移植とで異なることが示されている。

そこで, 生体肝グラフトの再生がシクロスポリン(ネオーラル)の代謝に及ぼす影響について検討し, 生体肝移植における therapeutic drug monitoring(TDM)に基づいたネオーラルの至適使用法の確立を目指した³⁾。

対象と方法

2001年4月から現在までネオーラル経口投与をベースとする免疫抑制療法を施行し, 薬物動態を解析できた10例の成人生体肝移植症例を対象

とした。

術前日, 術後3, 7, 21病日(POD)に経時的(ネオーラル投与後1, 2, 4, 6, 12時間後)に末梢静脈血および門脈血を採取し, area under the time concentration curve(AUC)を測定し, 7POD, 30PODに施行したCT volumetryによりグラフト肝容量を測定した。

免疫抑制はネオーラルとステロイドの2剤で行い, ネオーラル投与量は術前日5 mg/kg/dayで開始し, 術後4週間はトラフ値200~300 ng/mLを目標とした。

また, Level/Dose比(L/D比) = トラフ値 / 投与量, Δ AUC = 門脈血 AUC - 末梢血 AUC, 肝除去率 = Δ AUC / 門脈血 AUC, などについても検討した。

結果

(1) 周術期のAUC

術前はいずれの症例においてもC₂がピーク

表1 ネオーラル使用例の臨床成績: FK506との対比

(Mean follow up)	Neoral-based (24.3 months)	FK506-based (41.5 months)
Total cases (Male/Female)	10 (6/4)	15 (5/10)
Acute rejection	3 (30.0%)	6 (40.0%)
Infection	2 (20.0%) CMV enterocolitis, HCV acute hepatitis	3 (20%) CMV enterocolitis, sepsis, pulmonary aspergillosis
Side effects (others)	2 (20.0%) seizure, renal failure	2 (13.3%) renal failure
Graft or patient loss	1 (10.0%) PSC recurrence (reALDLT)	2 (13.3%) idiopathic cardiac failure, pulmonary aspergillosis

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値で、 C_2 、AUCは脳死肝移植で報告されているデータとほぼ同様であった($C_2 = 1,013 \pm 216$ ng/mL, $AUC_{0-12} = 6,952 \pm 1,143$ ng·hr/mL)。一方、3PODではピークが明らかでない症例や遅れる症例が多く、 C_{max} 484 ± 263 ng/mLと低かった。7PODでは術前のAUCに戻る傾向であったが、脳死肝移植での報告⁴⁾とくらべて C_0 、 C_2 、AUCともに低値であった。

術後1週間以内の AUC_{0-12} は C_2 のみならず C_0 ともよく相関し($r = 0.773$, $p = 0.0001$)、トラフ値でモニターしても支障のないことが示唆された。

(2) 肝再生に伴うシクロスポリンの代謝変動

シクロスポリンの肝除去率は7PODでは3PODにくらべ有意に増加し、グラフト容積の増加率と相関したが、プロトロンビン時間やASTなどの肝機能とは相関しなかった。30PODのL/D比はグラフト容積/標準肝容積(GV/SLV)と有意な負の相関($r = -0.699$, $p < 0.05$)を示し、肝再生に伴うグラフト容積の増大がシクロスポリンの代謝亢進に関与することが示唆された。

生体肝移植では、グラフトサイズがシクロスポリンの代謝に影響を与えることを考慮してネオー

ラルの投与量を加減する必要がある。

(3) 臨床成績

ネオーラルを*de novo*で使用した症例の成績は表1のごとくで、FK506をベースにした症例と拒絶率、合併症率、生存率などに差はなく、良好であった。

おわりに

ネオーラルは、生体肝移植においても安全・有効に使用できたが、脳死肝移植にくらべて吸収・代謝に違いがあり、生体肝移植独自の投与プロトコールが必要である。

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Quantitative Analysis of Anti-Hepatitis C Virus Antibody-Secreting B Cells in Patients With Chronic Hepatitis C

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To investigate the quantitative characteristics of humoral immunity in patients with hepatitis C, we established an enzyme-linked immunosorbent spot (ELISpot) assay for detection of anti-hepatitis C virus (HCV)-secreting B cells. Receiver operating characteristic curve analysis demonstrated 100% specificity and 58% to 92% sensitivity for detecting B-cell responses to NS5b, NS3, E2, and core antigens. The median sum of anti-HCV-secreting B cells to all HCV antigens tested was significantly higher in 39 patients with chronic hepatitis C (47.3 spot forming cells [SFCs]/10⁶ peripheral blood mononuclear cells [PBMCs]) than in 9 recovered subjects (15.3 SFCs/10⁶ PBMCs; $P = .05$) or 11 uninfected controls (5.3 SFCs/10⁶ PBMCs; $P < .001$); the significant difference ($P = .018$) in chronic versus recovered patients was in reactivity to nonstructural antigens NS3 and NS5b. Anti-HCV immunoglobulin M (IgM)-secreting B cells were also readily detected and persisted decades into HCV infection; there was no difference in IgM-positive cells between chronic and recovered patients. ELISpot reactivity to genotype 1-derived antigens was equivalent in patients of genotypes 1, 2, and 3. There was significant correlation between the numbers of anti-HCV IgG-secreting B cells and serum aminotransferase and to the level of circulating antibody. **In conclusion**, ELISpot assays can be adapted to study B-cell as well as T-cell responses to HCV. Measurement at the single-cell level suggests that humoral immunity plays a minor role in recovery from HCV infection and that B-cell immunity is strongest in those with persistent infection. (HEPATOLOGY 2006;43:91-99.)

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. More than half of patients with acute HCV infection develop chronic hepatitis, leading to cirrhosis and/or hepatocellular carcinoma in at least 20% of these patients.¹⁻³ Chronic HCV infection results in the induction of a strong humoral immune response, and measurement of anti-HCV antibodies in serum is widely used to screen for

HCV infection. Although several studies have examined the features of the humoral immune response to HCV,⁴⁻⁷ the quantitative characteristics of HCV-specific antibody production during infection remain undefined. In patients with acute hepatitis C, an early HCV-specific T-cell response is associated with viral clearance,⁸⁻¹¹ but the role of humoral immune responses in HCV clearance is unclear and appears to be subsidiary, because strong antibody responses are detected in all immunocompetent chronic HCV carriers. It is also unknown whether anti-HCV antibodies serve to control the level of viremia during chronic infection and whether they ameliorate horizontal or vertical transmission.

An enzyme-linked immunosorbent spot (ELISpot) assay for detecting individual B cells secreting specific antibodies has enabled investigators to study B-cell immunity at a cellular level in a variety of clinical applications.^{12,13} The advantages of the ELISpot assay are that it can detect even a single cell out of 10⁶ peripheral blood mononuclear cells (PBMCs), whose secretion level may not be sufficient for detection of circulating antibody, and distinguishes and quantifies only active immunoglobulin-secreting

Abbreviations: ELISpot, enzyme-linked immunosorbent spot; HCV, hepatitis C virus; SFC, spot-forming cell; PBMC, peripheral blood mononuclear cell; Ig, immunoglobulin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PBS, phosphate-buffered saline; ROC, receiver-operating characteristics; AUC, area under the curve; IQR, interquartile range.

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cells. This assay thus provides a useful tool for better understanding immunity to infectious diseases and improved analysis of the immune response to vaccination.¹⁴ Although studies of antigen-specific antibody-secreting cells in various viral infections have been conducted,¹⁵⁻¹⁹ there are no published data on detection and quantification of anti-HCV antibody-secreting B cells.

The objective of this study was to adapt the ELISpot assay for the detection of anti-HCV antibody-secreting B cells to (1) clarify the HCV-specific humoral immune responses in patients with chronic hepatitis C, (2) examine the correlation between the numbers of anti-HCV antibody-secreting B cells and clinical outcomes, and (3) examine humoral immune responses in patients with chronic hepatitis C compared with those who spontaneously clear HCV.

Patients and Methods

Subjects. Individuals who were identified by the Greater Chesapeake and Potomac Region of the American Red Cross as being positive for anti-HCV via enzyme immunoassay at the time of blood donation were referred to the Department of Transfusion Medicine at the National Institutes of Health for participation in a long-term study of the natural history of HCV infection^{20,21}; 750 participants were enrolled from 1990 through September 2003. Of these, 48 subjects were selected randomly to assess humoral immune responses at the B-cell level. The chronic hepatitis C group included 39 subjects who were positive for anti-HCV antibodies (EIA-2 and RIBA-3) and positive for HCV RNA. The recovered group included 9 anti-HCV-positive subjects who were HCV RNA-negative via qualitative polymerase chain reaction on at least two consecutive visits. The patients' characteristics are summarized in Table 1. Eleven volunteer blood donors without a history of HCV infection served as controls. All subjects were negative for hepatitis B surface antigen and antibodies to the human immunodeficiency virus. The study protocols were reviewed and approved by the appropriate institutional review boards, and all subjects gave written informed consent to participate in the study.

