ORIGINAL ARTICLE

Detection of HCV Antigens in Liver Graft: Relevance to the Management of Recurrent Post-Liver Transplant Hepatitis C

Alberto Grassi,[1] **Chiara Quarneti,**[1] **Matteo Ravaioli,**[2] **Francesco Bianchini,**[1] **Micaela Susca,**[1] **Antonia D'Errico,**[3] **Fabio Piscaglia,**[4] **Maria Rosa Tamè,**[4] **Pietro Andreone,**[1] **GianLuca Grazi,**[2] **Silvia Galli,**[5] **Daniela Zauli,**[1] **Antonio D. Pinna,**[2] **Francesco B. Bianchi,**[1] **and Giorgio Ballardini**[1]

[1]Department of Internal Medicine, Cardioangiology, Hepatology, University of Bologna-Sant'Orsola-Malpighi Hospital, Bologna, Italy; [2]Department of Surgery and Transplantation, Sant'Orsola Hospital, Bologna, Italy; [3]Department of Oncology and Haematology, Pathology Division of the "Felice Addarii" Institute, University of Bologna-Sant'Orsola-Malpighi Hospital, Bologna, Italy; [4]Department of Internal Medicine and Gastroenterology, University of Bologna, Bologna, Italy; [5]Department of Clinical and Experimental Medicine, University of Bologna-Sant'Orsola-Malpighi Hospital, Bologna, Italy

The aim of this study was to evaluate how the immunohistochemical detection of liver hepatitis C virus (HCV) antigens (HCV-Ag) could support the histologic diagnosis and influence the clinical management of post-liver transplantation (LT) liver disease. A total of 215 liver specimens from 152 HCV-positive patients with post-LT liver disease were studied. Histologic coding was: hepatitis (126), rejection (34), undefined (24; coexisting rejection grade I and hepatitis), or other (31). The percentage of HCV-Ag infected hepatocytes were evaluated, on frozen sections, by an immunoperoxidase technique. HCV-Ag were detectable early in 57% of cases within 30 days post-LT, 92% of cases between 31 and 180 days, and 74% of cases after more than 180 days. Overall, HCV-Ag were detected more frequently in histologic hepatitis as compared to rejection ($P <$ 0.0001) with a higher percentage of positive hepatocytes ($P <$ 0.00001). In 16 patients with a high number of HCV-Ag-positive hepatocytes (65%; range 40-90%) a clinical diagnosis of recurrent hepatitis (RHC) was made despite inconclusive histopathologic diagnosis. Multivariate analysis identified the percentage of HCV-Ag-positive hepatocytes and the time post-LT as independent predictors for RHC ($P =$ 0.008 and $P =$ 0.041, respectively) and the number of HCV-Ag-positive hepatocytes $\geq$50% as the only independent predictor for nonresponse ($P <$ 0.001) in 26 patients treated with α-interferon plus ribavirin. In conclusion, HCV reinfection occurs early post-LT, reaching its peak within 6 months. Immunohistochemical detection of post-LT HCV reinfection support the diagnosis of hepatitis when the histologic features are not conclusive. A high number of infected cells, independently from the genotype, represents a negative predictive factor of response to antiviral treatment. *Liver Transpl 12:1673-1681, 2006.* © 2006 AASLD.

Hepatitis C virus (HCV)-related end-stage liver disease represents one of the most common indications for liver transplantation (LT) worldwide.[1] Almost all patients undergoing LT for HCV-related liver disease show an early graft reinfection, with HCV-ribonucleic acid (RNA) viremia reaching and exceeding pre-LT levels.[1-9] In this setting, histologic progression of chronic hepatitis C is more aggressive, resulting in a reduction of survival with respect to non-HCV transplanted patients and may often require retransplantation.[10-13]

Histopathologic assessment is considered essential for differentiating recurrent hepatitis C (RHC) from

acute or chronic rejection. Although typical histologic features of hepatitis C are well defined in the immunocompetent patient, the distinction of the 2 pathologic patterns may be often very difficult in the post-LT immunosuppressed patient. Indeed, both RHC and rejection are associated with the presence of a lymphoid infiltrate and signs of biliary damage, and strong immunosuppressive therapy in the first months after LT can alter the typical histologic features of hepatitis C. Some authors suggest that the presence of an important eosinophil infiltrate[14] or the presence of activated and blast lymphoid cells[15] might better characterize rejection, but, considering that rejection and RHC may often coexist, the differential diagnosis remains difficult.[4,16,17]