Laboratory Testing. Antibodies to HCV were measured in serum samples via second-generation enzyme immunoassay (EIA-2; Abbott Laboratories, North Chicago, IL). EIA-2 reactive samples were subsequently tested via third-generation recombinant immunoblot assay (RIBA-3; Chiron Corp., Emeryville, CA). Reactivity to at least two of four HCV antigens (5-1-1/C100-3, C33, C22, and NS5) was considered a positive RIBA-3 result, no reactivity was considered a negative result, and reactiv-

Table 1. Demographic and Clinical Characteristics of Patients With HCV Infection

Characteristics	All (N = 48)	Chronic (n = 39)	Recovered (n = 9)	P Value
Mean age, yrs (range)	51 (33-83)	52 (37-83)	49 (33-78)	.46
Male, n (%)	23 (48)	17 (44)	6 (67)	.28
Race, n (%)				
White	43 (90)	35 (90)	8 (89)	1.00
Black	5 (10)	4 (10)	1 (11)	
Source of infection, n (%)				
Transfusion	16 (33)	14 (36)	2 (22)	.30
Injection drug use	19 (40)	15 (38)	4 (44)	
Nasal cocaine use	4 (8)	2 (5)	2 (22)	
Occupational	6 (13)	6 (15)	0 (0)	
Unknown	3 (6)	2 (5)	1 (11)	
Genotype, n (%)				
1	25 (52)	24 (62)	1 (11)	.074
2	7 (15)	6 (15)	1 (11)	
3	2 (4)	1 (3)	1 (11)	
Unknown	14 (29)	8 (21)	6 (67)	
Mean values (range)				
ALT (IU/L)	52 (15-251)	58 (28-251)	25 (15-52)	.001
AST (IU/L)	43 (12-145)	48 (12-145)	24 (13-37)	.001
ALP (IU/L)	69 (32-171)	71 (35-171)	59 (32-74)	.20
Total bilirubin (mg/dL)	0.7 (0.3-1.5)	0.7 (0.3-1.5)	0.7 (0.4-1.4)	.72
Albumin (g/dL)	3.9 (3.3-4.5)	3.9 (3.3-4.5)	4.0 (3.6-4.3)	.53
GGTP (g/dL)	44 (8-286)	48 (8-286)	27 (8-102)	.025
HCV RNA level (10 ⁶ IU/mL)	11.2 (<0.5-73)	14.1 (<0.5-73)	ND	<.001
Recombinant strip immunoblot assay				
C100	3.0 (0-4)	3.2 (0-4)	2.1 (0-4)	.042
C33	3.5 (1-4)	3.7 (1-4)	2.6 (1-4)	.011
C22	3.8 (0-4)	3.9 (1-4)	3.1 (0-4)	.068
NS5	2.1 (0-4)	2.3 (0-4)	1.3 (0-4)	.18

Abbreviations: ALP, alkaline phosphatase; GGTP, γ -glutamyltransferase; ND, below the limits of detection.

ity to only one antigen was considered an indeterminate result. The serum levels of HCV RNA were determined using the qualitative and quantitative COBAS AMPLICOR assays (Roche Diagnostic Systems, Branchburg, NJ), which amplify HCV RNA via reverse-transcription polymerase chain reaction. HCV genotypes were determined using INNO-LiPA HCV II (Innogenetics, Gent, Belgium). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and other relevant biochemical tests were performed using standard methods.

PBMCs. PBMCs were isolated from whole blood using cellular preparation tubes (Becton Dickinson, Franklin Lakes, NJ), washed one time in phosphate-buffered saline (PBS) and three times in medium (RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 5×10^{-5} mol/L 2 mercaptoethanol, 50 U/mL penicillin, 50 μ g/mL streptomycin, and 10% fetal bovine serum), and were either studied immediately or cryopreserved in media containing 50% fetal bovine serum, 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO), and 10% RPMI 1640.

HCV Proteins. The recombinant full-length HCV core protein (amino acid residues 1-191), E2 protein

(amino acid residues 384-746), NS3 protein (amino acid residues 1027-1657), and NS5B protein (amino acid residues 2421-3011) were expressed and purified from *Escherichia coli* using the expression vector as previously described.^{22,23} Control proteins were expressed as carboxy-terminal fusion proteins with human superoxide dismutase in *E. coli*.

ELISpot Assay. Ninety-six-well plates containing high-protein binding membranes (MAIP S4510; Millipore Co., Bedford, MA) were coated with a 10- μ g/mL purified recombinant HCV core, E2, NS3, NS5b, or control antigens in carbonate coating buffer (0.1 mol/L Na_2CO_3 , 0.1 mol/L NaHCO_3 ; pH 9.6). After incubation at 4°C overnight, the plates were washed twice with PBS and blocked with 3% bovine serum albumin for more than 30 minutes at 37°C. Cryopreserved PBMCs were thawed and incubated for 44 hours at 37°C in a humidified atmosphere of 5% CO_2 at 1.25×10^5 or 2.5×10^5 cells/well in AIM V Media (Invitrogen, Carlsbad, CA). All determinations were run in triplicate. After incubation, the cells were removed by washing 6 times with PBS containing 0.05% NP-40, and the plates incubated with horseradish peroxidase-linked anti-human IgG or IgM antibodies (1:1,000; KPL, Gaithersburg, MD) at 37°C for 2 hours. After the plates were washed twice with PBS and 6 times with PBS containing 0.05% NP-40, an optimal 4CN peroxidase substrate (Bio-Rad, Hercules, CA) was added and incubated for 20 to 30 minutes at room temperature to develop the spots. The reaction was stopped by washing with distilled water. The plates were dried overnight, and the spots were counted automatically by an ELISPOT reader (Carl Zeiss Vision, Hallbergmoos, Germany). The frequencies of anti-HCV antibody-secreting B cells were calculated by subtracting the mean number of spots in the control wells from the HCV antigen-coated wells, and expressed as the mean of triplicates of spot-forming cells (SFCs) per 10^6 PBMCs. Assays with a high background (>5 spots/well in the negative control) were excluded.

Assay of Anti-HCV/NS3 Antibodies. Anti-HCV/NS3 IgG was assayed via ELISA as described previously.²³ Briefly, MaxiSorp Nunc-Immuno plates were coated with recombinant HCV NS3 protein at 6 μ g/mL in coating buffer (20 mmol/L sodium bicarbonate buffer [pH 9.6], 0.15 mol/L NaCl) and overcoated with 0.1% bovine serum albumin in PBS buffer (pH 7.4). The sera were tested via two-fold serial dilution in 0.3% IGEPAL CA-630 (Sigma), 5% milk diluent (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and PBS [pH 7.4], with initial dilution at 1:250. Biotinylated anti-human IgG γ (Kirkegaard & Perry Laboratories) and streptavidin-horseradish peroxidase (Kirkegaard & Perry Laboratories)

were added sequentially. One hundred microliters per well ABTS microwell peroxidase substrate was used to develop the color and 100 μ L per well peroxidase stop solution (Kirkegaard & Perry Laboratories) was added to stop the reaction. Absorbance was read at 405 nm. The IgG titer was determined via end point dilution.

Statistical Analysis. The Mann-Whitney *U* test or Student *t* test was used to analyze continuous variables as appropriate. Spearman's rank order correlations were used to evaluate the frequencies of anti-HCV antibody-secreting B cells to each antigen and to the clinical features. A *P* value of .05 or less was considered significant. Although SFCs/ 10^6 PBMCs were expressed in this study, the statistics were significant whether this was used or the raw counts were used. Statistical analyses were performed using SigmaStat (version 2.03; SPSS, Chicago, IL). Receiver-operating characteristic (ROC) curve analysis was performed using MedCalc 7.0 software (<http://www.medcalc.be>). The best cutoff values of the ELISpot assays were chosen automatically by MedCalc 7.0 as the SFCs with the highest diagnostic accuracy (*i.e.*, the sum of the false-negative and false-positive rates was minimized). The respective overall diagnostic values were expressed using the area under the curve (AUC).

Results

Optimal Cutoff Values for ELISPOT Assay. To determine the optimal cutoff values for the B-cell ELISpot assay in differentiating patients with HCV infection from HCV seronegative blood donors, ROC curve analysis was performed. The ROC curves for the ELISpot assay detecting anti-HCV IgG-specific B cells were obtained via calculations made using the values obtained from 48 patients with HCV infection and the 11 HCV-negative volunteer blood donors. The selection of the optimal cutoff point value was based on the level at which the accuracy was maximum (see Patients and Methods). The optimal cutoff values, sensitivity, specificity, positive predictive values, negative predictive values, and calculated AUCs to all HCV antigens are listed in Table 2. In our ELISpot assay, the values of sensitivity ranged from 58% to 92%, and the values of specificity were 100%. The AUC results were constantly high in the ELISpot assays for all antigens, and AUC values were between 0.71 (NS5B antigen) and 0.94 (core and E2 antigens).

After we defined the optimal cutoff value for each antigen, we determined the frequencies of anti-HCV IgG-secreting B cells in 48 patients with HCV infection. The prevalence of anti-HCV IgG-secreting B cells during HCV infection specific for the various antigens were:

Table 2. Optimal Cutoff Values, Sensitivity, Specificity, AUC, and Predictive Values of Anti-HCV IgG-Secreting B Cells in ELISpot Assay in 48 Patients With Chronic Hepatitis C and 11 Volunteer Blood Donors

Antigen	Cutoff Value	Sensitivity, % (95% CI)	Specificity, % (95% CI)	AUC (95% CI)	PPV, %	NPV, %
Core	13.4	92 (80-98)	100 (71-100)	0.94 (0.84-0.98)	100	73
E2	10.7	92 (80-98)	100 (71-100)	0.94 (0.85-0.99)	100	73
NS3	5.4	77 (63-88)	100 (71-100)	0.83 (0.71-0.92)	100	50
NS5B	5.4	58 (43-72)	100 (71-100)	0.71 (0.58-0.82)	100	36

NOTE. All AUC values were significantly higher than a 0.50 nonpredictive value ($P < .001$ for all comparisons).

Cutoff values were determined by making ROC curves and are expressed as SFCs/ 10^6 PBMCs.

Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

core, 92%; E2, 92%; NS3, 77%; and NS5B, 58% (Table 2).

We further assessed the optimal cutoff values for the ELISPOT assay detecting anti-HCV IgM-secreting B cells using ROC curve analysis in 43 patients with HCV infection and in 6 HCV-negative blood donors (Table 3). The AUC values ranged from 0.73 (NS5B antigen) to 0.94 (core antigen). The prevalence of anti-HCV IgM-secreting B cells ranged from 54% (NS5B antigen) to 84% (core antigen) (Table 3).

Detection and Quantitation of Anti-HCV Antibody-Secreting B Cells. Forty-eight PBMC samples obtained from patients with HCV infection and 11 samples from healthy volunteer blood donors were examined for detection of anti-HCV IgG-secreting B cells. The median numbers of the sum of anti-HCV IgG-secreting B cells to all HCV antigens were significantly higher in patients with HCV infection (38.3 SFCs/ 10^6 PBMCs; interquartile range [IQR], 10.7-149.3) compared with control anti-HCV negative donors (5.3 SFCs/ 10^6 PBMCs; IQR, 2.7-8.0; $P < .001$). Figure 1A shows box plots for the numbers of anti-HCV IgG-secreting B cells to all 4 HCV antigens in patients with HCV infection and in the controls. Among 48 patients with HCV infection, the median numbers of anti-HCV IgG-secreting B cells ranged from 10.7 SFCs/ 10^6 PBMCs (NS5B antigen) to 119.0 SFCs/ 10^6 PBMCs (E2 antigen). The median numbers of anti-HCV IgG-secreting B cells in patients with HCV infection were significantly higher than those in controls for each HCV antigen (Fig. 1A).

Subsequently, we developed an ELISpot assay for detecting anti-HCV IgM-secreting B cells. Detection of the anti-HCV IgM-secreting B cells was performed in 43 patients with HCV infection and in 6 anti-HCV negative blood donors (Fig. 1B). The median numbers of the sum of anti-HCV IgM-secreting B cells to all HCV antigens were significantly higher in patients with HCV infection (21.3 SFCs/ 10^6 PBMCs; IQR, 9.2-48.0) compared with the controls (8.0 SFCs/ 10^6 PBMCs; IQR, 0.0-10.7; $P < .001$). The median numbers of anti-HCV IgM-secreting B SFC to the core (31.1 vs. 4.0 SFCs/ 10^6 PBMCs; $P < .001$) and E2 (32.0 vs. 8.0 SFCs/ 10^6 PBMCs; $P = .005$) antigens in patients with HCV infection were significantly higher than those in controls. (Fig. 1B).

Relationship Between Anti-HCV Antibody-Specific B Cells and HCV Genotypes. Because the antigens used were derived from HCV genotype 1a, the numbers of anti-HCV IgG-secreting B cells were compared between 25 patients with HCV genotype 1 infection (10 with 1a, 11 with 1b, and 4 not subtyped) and 9 infected with another single genotype (1 with 2a, 4 with 2b, 2 with 2 untyped, and 2 with 3a). The median value of the anti-HCV IgG-secreting B cells to each antigen was not statistically different between the genotype 1 group and the other genotype groups (Fig. 2). In addition, there were no statistically significant differences in detecting anti-HCV IgM-secreting B cells to all HCV antigens in those with genotype 1 versus non-1 infections (data not shown).

Table 3. Optimal Cutoff Values, Sensitivity, Specificity, AUC, and Predictive Values of Anti-HCV IgM-Secreting B Cells in ELISpot Assay in 43 Patients With Chronic Hepatitis C and 6 Volunteer Blood Donors

Antigen	Cutoff Value	Sensitivity, % (95% CI)	Specificity, % (95% CI)	AUC (95% CI)	PPV, %	NPV, %
Core	12.1	84 (69-93)	100 (54-100)	0.94 (0.84-0.99)	100	46
E2	17.4	72 (56-85)	100 (54-100)	0.86 (0.73-0.94)	100	33
NS3	10.7	70 (54-83)	100 (54-100)	0.74 (0.60-0.86)	100	32
NS5B	8.1	54 (38-69)	100 (54-100)	0.73 (0.58-0.85)	100	23

NOTE. All AUC values were significantly higher than a 0.500 nonpredictive value ($P < .001$ for all comparisons). Cutoff values were determined by making ROC curves and are expressed as SFCs/ 10^6 PBMCs.

Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

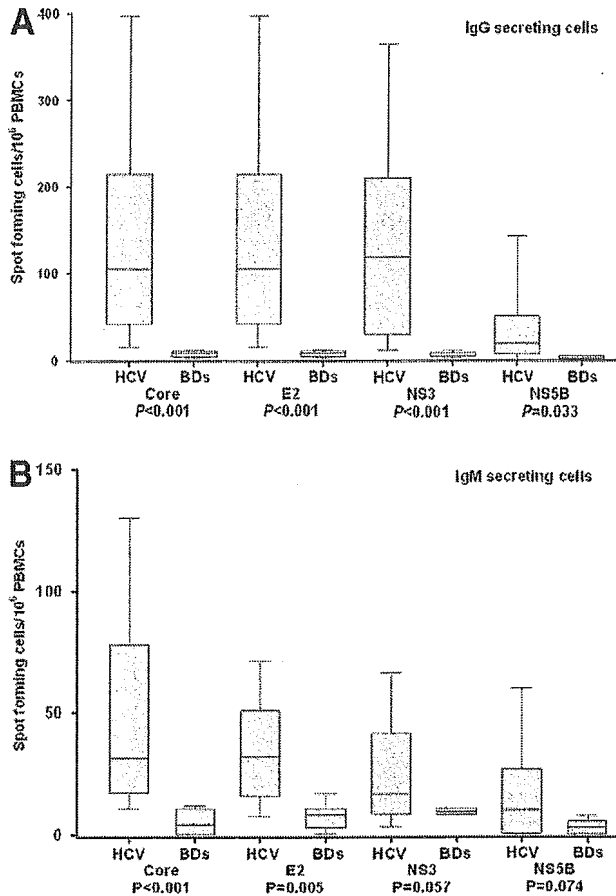


Fig. 1. Detection of anti-HCV antibody-secreting B cells in patients with HCV infection and volunteer blood donors. Boxes represent the IQR of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. (A) The frequencies of anti-HCV IgG-secreting B cells to 4 HCV antigens were detected in 48 patients with HCV infection and in 11 volunteer blood donors. (B) The frequencies of anti-HCV IgM-secreting B cells were detected in 43 patients with HCV infection and in 6 volunteer blood donors. PBMCs, peripheral blood mononuclear cells; IgG, immunoglobulin G; HCV, hepatitis C virus; BDs, blood donors; IgM, immunoglobulin M.

Correlation Between Anti-HCV IgG-Secreting B Cells and Clinical Features in Patients With HCV Infection.

Several demographic (age and sex) and clinical (viral load, genotype, ALT, AST, alkaline phosphatase, total bilirubin, albumin, γ -glutamyltransferase, intensity of RIBA assay, and anti-HCV antibodies) findings were examined for their correlation with anti-HCV IgG-secreting B-cell frequency in patients with HCV infection. The circulating anti-HCV IgG-secreting B-cell frequency to the core antigen (Fig. 3A) was significantly correlated with the value of ALT ($P = .048$, $r = 0.29$) and inversely correlated with serum albumin ($P = .048$, $r = -0.33$). Similarly, the number of anti-HCV IgG-secreting B cells to the E2 antigen was significantly correlated with the

value of ALT ($P = .037$, $r = 0.30$) (Fig. 3B) and AST ($P = .033$, $r = 0.31$) (Fig. 3C) and was inversely correlated with serum albumin ($P = .029$, $r = -0.36$). Furthermore, the number of SFCs to the NS3 antigen was significantly correlated with the circulating antibody level to the NS3 antigen in 38 patients with available serum samples ($P = .008$, $r = 0.43$) (Fig. 3D). There was no significant correlation between the numbers of anti-HCV IgG-secreting B cells to NS3 or NS5b antigens and any of the biochemical, demographic, or clinical parameters specified above.