A reliable histopathologic diagnosis is a crucial step in the therapeutic decision process. A wrong diagnosis of rejection may lead to pushing immunosuppression, thus worsening RHC and facilitating a more rapid progression to cirrhosis. Vice-versa, an improper diagnosis of RHC in patients with rejection may lead to tapering immunosuppressive therapy, consequently accelerating the rejection process.[16-18] Unfortunately, the reliability of histopathologic assessment in differentiating between RHC and rejection is quite unsatisfactory given the relatively low inter- and intraobserver agreement rates.[19-21] In conclusion, histopathology alone cannot be considered the gold standard in this context.

In order to validate the histopathologic diagnosis, virus-related parameters have been evaluated. Some investigators have shown that graft hepatitis is significantly related to a high post-LT viremia,[3,5,7,22] but others either have not confirmed those results[23,24] or have found a correlation with viremia but not with intrahepatic HCV replication.[2] In addition, it has been reported that genotype 1 is more pathogenic as compared with other genotypes in the post-LT setting,[5,6,25-27] but this has not been confirmed by other studies.[1,28,29]

Because patients usually remain persistently viremic after LT, the demonstration of the actual graft reinfection requires the direct identification of HCV within hepatocytes. The presence of viral RNA in liver samples has been demonstrated by in situ hybridization[30] and reverse transcriptase in situ polymerase chain reaction.[31,32] These methods, however, suffer from technical difficulties and cannot be used routinely.

More data are available on the possibility of identifying HCV antigens (HCV-Ag) in the liver by immunohistochemistry.[32-36] Few data, however, exist in the post-LT liver. Some authors suggest that a high expression of HCV-Ag seems to predict virus-mediated liver damage,[5-7] but others disagree.[2] Using this technique, we have previously demonstrated that the detection of liver HCV-Ag is sensitive enough to be used in clinical practice as a diagnostic tool to detect reinfection of the transplanted liver and that the number of infected hepatocytes correlates with the type of histologic damage.[37]

The aim of the present study was to evaluate in a large series of post-LT patients how the immunohistologic detection of HCV-Ag could support the histologic diagnosis and, consequently, guide the clinical management of post-LT liver disease.

PATIENTS AND METHODS

Patients and Liver Specimens

We studied 215 consecutive frozen liver specimens from 152 patients (104/48 males/females) that were performed from 6 days to 4 years post-LT (from January 1996 to September 2005), due to the onset of increased serum alanine transaminase and/or bilirubin. All patients were HCV-RNA-positive before LT and all biopsies were obtained before starting any post-LT antiviral treatment. In 49 of these patients at least 2 biopsies were available: 2 in 37 patients, 3 in 10 patients, and 4 in 2 patients. In 118 cases the HCV genotyping was performed and detected genotype 1 in 79 patients, genotype 2 in 25 patients, genotype 3 in 11 patients, and genotype 4 in 3 patients.

The rate of end-of-treatment response was evaluated in 26 patients undergoing liver biopsy within 3 days before starting a 24/48 week antiviral treatment. An end-of-treatment biopsy (not included in the previous series) was available in 14 out of 26 patients.

All patients gave their written informed consent to liver biopsy, which was part of the diagnostic protocol. Liver biopsy specimens were performed percutaneously with 1.4- or 1.2-mm needles. Liver fragments were divided into 2 parts: the first (at least 20 mm) was fixed in formalin and embedded in paraffin for conventional histologic evaluation, the second (length of 7-15 mm) was snap-frozen in embedding media (Tissue Tek O.C.T.; Miles Inc. Elkhart, IN) and stored at −80°C. From each frozen liver sample serial 5-μm-thick sections were obtained. Sections were air dried, fixed in acetone for 5 minutes, wrapped with aluminum foil, and stored at −20°C until use.