Comparison of the Number of Anti-HCV Antibody-Secreting B Cells Between Patients With Chronic Hepatitis C and Patients Who Recovered. As shown in Table 1, patients with chronic hepatitis C had significantly higher mean serum levels of ALT (58 vs. 25 IU/L; $P = .001$), AST (48 vs. 24 IU/L; $P = .001$), and γ -glutamyltransferase (48 vs. 27 IU/L; $P = .025$) compared with the recovered patients. The mean HCV RNA level in the chronic group was 14.1×10^5 IU/mL. There were significant differences in the mean intensity of the RIBA assay against the C33 and C100 proteins in chronic vs. recovered subjects (C33, 3.2 vs. 2.1, $P = .042$; C100, 3.7 vs. 2.6, $P = .011$). We found no significant difference between patients with chronic hepatitis C and patients who had recovered when their age, sex, race, source of infection, HCV genotypes, total bilirubin, or albumin were compared.

The median numbers of the sum of anti-HCV IgG-secreting B cells to all HCV antigens were significantly higher in patients with chronic hepatitis C (47.3 SFCs/

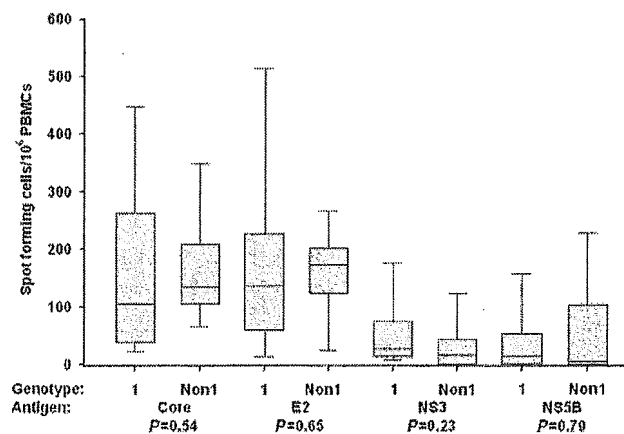


Fig. 2. Detection of anti-HCV IgG-secreting B cells in patients infected with HCV of genotype 1 and nongenotype 1. Boxes represent the IQR of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. The frequencies of anti-HCV-secreting B cells were detected in patients infected with genotype 1 ($n = 25$) and in those with other genotypes ($n = 9$). PBMCs, peripheral blood mononuclear cells; Non1, nongenotype 1.

10^6 PBMCs; IQR, 13.3-149.7) than in recovered patients (15.3 SFCs/ 10^6 PBMCs; IQR, 3.3-142.7; $P = .05$) and normal controls (5.3 SFCs/ 10^6 PBMCs; IQR, 2.7-8.0; $P < .001$). The median numbers of the sum of anti-IgG-

secreting B cells to structural antigens were not significantly higher in patients with chronic hepatitis C (108.3 SFCs/ 10^6 PBMCs) than in those who recovered (97.4 SFCs/ 10^6 PBMCs) (Fig. 4A). In contrast, the median numbers of the sum of anti-HCV IgG-secreting B cells to nonstructural antigens were significantly higher in patients with chronic hepatitis C (19.0 SFCs/ 10^6 PBMCs) than in patients who recovered (4.9 SFCs/ 10^6 PBMCs; $P = .018$), particularly for NS3 antigen (26.7 vs. 5.3 SFCs/ 10^6 PBMCs; $P = .032$) (Fig. 4B). Furthermore, patients with chronic hepatitis C had a significantly higher frequency of anti-HCV IgG-secreting B cells to the NS3 antigen than those who recovered (85% vs. 44%; $P = .02$) (Fig. 4C).

The median numbers of the sum of anti-HCV IgM-secreting B cells to all HCV antigens were similar in patients with chronic hepatitis C (22.0 SFCs/ 10^6 PBMCs; IQR, 8.2-49.3) and recovered patients (20.7 SFCs/ 10^6 PBMCs; IQR, 12.2-36.7) and were significantly higher than in the controls (8.0 SFCs/ 10^6 PBMCs; IQR, 0.0-10.7; $P < .001$) (Fig. 4A). When the responses were analyzed for structural and nonstructural antigens, the median numbers of the sum of anti-HCV IgM-secreting B cells were not significantly different in patients with chronic hepatitis C and recovered subjects for either structural antigens (30.7 vs. 31.6 SFCs/ 10^6 PBMCs) or nonstructural antigens (20.7 vs. 12.7 SFCs/ 10^6 PBMCs) (Fig. 4A).

Discussion

We developed an ELISpot assay for sensitive quantitative assessment of anti-HCV antibody-secreting B cells in PBMCs from patients with HCV infection and used this technique to analyze the induction of humoral immune responses at the single-cell level. IgG and IgM anti-HCV antibody secreting B cells to core, E2, NS3, and NS5 were detected and quantified in patients with chronic HCV infection and compared with recovered patients and uninfected controls. The key findings were: (1) anti-HCV secreting B-cell responses were greater in chronically infected patients than in recovered patients, suggesting that antibody does not play a major role in recovery from acute

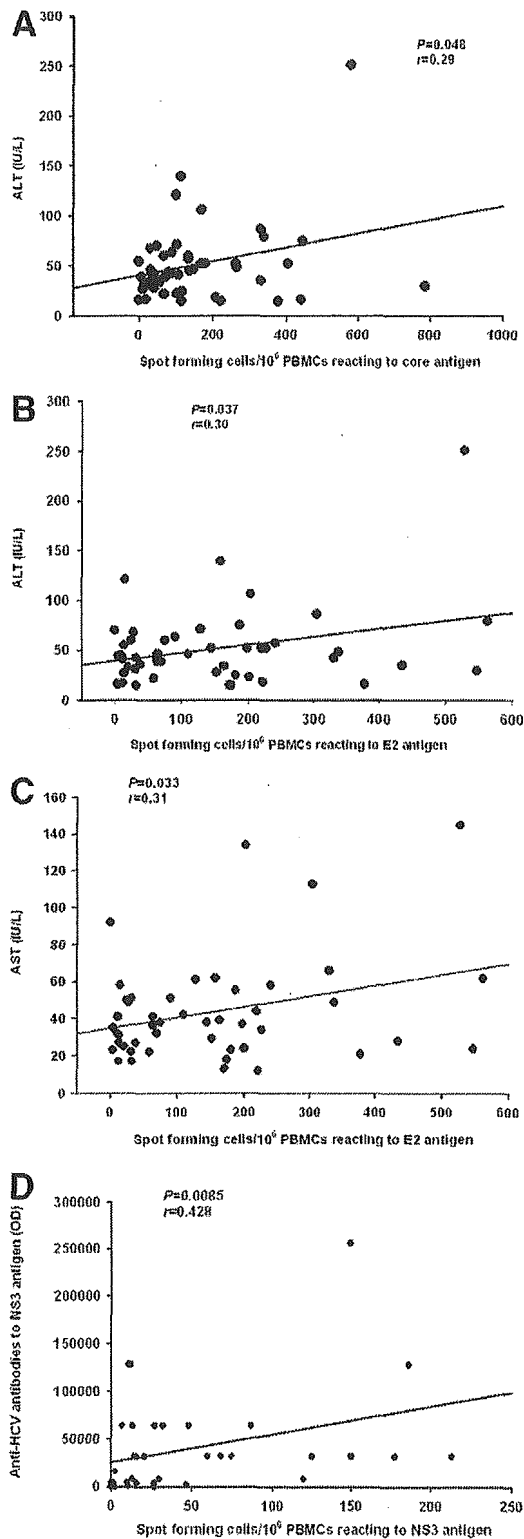


Fig. 3. Correlation of the number of anti-HCV IgG-secreting B cells and clinical characteristics in 48 patients with HCV infection. (A) Frequency of circulating anti-HCV IgG-secreting B cells to core antigen was significantly correlated with the value of ALT ($r = 0.29$, $P = .048$). (B-C) Frequency of circulating anti-HCV IgG-secreting to E2 antigen was correlated with the value of (B) ALT ($r = 0.30$, $P = .037$) and (C) AST ($r = 0.31$, $P = .033$), respectively. (D) Frequency of circulating anti-IgG-secreting B cells to NS3 antigen was correlated with the value of anti-HCV antibodies to NS3 antigen ($r = 0.43$, $P = .0085$). ALT, alanine aminotransferase; PBMCs, peripheral blood mononuclear cells; AST, aspartate aminotransferase; HCV, hepatitis C virus.

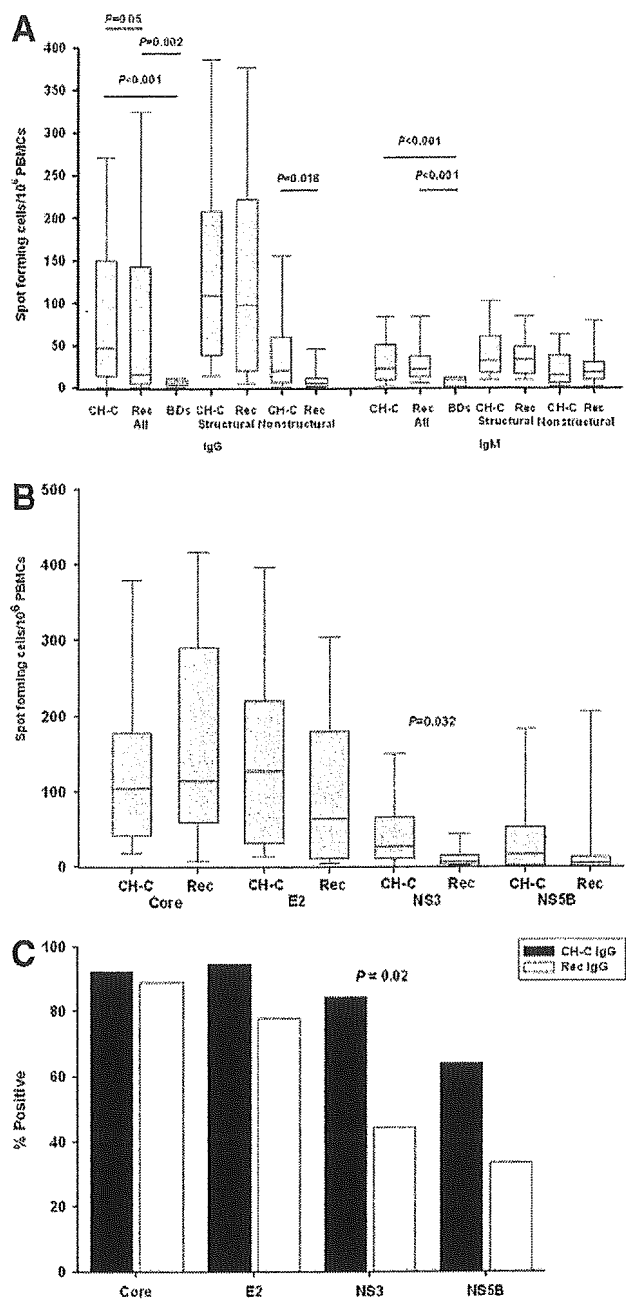


Fig. 4. Detection of anti-HCV antibody-secreting B cells in patients with chronic hepatitis C and in patients who had recovered from HCV. (A) Circulating anti-HCV IgG-secreting B cells were detected in 39 patients with chronic hepatitis C, 9 patients who had recovered from HCV infection, and 11 volunteer blood donors. Circulating anti-HCV IgM-secreting B cells were detected in 34 patients with chronic hepatitis C, 9 patients who had recovered from HCV infection, and 6 volunteer blood donors. (B) Frequency of circulating anti-HCV IgG-secreting B cells to 4 HCV antigens were detected in 39 patients with chronic hepatitis C and in 9 recovered patients. (C) The prevalence of anti-HCV IgG-secreting B cells in 39 patients with chronic hepatitis C and 9 recovered patients. PBMCs, peripheral blood mononuclear cells; CH-C, chronic hepatitis C; BDs, blood donors; Rec, recovered; IgG, immunoglobulin G; IgM, immunoglobulin M.

HCV infection, as also indicated by recently developed pseudotype assays for HCV-neutralizing antibodies^{24,25}; (2) the primary difference between chronically infected and recovered subjects was in the greater reactivity of the former to nonstructural antigens; (3) in chronic infection, HCV antibodies were cross-reactive against genotypes, again consistent with recent findings by neutralizing antibody assays^{26,27}; (4) the ELISpot assay can measure IgM as well as IgG responses at the single-cell level, providing a new means to measure the more elusive IgM response; (5) IgM responses were surprisingly well maintained during chronic infection; and (6) IgG responses correlated positively with serum transaminase levels.

In this study, the B-cell ELISpot assay showed high specificity (91% to 100%) and sensitivity (58% to 92%) to all HCV antigens through analysis of the ROC curves and thus achieved high diagnostic accuracy. Although there was a general problem that raw numbers of SFCs were low, statistical analysis and prior publications^{28,29} suggest that these small differences are consistent and relevant. Of note, individuals infected with nongenotype 1 variants were strongly positive in this assay, which used only genotype 1-derived antigens. This suggests that genotype 1 contains conserved epitopes that will allow the ELISpot assay to assess humoral immune responses to HCV irrespective of genotype (Fig. 2), with the caveat that we did not assess genotypes 4, 5, and 6, all of which are rare in the United States.

ELISpot assay has been used as a sensitive and specific tool to measure B-cell responses in autoimmune diseases^{28,29} and viral infections such as cytomegalovirus,¹⁵ rotavirus,¹⁶ measles virus,¹⁷ and hepatitis B virus,^{18,19} as well as to evaluate responses to bacterial³⁰ and viral vaccines.^{18,19} Other reports demonstrate that ELISpot is able to detect and numerate antigen-specific memory B cells in PBMCs after *in vitro* stimulation in both autoimmune diseases and viral infection.^{31,32} Thus, the B-cell ELISpot assay might be a useful tool to detect anti-HCV-specific memory B cells, and to monitor the efficacy of future HCV vaccines.

Interestingly, this study showed a strong correlation between the numbers of anti-HCV IgG-secreting B cells to the core and E2 antigens and the values of serum transaminases. The clinical significance of these observations is unknown, but raises the possibility that antibodies can contribute to liver cell injury. In addition, Ni et al.³³ recently reported that 10 of 36 hepatitis C patient samples showed increased B-cell frequencies that correlated with the degree of hepatic fibrosis. There are insufficient histological data in our study to assess whether the numbers of anti-HCV antibody-secreting B cells correlate with the degree of fibrosis as well as biochemical evidence of inflammation.

The median numbers of the sum of anti-HCV IgG-secreting B cells to nonstructural antigens were significantly higher in patients with chronic hepatitis C than in recovered patients. Similarly, an HCV-specific B-cell response was more frequently detected in patients with chronic hepatitis C than in recovered subjects (92% vs. 56%; $P = .017$) and was directed against a broader range of HCV antigens, particularly to NS3. In contrast, CD4 T-cell responses to NS3 epitopes are greatest in patients who recover from HCV infection.^{34,35}

We have also developed and evaluated the ELISpot assay for detecting anti-HCV IgM-secreting B cells. It has been reported that IgM anti-HCV in serum might be predictive of viral clearance in acute hepatitis C or response to interferon therapy.³⁶⁻⁴⁰ However, these results have been controversial and other studies have shown a significant correlation between IgM anti-HCV levels in serum and the recurrence of hepatitis C after liver transplantation.^{41,42} In this study, we found that IgM-secreting B cells persisted during chronic infection so that the usefulness of IgM detection for assessing acute versus chronic HCV infection would have to depend on quantitative differences in IgM level rather than the simple presence or absence of IgM antibody. The fact that there are no standardized assays for measuring IgM anti-HCV in serum and the ready detection of IgM-secreting B cells in this study suggests that the ELISpot assay could be used to better define the clinical relevance of IgM antibody in acute and chronic HCV infection.

Overall, this study, as do studies of HCV-specific neutralizing antibodies,^{26,27} suggest that the humoral arm of the HCV immune response is not a critical element of spontaneous viral clearance. However, because of the difficulty in obtaining serial acute-phase PBMC collections from recovering subjects, our study does not exclude a role for antibody-mediated viral clearance early in HCV infection. Sequential acute phase ELISpot IgM testing of PBMCs is planned in forthcoming chimpanzee infectivity studies. Nonetheless, studies of neutralizing and anti-envelope antibodies that measured serial acute phase serum samples from recovering subjects^{26,27} did not show that such antibodies correlated with viral clearance. Rather, it appears in those studies and the current study that antibodies to HCV increase in strength and broadness of reactivity during the course of chronic infection, presumably because of persistent antigenic stimulation. This is in contrast to cell-mediated immunity that is markedly diminished in chronically infected compared with recovered subjects. This dichotomy between the humoral and cellular immune response to HCV is intriguing and suggests T-cell tolerance in the absence of B-cell tolerance.