Histology coding was made considering the prevalent pattern of liver damage, as follows: hepatitis, rejection, "other" (15 cholangitis/cholestasis, 4 reperfusion damage, 3 nonalcoholic steatohepatitis, 2 steatosis, 2 remodeling, 2 minimal lesions, 1 graft dysfunction, 1 lymphoma, and 1 toxic damage), or undefined (when rejection grade II-III was excluded but both hepatitis and rejection grade I patterns coexisted).

Hepatitis and rejection were defined according to accepted criteria.[38,39] In particular, recurrence of HCV was diagnosed when lobular or interface necroinflammatory activity was more prevalent and prominent than bile duct inflammation and damage. In this study we made no morphological distinction between "acute" and "chronic" hepatitis. In 4 cases out of 34, chronic rejection was identified, in 3 of 4 more than 6 months after LT.

Immunohistochemical detection of HCV-Ag

Before staining, sections were fixed for 5 minutes in chloroform at room temperature. HCV-Ag were detected
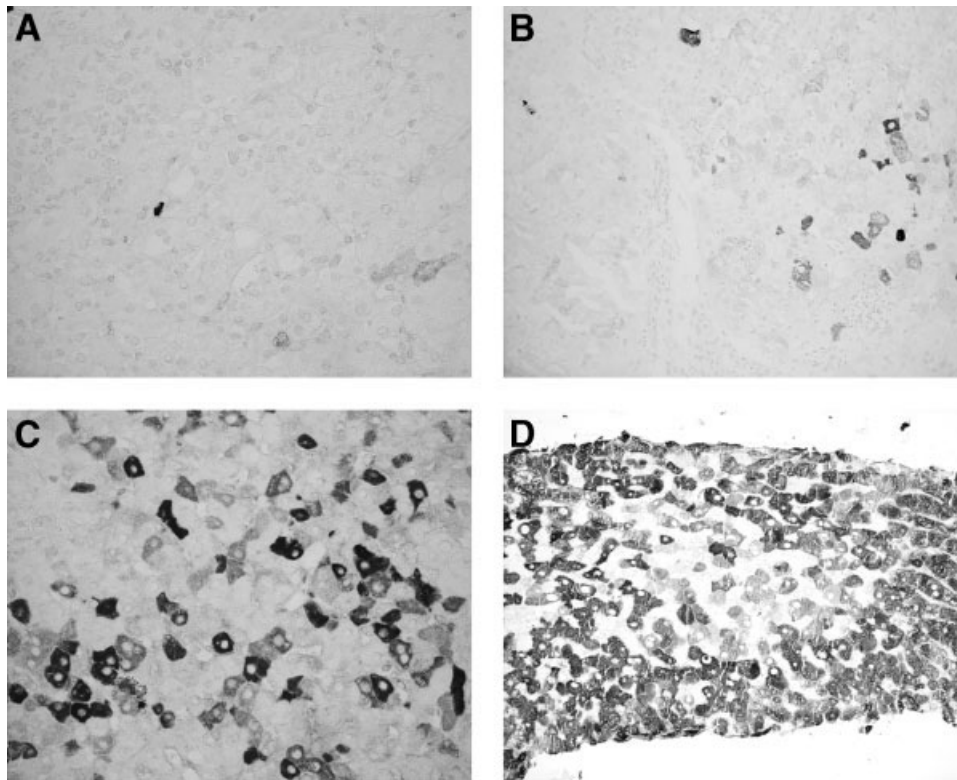
Figure 1. Lobular areas from different liver biopsies stained for HCV-Ag by immunohistochemistry. Cytoplasmic positivity of hepatocytes with different intensity of staining. Original magnification: (A,C) 40X and (B) D 20X. (A) Few hepatocytes faintly positive. (B) A small group of hepatocytes with different intensity of staining. (C) Widespread positivity with different intensity of staining. (D) Widespread positivity, with prevalent strong intensity of staining.
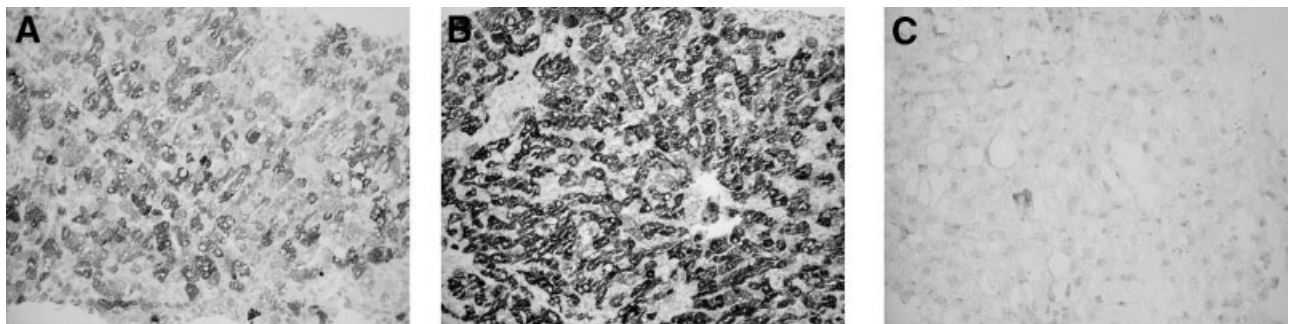

Figure 2. Lobular areas from serial biopsies of the same patient stained for HCV-Ag by immunohistochemistry. Original magnification: 20X. (A) At 24 days post-LT, widespread positivity, with medium intensity of staining. (B) At 47 days post-LT, widespread positivity, with strong intensity of staining. (C) At 146 days post-LT (after 90 days therapy with standard-interferon plus ribavirin), 1 residual hepatocyte faintly positive.

using a 4-step immunoperoxidase technique with fluorescein isothiocyanate-conjugated human immunoglobulin G from an anti-HCV-positive patient, obtained as previously described.[33,35,38] The reagent was able to recognize the 4 HCV antigens of RIBA3 (Ortho Diagnostic Systems, Raritan, NJ) strips.[40]

The following immunohistochemical reactions were performed:

Fluorescein isothiocyanate-anti-HCV immunoglobulin G, diluted 1:15 with 30% normal AB human serum in phosphate-buffered saline (PBS), incubated 60 minutes in a damp chamber at 4°C, followed by a 30-minute PBS wash;

Anti-fluorescein mouse monoclonal antibody (DAK-FITC4; Dako A/S, Glostrup, Denmark) 1:50 in PBS, incubated 30 minutes at room temperature in a damp chamber, followed by a 15-minute PBS wash;

Anti-mouse, peroxidase-conjugated, rabbit immunoglobulins (Dako A/S, Glostrup, Denmark) 1:50 in PBS containing 5% normal AB blood group human serum, 30 minutes at room temperature followed by a 15-minute PBS wash;

TABLE 1. Immunohistochemical Data of HCV-Ag Expression in 215 Biopsies Grouped According to the Histologic Diagnosis

Histologic diagnosis	<30 days post-LT			30-180 days post-LT			>180 days post-LT			Total		
	No.	No.+ (%)	m % (range)	No.	No.+ (%)	m % (range)	No.	No.+ (%)	m % (range)	No.	No.+ (%)	m % (range)
Hepatitis	4	3 (75%)	11 (0-70)	54	54 (100%)	60 (1-98)	68	55 (81%)	30 (0-90)	126	112 (89%)	40 (0-98)
Rejection	10	6 (60%)	1 (0-20)	8	6 (75%)	10 (0-90)	16	8 (50%)	0.5 (0-90)	34	20 (59%)	1 (0-90)
Undefined†	12	6 (50%)	2 (0-80)	8	7 (88%)	45 (0-90)	4	2 (50%)	27.5 (0-60)	24	15 (63%)	4 (0-80)
Other*	9	5 (56%)	1 (0-70)	13	9 (69%)	15 (0-70)	9	7 (77%)	4 (0-80)	31	21 (68%)	3 (0-80)
Total	35	20 (57%)	1 (0-80)	83	76 (92%)	50 (0-98)	97	72 (74%)	20 (0-90)	215	168 (78%)	20 (0-98)

Abbreviations: No., number of cases; No.+, number of HCV-Ag-positive cases; m %, median percentage of HCV-Ag-positive hepatocytes; †Undefined, rejection grade II-III was excluded but both hepatitis and rejection grade I patterns coexisted.
*Other: 15 cholangitis/cholestasis, 4 reperfusion damage, 4 reperfusion damage, 3 nonalcoholic steatohepatitis, 2 steatosis, 2 remodeling, 2 minimal lesions, 1 graft dysfunction, 1 lymphoma, and 1 toxic damage.