It is interesting to speculate on the role that antibodies might play in HCV infection. First, it seems reasonable that such antibodies complexed to virus would reduce the level of free virus and diminish transmission to others. This reduction in free virus in addition to lowered viral load might explain the relative rarity of sexual and perinatal transmission during chronic HCV infection. More intriguing is whether such antibodies establish the set point for viral load during chronic infection. It is known that viral loads are highest early in HCV infection prior to the appearance of antibody⁴³ and that chronically infected patients establish a lower and relatively constant level of viremia.⁴⁴ It appears that production and elimination of virus achieve a steady state. This steady state is probably multifactorial in origin, but antibody may play a key role. When patients in a steady state are immunosuppressed at the time of transplantation⁴⁵ or when coinfecting with human immunodeficiency virus,⁴⁶ the viral load increases, supporting an immunological role for viral containment even in the absence of clearance. A deleterious function of anti-HCV is that it serves to drive quasi-species evolution making it increasingly hard for the immune system to achieve viral clearance. Farci et al.⁴⁷ have shown in both humans and chimpanzees that the appearance of antibody coincides with increasing viral diversity and complexity and predicts progression to chronic infection.

In conclusion, there is much to explore regarding the function and relevance of IgG and IgM antibodies in HCV infection, and we believe the ELISpot assay, by measuring antibody production at the single-cell level, provides a new and useful tool for understanding the complex interplay between HCV and the host immune response.

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Identification of Novel HCV Subgenome Replicating Persistently in Chronic Active Hepatitis C Patients

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In an effort to clarify the life cycle of HCV, the HCV genome in liver biopsies taken from chronic active hepatitis C patients undergoing interferon treatment was investigated. Molecular cloning by long distance reverse-transcription polymerase chain reaction (RT-PCR) revealed that the HCV genome in two patients with high viral loads in the liver had in-frame deletions of approximately 2 kb between E1 and NS2, which encode the E1–NS2 fusion protein and six other HCV proteins: core, NS3, NS4A, NS4B, NS5A, and NS5B. Among the remaining 21 chronic active hepatitis C patients, these types of deletion were found in another two patients and in two hepatocellular carcinoma patients. Out-of-frame deletions in the structural region were isolated from the other five patients, but the dominant RT-PCR products were non-truncated genomes. Retrospective analysis of a series of serum samples taken from a patient carrying the subgenome with the in-frame deletion revealed that both the subgenome and the full genome persisted through the 2-year period of investigation, with the subgenome being predominant during this period. Sequence analysis of the isolated cDNA suggested that both the subgenome and the full genome evolved independently. Western blotting analysis of HCV proteins from the HCV subgenome indicated that they were processed in the same way as those from the full genome. HCV subgenomes thus appear to be involved in the HCV life cycle. *J. Med. Virol.* 77:399–413, 2005. © 2005 Wiley-Liss, Inc.

KEY WORDS: HCV; deletion; replication; biopsy

INTRODUCTION

Hepatitis C virus (HCV) is primarily transmitted via blood and blood-derived materials [Alter et al., 1989]

and often causes chronic hepatic diseases that progressively worsen to chronic active hepatitis, cirrhosis, and finally to hepatocellular carcinoma (HCC) [Kiyosawa et al., 1990, 1994, 2004; Alter and Seeff, 2000]. Interferon (IFN) and interferon with ribavirin treatment are effective in eradicating HCV from patients [Iino et al., 1994; McHutchison and Fried, 2003], improving liver histological findings, and in prolonging life in patients with hepatitis C [Yoshida et al., 1999; Kasahara et al., 2004]; however, their efficacy is limited.

HCV was first identified as cDNA clones, and was characterized molecularly using cDNA isolates [Choo et al., 1989; Kuo et al., 1989]. The HCV genome is single-stranded RNA of about 9,600 nucleotides with an untranslated region (UTR) at each end, and encodes a polyprotein of about 3,010 amino acids [Choo et al., 1989; Kato et al., 1990], which is processed into 10 proteins by a host peptidase and two HCV proteases [Hijikata et al., 1991, 1993; Grakoui et al., 1993a]; Core, E1, and E2 are structural proteins for virion formation, and NS3, NS4A, NS4B, NS5A, and NS5B are components of the replication machinery for the RNA genome [Houghton et al., 1994]. However, isolation of virion particles has been difficult owing to a lack of in vitro culture systems for HCV.

HCV replication in chimpanzee following intrahepatic injection of an RNA transcript from HCV genomic cDNA proved that a molecular clone could represent a functional HCV genome [Yanagi et al., 1997]. Lohmann

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et al. [1999] established a dicistronic subgenomic RNA that replicates in a hepatoma cell line (Huh7) and consists of the HCV NS protein coding region, the 5'- and 3'-UTR of HCV and a selective marker gene. HCV subgenomic RNA replicon systems are vital to the study of the mechanisms of HCV RNA replication, but there remain problems with regard to viral replication. Information obtained from liver biopsies of hepatitis C patients thus remains important in clarifying the life cycle of HCV.

Histological grading for diagnosis [Perrillo, 1997], immunohistochemical analysis, immuno-staining [Infantolino et al., 1990; Hiramatsu et al., 1992], electron microscopic analysis [Fagan et al., 1992], in-situ hybridization and in-situ reverse transcription polymerase chain reaction (RT-PCR) [Lau et al., 1996; Dries et al., 1999], and quantitation of HCV RNA in liver biopsy specimens [Sakamoto et al., 1994; Nuovo et al., 2002] have all been used to demonstrate HCV replication in liver. However, molecularly characterized data have been limited; HCV RNA isolated from liver was found to be equivalent in size to the well-characterized RNA seen in circulating HCV [Nielsen et al., 2004], thus confirming HCV replication in liver.

In order to obtain data to elucidate the nature of HCV in liver, viral loads and the structure of the HCV genome in patient liver biopsy specimens were examined. A highly sensitive ELISA for quantitation of the HCV core antigen [Aoyagi et al., 1999; Tanaka et al., 2000] and a quantitative RT-PCR system were applied to estimate viral loads in serum and liver biopsies. For structural analysis of the whole genome in specimens, molecular clones were used. Surprisingly, it was found that novel HCV subgenomes were predominant in several patients and, in one patient, these persisted for several years. The nature of these subgenomes are described and discussed in this paper.

MATERIALS AND METHODS

Samples and Antibodies

Serum and liver biopsy specimens were taken from patients undergoing IFN- α treatment at Shinshu University Hospital. Informed consent was obtained from all patients from whom samples were taken. A 7.2 mega-unit dose of IFN- α was administered daily for 2 weeks, followed by three times per week for 22 weeks. Serum samples were collected 1 or 2 months before administration of IFN- α . A series of serum specimens was collected from one patient from before IFN treatment until 1 year after treatment. Liver biopsy specimens from two HCC patients were obtained from surgically removed cancerous liver tissues.

An anti-core monoclonal antibody (5E3) has been described previously [Kashiwakuma et al., 1996]. Anti-E1 monoclonal antibody was raised against recombinant E1 and E1/E2 proteins expressed in Sf-9 cells infected with recombinant baculoviruses (Yamaguchi unpublished). Anti-NS3 rabbit polyclonal antibody was purified from the serum of rabbits immunized with the

recombinant NS3 antigen expressed in *E. coli* [Saito et al., 1992]. Anti-mouse and rabbit immunoglobulin antibody conjugated with HRP were purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD) and Bio-Rad Laboratories (Tokyo, Japan), respectively. All primers were purchased from Sigma Genosys (Tokyo, Japan) and Texas Genomics Japan (Tokyo, Japan), and sequences are available on request.

Quantitation of HCV Core Antigen

Quantities of HCV core antigen were measured by EIA as described previously [Aoyagi et al., 1999; Kato et al., 2003]. The concentration of core antigen was expressed in fmol/L, and the cut-off value of the assay was set at 7.5 fmol/L. For quantitation of the core antigen in liver, extracts were diluted to 100 μ g of liver protein per milliliter with negative control serum before pretreatment of the samples. Samples were heated at 56°C for 30 min with pretreatment solution containing SDS, CHAPS, and Triton X-100, and were then added to wells pre-coated with anti-HCV core antibodies, and reaction buffer was used to fill the wells. Captured core antigen after 1-hr incubation was reacted for 30 min with anti-HCV antibodies conjugated with horseradish peroxidase after stringent washing. Bound enzyme activities were measured using a Fusion plate reader (PerkinElmer, Tokyo, Japan) with a chemiluminescent reagent (SuperSinal Pico ELISA, Pierce, Rockford, IL)

Real-Time PCR Assay for HCV RNA

HCV RNA was recovered from samples by using the QIAamp viral RNA kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instructions. HCV RNA was reverse-transcribed and amplified using QuantiTect One-Step RT-PCR kit (QIAGEN) with primers. For quantitation of the 5'-UTR, the forward primer, chiba-s (5'-TAGTGGTCTGCGGAACCGGT-3'), and reverse primer, chiba-as (5'-TGCACGGTCTACGAGACCT-3'), yielded fragments corresponding to nucleotides 141–339 of HCV RNA. In the case of the E2 region, HC1986S (5'-TGGTTCGGCTGYACATGGATGAA-3') and HC2199AS (5'-GGRTAGTGCCARAGCCTGTATGGGTA-3') primers were used. Reactions were performed with a LightCycler system (Roche Diagnostics K.K., Tokyo, Japan), and fluorescence by SYBR green was monitored after each elongation reaction for real-time monitoring of DNA products during PCR. The amount of HCV RNA was calculated according to the calibration curve produced with serial dilutions of standard RNA synthesized by T7 RNA polymerase (Ambion, Inc., Austin, TX) from plasmids carrying the HCV cDNA isolate (genotype 1b). To examine the specificity of PCR, the melting point of DNA products was analyzed by melting curve analysis using LCDA software (Roche Diagnostics).