Anti-rabbit peroxidase-conjugated swine immunoglobulins (Dako A/S, Glostrup, Denmark), diluted 1:100 in PBS containing 5% normal AB human serum, for 30 minutes at room temperature followed by a 15-minute PBS wash; Tetrahydrochloride diaminobenzidine, 0.5 mg/mL (Sigma Chemical Co., St. Louis, MO) in Tris HCl buffer 0.05 M, pH 7.8, containing H_2O_2 0.003% for 7 minutes in the dark. Sections were then dehydrated in the series of alcohols and mounted with Eukit (O. Kindler GmbH, Freiburg, Germany).

Controls of specificity were conducted as previously reported and included both blocking experiments, using, in the first step of the procedure, high-titer anti-HCV-positive sera (which abolished the reaction) instead of the human AB blood group serum, and reproduction of the staining pattern using fluorescein isothiocyanate-conjugated immunoglobulins eluted from RIBA3 strips.[35,40]

The presence of positive hepatocytes, the percentage of infected cells, and the intensity of staining (scored: 1, faint; 2, medium; and 3, strong) were evaluated by 2 observers (A.G. and G.B.) (Figs. 1 and 2). The data collected by the 2 observers were compared using Friedman analysis: no significant differences were found and mean values were used.

The pathologist (A.D.) who evaluated liver histology was blinded to the result of HCV-Ag immunohistochemical evaluation. Similarly A.G. and G.B. were blinded to the histopathological diagnosis.

Statistical Analysis

Prevalence and median values of different parameters have been compared with chi-squared, Fisher's exact, and Mann-Whitney tests, when appropriate. Linear correlation was evaluated when needed. After univariate analysis, multivariate logistic regression analysis was used to assess the accuracy of variables as predictors of recurrent hepatitis and response to therapy. Differences were considered significant for P less than 0.05. Statistical analysis was carried out with SPSS (SPSS Base 10.0, Application Guide; SPSS Inc., Chicago, IL, 1998).

RESULTS

Data about HCV-Ag expression in 215 specimens are summarized in Table 1. Biopsies were grouped based on the timing after LT: within 30 days ("reinfection" phase), between 30 and 180 days ("acute" phase), and after more than 180 days ("chronic" phase) and were divided according to the histologic diagnosis.

HCV-Ag were detected in 78% of all liver biopsies (57% and 92% of cases in "reinfection" and "acute" phases, respectively). HCV-Ag were always detectable during the "acute" phase, with a high median percentage of infected hepatocytes (60%, range 1-98%) when the histologic diagnosis was hepatitis. The median percentage of liver HCV-Ag-positive hepatocytes was lower

TABLE 2. Patients With a Final Clinical Diagnosis of Hepatitis Despite an Initial Inconclusive or Discordant Histologic Diagnosis

Patients	Days post-LT	Histological diagnosis	% HCV-Ag-positive hepatocytes	HCV-RNA (Meq/mL)
1	12	Undefined	50	N.A.
2	15	Undefined	80	>40
3	23	Undefined	70	>40
4	24	Other (remodeling)	70	>40
5	32	Other (minimal lesions)	60	N.A
6	39	Undefined	50	5.3
7	40	Undefined	70	N.A
8	49	Other (cholestasis)	70	N.A
9	51	Undefined	90	0.2
10	97	Other (cholestasis)	70	13.4
11	105	Other (cholestasis)	40	N.A
12	107	Undefined	60	5.2
13	111	Undefined	40	5.7
14	113	Other (reperfusion damage)	40	N.A
15	153	Rejection	90	N.A
16	249	Undefined	60	17.9

Abbreviations: N.A., not available within 3 days from liver biopsy; Undefined: rejection grade II-III was excluded but both hepatitis and rejection grade I patterns coexisted.

TABLE 3. Immunohistochemical HCV-Ag Expression According to HCV Genotype

Days post-LT	Genotype	Number	% of HCV-Ag-positive cases	Median % of HCV-Ag-positive hepatocytes (range)
<30	1	11	45%	0 (0-80)
	Non-1	9	44%	0 (0-70)
30-180	1	37	97%	50 (0-95)
	Non-1	16	94%	20 (0-80)
>180	1	31	87%	30 (0-90)
	Non-1	14	64%	17.5 (0-90)
Total	1	79	86%	40 (0-95)
	Non-1	39	72%	10 (0-90)

when the histologic diagnosis was rejection, undefined or other (10%, 45%, and 15%, respectively) (Table 1).