Cloning and Analysis of HCV cDNA

HCV cDNA was amplified by long distance RT-PCR (LD-RT-PCR) as described previously [Tellier et al., 1996;

TABLE I. Viral Data of Patients

Patient No.	HCV genotype	Viral loads				PCR primer sets for positive results		
		Serum		Liver biopsy		Non-truncated genome	Truncated genome	Test primer sets
		Core antigen (fmol/L)	HCV RNA (copies/ml)	Core antigen (fmol/g protein)	HCV RNA (copies/g protein)			
368	1b	17,108.5	1.73×10^5	5,462.4	4.71×10^8	—	j	j
207	1b	12,695.2	1.44×10^5	30,792.3	1.43×10^{10}	NT	NT	NT
204	1b	5,082.4	5.74×10^4	8,779.7	2.22×10^9	i	—	i
274	1b	1,034.4	4.24×10^3	2,651.7	3.56×10^7	a, b, c, d	e	a-e
193	1b	988.8	3.09×10^4	14,519.9	1.07×10^9	a, b, c, d, e	—	a-e
331	1b	922.2	2.03×10^3	2,387.1	2.84×10^8	a, c, d	b, e	a-e
325	1b	623.5	3.82×10^3	10,127.9	7.28×10^7	a, b, c, d, e	d	a-e
288	1b	254.5	1.00×10^1	4,037.9	9.50×10^6	a, b, c, d, e	d, e	a-e
299	1b	166.6	1.14×10^3	1,287.8	5.35×10^7	c, d	—	a-e
295	1b	1.0	5.11×10^1	261.5	2.62×10^7	a, b, c, d, e	b	a-e
171	1b	1,077.3	6.42×10^3	3,781.8	6.91×10^6	c, d	b	a-e
257	1b	12.7	1.06×10^2	568.5	2.78×10^7	d	—	a-e
372	1b	723.7	2.28×10^4	1,784.1	3.35×10^8	a, b, c, d, e	—	a-e
373	1b	597.0	8.31×10^3	33,919.0	2.65×10^9	—	a, c, d	a-e
248	2a	209.3	2.58×10^2	4,417.1	3.70×10^8	—	—	a-e
235	2a	3,616.2	3.66×10^2	7,462.1	1.55×10^9	c	—	a-e
203	2b	95.1	1.46×10^2	5,590.9	1.82×10^9	—	b, d	a-e
178	2b	34.5	8.08×10^1	609.1	4.51×10^7	—	—	a-e
297	2	3,112.7	8.35×10^3	2,883.6	1.14×10^8	—	—	a-e
298	2a	180.0	8.09×10^2	3,015.0	1.76×10^9	b	—	a-e
305	2a	173.6	1.12×10^3	1,782.8	5.96×10^7	b	—	a-e
201	2a	127.6	2.40×10^3	497.6	1.87×10^7	—	—	a-e
357	2	227.2	3.11×10^3	321.9	2.29×10^7	—	—	a-e

NT: not tested.

Yanagi et al., 1998]. HCV cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen K.K., Tokyo, Japan) with HC1b9405R primer (5'-GCCTA-TTGGCCTGGAGTGT'TTAGCTC-3'). After RNase H

(Invitrogen) treatment at 37°C, a cDNA mixture was subjected to PCR with KlenTaq DNA polymerase (BD Biosciences Clontech, Tokyo, Japan), HClong A1 primer (5'-GCCAGCCCCCTGATGGGGGCGACA-

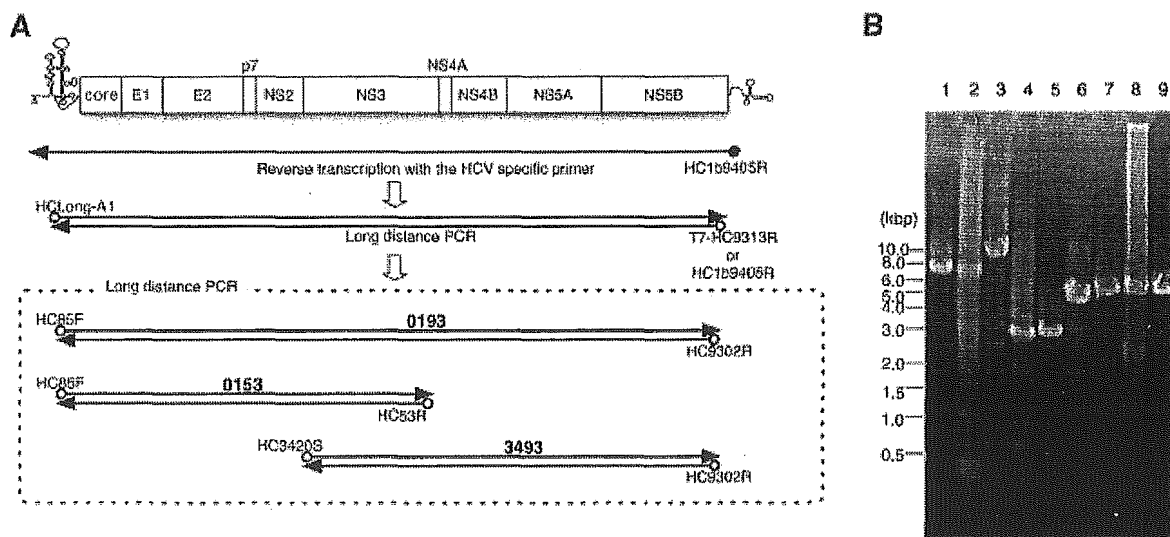


Fig. 1. Molecular cloning of HCV genome by long distance reverse-transcription PCR. A: Schematic view of HCV RNA is shown at the top of the figure. HCV cDNA, which was synthesized from total RNA from liver using reverse transcriptase with HCV-specific primers for the 3'-UTR, was amplified by nested PCR with HCV-specific primers. The longest LD-RT-PCR product, 0193, covered 99% (amino acids 1-2,987) of the HCV polyprotein coding sequence of genotype 1b HCV (length: 3,011 amino acids). Fragments obtained by LD-RT-PCR using HC85F

and HC9302R, HC85F, and HC53R; and HC3420S and HC9302R were designated 0193, 0153, and 3493, respectively. After agarose gel electrophoresis, LD-RT-PCR products from liver biopsy samples were stained with ethidium bromide. B: Lanes 1-3, lanes 4-6, and lanes 7-9 represent 0193, 0153, and 3493 fragments from Patient 207 (lanes 1, 4, and 7), 373 (lanes 2, 5, and 8), and control HCV cDNA (lanes 3, 6, and 9), respectively. The positions of markers are indicated at the left side of the image.

CTCCACC-3') and T7-HC9313R primer (5'-TCTAGTC-GACGGCCAGTGAATTGTAATACGACTCACTCTAG-GGCGGGGGTTCGGGCWCGNGACABGCTGTGA-3') or HC1b9405b for 35 cycles of denaturation at 94°C for 20 sec and extension at 68°C for 9 min. Second-round PCR was carried out with the primers, HC85F (5'-ATGGCGTTAGTATGAGTGTCTGTCGTCAGCCT-3') and HC9302R (5'-TCGGGCACGAGACAGGCTGTGATA-TATGTCT-3'), HC85F and HC53R (5'-GCTTAAGTG-ACGACCTCCAGTCCAGCCGACAT-3'), and HC3420S (5'-GCGCCCATCACGGCTACTCCCAACAA-3') and HC9302R, for 20 cycles under the same conditions as

first-round PCR. PCR products were purified from the gel using a QIA-quick gel kit (QIAGEN), and were then cloned into the pGEM-T easy vector (Promega K.K., Tokyo, Japan). The cDNA clones, LV207-0193-1, -3, -15, and -6, and LV373-0193-10, LV373-0153-5, and LV373-0153-6 were obtained from liver biopsy samples from Patient 207 and 373, respectively.