In the "chronic" phase 74% of biopsies were found positive for HCV-Ag. In the case of hepatitis, this percentage was 81%, with a reduction in the median percentage of positive hepatocytes (30%; range 0-90%) with respect to the "acute" phase. The median percentage of liver HCV-Ag-positive hepatocytes was lower when the diagnosis was rejection, undefined, or other (0.5%, 27.5%, and 4%, respectively) as compared to the "acute" phase.

On the whole, histologic hepatitis was significantly more frequently HCV-Ag-positive when compared with rejection (89% vs. 59%; $P < 0.0001$), with a significantly higher median percentage of positive hepatocytes (40% vs. 1%; $P < 0.00001$).

The evaluation of follow-up clinical and biochemical parameters was concordant with the initial histopathological diagnosis in all but 16 patients (initial diagnosis: undefined in 9, other in 6, and rejection in 1 case), in which conclusive diagnosis was changed into recurrent

hepatitis: in all these cases, a high number of HCV-Ag-positive hepatocytes (65%; range 40-90%) was recorded (Table 2).

The dynamics of HCV reinfection was similar in the different genotypes (Table 3), although a trend toward a higher median number of HCV-Ag-positive hepatocytes was found in genotype 1 (40%; range 0-95%) with respect to non-1 (10%; range 0-90%) ($P = 0.088$).

Comparing genotype 1 with non-1, no significant differences were found concerning the final diagnosis of hepatitis (76% genotype 1 and 62% other genotypes; $P = 0.16$). Moreover, when the diagnosis was hepatitis, the percentage of HCV-Ag-positive cases (95% and 88%, respectively; $P = 0.46$) and the median percentage of HCV-Ag-positive hepatocytes (50% and 27.5%, respectively; $P = 0.06$) were not significantly different in both groups. The frequency of a final diagnosis of rejection was similar in genotype 1 (11%) and non 1 patients (10%) (Table 4).

Quantitative HCV viremia at the time of biopsy was available in 124 cases. Qualitative and quantitative se-

TABLE 4. Immunohistochemical HCV-Ag Expression in Biopsies From Patients With a Final Diagnosis of Hepatitis or Rejection Grouped by Genotype

Diagnosis	Genotype	Number (%)	% of HCV-Ag-positive cases	Median % of HCV-Ag-positive hepatocytes (range)
Hepatitis	1	60 (76%)	57 (95%)	50 (0-98)
	Non-1	24 (62%)	21 (88%)	27.5 (0-80)
Rejection	1	9 (11%)	5 (56%)	10 (0-70)
	Non-1	4 (10%)	3 (75%)	2.5 (0-90)

rum HCV-RNA was negative in 15 cases, which were also all negative for tissue HCV-Ag. A total of 100 (92%) out of 109 HCV-RNA-positive cases were positive for liver HCV-Ag by immunohistochemistry. A significant correlation between quantitative serum HCV-RNA and the percentage of HCV-Ag-positive hepatocytes was found (r = 0.41; $P < 0.001$) in the 109 HCV-RNA-positive cases. This correlation was high during the "reinfection" phase (r = 0.80; $P < 0.001$) and in the "chronic" phase (r = 0.44; $P < 0.01$), whereas no statistically significant correlation was found in the "acute" phase (r = 0.27; $P > 0.05$).

When specimens obtained within 180 days post-LT ("reinfection" and "acute" phase) were considered, univariate analysis indicated that recurrent hepatitis was significantly associated with serum HCV-RNA levels ($P < 0.00001$), percentage of HCV-Ag-positive hepatocytes ($P < 0.00001$), the intensity of immunostaining ($P < 0.00001$), the time post-LT ($P < 0.00001$), and serum bilirubin levels ($P < 0.003$). Multivariate analysis identified the percentage of HCV-Ag-positive hepatocytes and the time post-LT as the only significant independent predictors of recurrent hepatitis ($P = 0.008$; confidence interval 1.014-1.094 and $P = 0.041$, confidence interval 1.001-1.038, respectively). The cutoff percentage of ≥30% HCV-Ag-positive hepatocytes showed a sensitivity of 78%, a specificity of 98%, a negative predictive value of 73%, and a positive predictive value of 98% for the diagnosis of recurrent hepatitis. A number of HCV-Ag-positive hepatocytes (<30%) showed a sensitivity of 100%, a specificity of 57%, a negative predictive value of 100%, and a positive predictive value of 28% for the diagnosis of rejection.