Nucleotide sequences of the cloned cDNA fragments were determined using a CEQ-2000 XL analysis system with a DTCS quick start kit and HCV-specific primers according to the manufacturer's instructions. Sequence data were analyzed on Macintosh computers with the

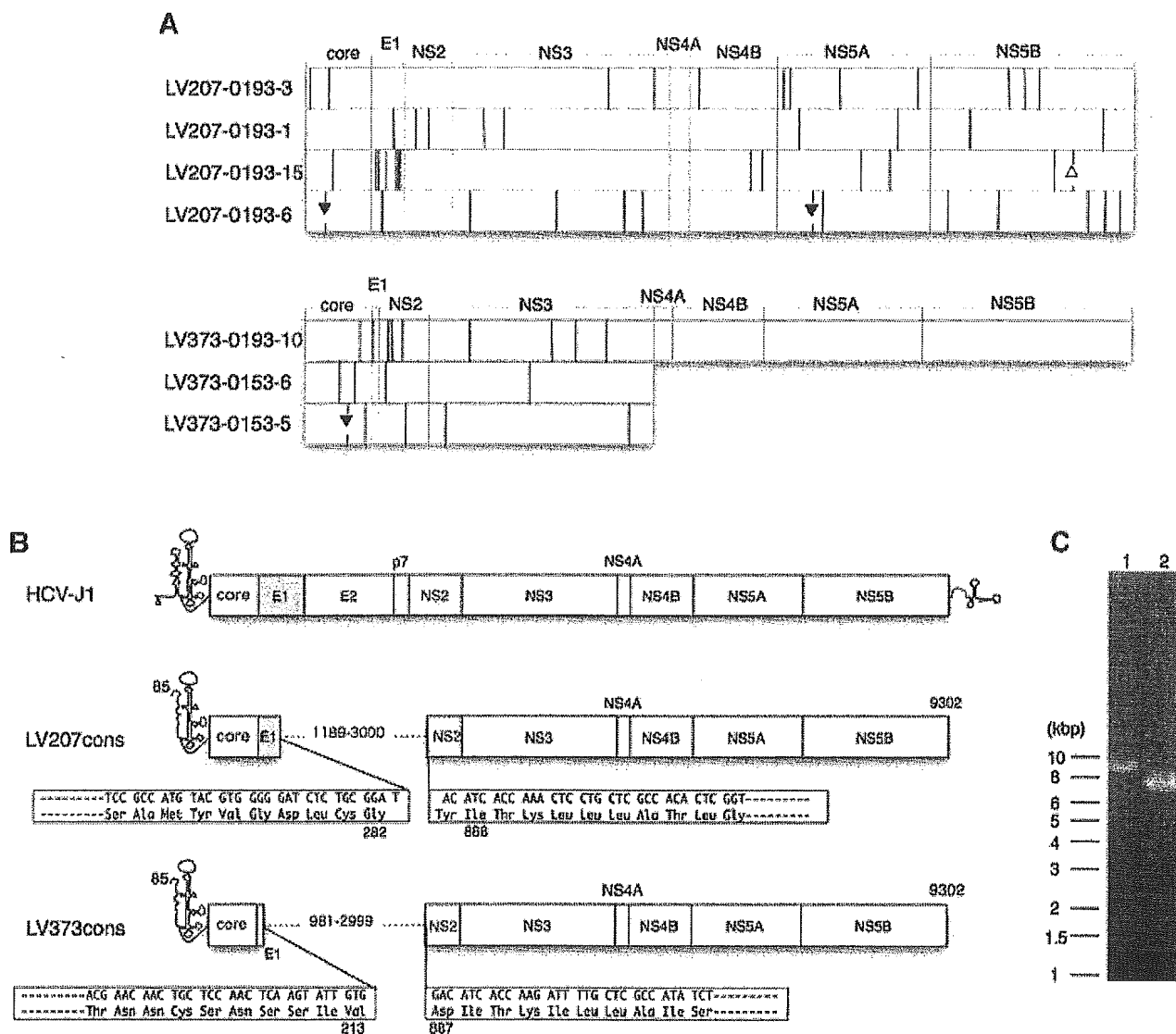


Fig. 2. Schematic presentation of HCV subgenomes from livers containing in-frame deletion. A: Comparisons of the deduced amino acid sequences of LD-RT-PCR fragments cloned with pGEM-T Easy are depicted. The cDNA isolates from liver biopsy are designated by the prefix LV followed by Patient numbers and fragment names described in legend of Figure 1. Bold bars indicate the positions of amino acids that differed. Closed triangles show the positions of stop codons, and open triangles indicate base deletions. B: Schematic HCV cDNA structures with their nucleotide sequences and deduced amino acid

sequences around the boundary of the deletions are depicted. The deleted regions were represented as nucleotide positions as those corresponding to HCV-J1 by numbers between the dotted tagged boxes. Boundaries of the deletions are shown as amino acid positions corresponding to those of HCV RNA (HCV-J1) on the bottom line of the graphs. C: Images of agarose gel electrophoresis of LD-RT-PCR products amplified from in vitro non-truncated (lane 1) G14 and truncated (lane 2) RNA transcripts from Donor G14 and Patient 207 cDNAs, respectively.

Sequencer (Gene Code Corporation, Ann Arbor, MI), MacVector (Accelrys K.K., Tokyo, Japan), and EMBOSS [Rice et al., 2000] software packages.

Construction of HCV cDNA Expression Vectors

HCV RNA reverse transcribed with HC1b9405R from Patient 207 liver biopsy was subjected to PCR using the primers T7-HCLongH1 (5'-TCTAGTCGACGGCCAG-TGAATTGTAATACGACTCACTATAGGGCGGCCAG-CCCCCTGATGGGGGCGACACTCCACC-3') and core-cla-as (5'-GCCGCATGTAAGGTATCGATGACC-3') in order to amplify the entire 5'-UTR, and cloned (LV207-H1-Cla). To obtain the 3'-UTR cDNA (LV373-3'UTR), cDNA was synthesized with HC8913F primer (5'-CTTGAAAAAGCCCTGGATTGTCAGAT-3') from the minus strand RNA of Patient 373 liver sample. cDNA was amplified by PCR with HC8913F and RP2 (5'-ACATGATCTGCAGAGAGGCC-3'), and followed by PCR with HC8939F and R1 (5'-ACATGATCTGCAGAGAGGCCAGTATCAGCACTCTC-3').

Ligation of the fragments from LV207-0193-1, -15, and -6, the 5'-UTR-core fragment and the 3'-UTR fragments gave the chimeric HCV cDNA (LV207cont). The cDNA encoding the HCV subgenome was inserted into pcDNA3.1 (Invitrogen) to construct pcD/LV207cont. A Cla I site in the core region and an Asc I site in the NS2 region of pcD/LV207cont were introduced using Quick-Change II site-directed mutagenesis kits with primers. Insertion of the corresponding fragment from E1 to NS2, which was obtained from HCV-J1 cDNA [Aizaki et al., 1998] by PCR with core-cla-s (5'-GGTCATCGA-TACCCCTTACATGCGGC-3') and Asc-M-as (5'-CCTTC-CTCGGCGCGCCGAGACRGGTAGACCCRAGATGATGTCCCCACA-3') generated pcD/J1NLV.

In Vitro Synthesis of Truncated and Non-Truncated RNA Transcripts and Subsequent RT-PCR

Truncated cDNA, LV207cont, having the T7 promoter sequence was inserted into pBluescript II (Stratagene) to give pLV207cont. A plasmid carrying non-truncated HCV cDNA was constructed by inserting full-length cDNA derived from G14 plasma, in which only full-length HCV RNA was detected, into pBluescript II. Truncated and non-truncated RNA was synthesized using MEGAscript T7 kit (Ambion, Inc.) according to the manufacturer's instructions.

RNA transcripts (10⁴ copies) were mixed with RNA extracted from uninfected liver tissue, and were reverse-transcribed and amplified by same protocol used to obtain truncated cDNAs. In addition, RNA transcripts and extracted RNA from plasma or serum were amplified by RT-PCR using primers targeting the junction site of LV207 cDNA. RNA was reverse-transcribed and amplified using QuantiTect SYBR GREEN RT-PCR Kit (QIAGEN) with sense, LV207-1S (5'-GCGTCCCC-ACTAAGGCAATA-3'), and antisense primers, LV207-3AS (5'-AGCAGGAGTTTGGTGATGATCCG-3'), for the

TABLE II. List of Primers Used for Detecting Truncated and Non-Truncated HCV Genomes

Reverse transcription	Primer sets										
	a	b	c	d	e	f	g	h	i	j	
Sense	HC3945R	HC3481R	HC3945R	HC3945R	HC3297R	HC3945R	HC3945R	HC3945R	HC3945R	HC3945R	HC3481R
Antisense	HCLongA1	HCLongA1	HC813S	HC813S	HCLongA1	HCLongA1	HCLongA1	HCLongA1	HC813S	HC813S	HCLongA1
Sense	HC3945R	HC3481R	HC3945R	HC3945R	HC3297R	HC3945R	HC3945R	HC3945R	HC3297R	HC3297R	HC3481R
Antisense	HC85F	HC85F	HC841S	HC841S	HC85F	HC85F	HC85F	HC841S	HC841S	HC841S	HC85F
	HC3297R	HC3297R	HC3759R	HC311AS	HC3174AS	HC3297R	HC3759R	HC3759R	HC3174S	HC3174S	HC3297R