Evaluating the dynamic modifications of HCV-Ag expression in patients who underwent more than 1 biopsy, we found an increase in the median percentage of positive hepatocytes between the first (1%; range 0-70%) and the second biopsy (50%; range 0-95%) in the 16 patients who obtained the first biopsy within 30 days after LT. On the contrary, a decrease of the median percentage of positive hepatocytes between the first (55.0%; range 0-90%) and the second biopsy (27.5%; range 0-85%) was observed in the 22 patients who underwent the first biopsy between 30 and 180 days after LT (Fig. 3).

A total of 26 patients completed a 24/48 week course of therapy with standard or pegylated interferon plus ribavirin. Of these, 11 turned to be HCV-RNA-negative at the end of therapy (end-of-treatment
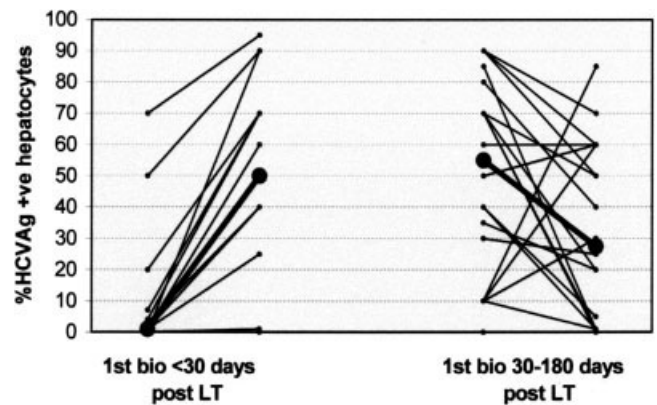

Figure 3. Temporal modifications of the number of HCV-Ag-positive hepatocytes in patients with 2 serial biopsies. On the left, 16 patients first biopsied within 30 days post-LT; on the right, 22 patients first biopsied between 30 and 180 days post-LT. Median values are reported in bold and black.

response) (9 within 12 weeks of treatment), while 15 remained HCV-RNA-positive (nonresponders) (Table 5). Pretreatment histology showed hepatic damage of various degree without differences in the 2 groups; in particular, no patient with the severe cholestatic form was treated.

Univariate analysis indicated that nonresponse was significantly associated with HCV-Ag positivity ≥50% ($P = 0.011$), serum HCV-RNA >4 Meq/mL ($P = 0.022$), and genotype 1 ($P = 0.038$); multivariate analysis identified percentage of HCV-Ag ≥50% as the only significant independent predictor for nonresponse ($P < 0.01$; odds ratio, 11.11; 95% confidence interval, 1.79-68.89). When considering only the 18 genotype 1 patients, nonresponse was significantly associated with HCV-Ag positivity ≥50% ($P = 0.044$) but not with serum HCV-RNA >4 Meq/mL ($P = 0.065$).

In 4 end-of-treatment-response patients and 10 nonresponders, a biopsy performed at the end of therapy was available: liver HCV-Ag expression was negative in all the end-of-treatment-response patients, and positive in all the nonresponders (median 50%; range 5-70). The number of positive hepatocytes, however, decreased in 6 of the 10 nonresponder cases, whereas it was unmodified in 4 cases.

DISCUSSION

It is widely accepted that graft reinfection after LT for HCV-related liver disease occurs in the vast majority of

TABLE 5. Patients Who Completed a 24/48-Week Course of Antiviral Therapy			
	End-of-treatment virological response to therapy		
	Responders	Nonresponders	P
Number	11	15	
Male/Female	8/3	12/3	0.51
Age; median (range)	56 (44-67)	51 (22-63)	0.09
Start-of-treatment: days post-LT; median (range)	112 (23-1,187)	124 (43-1,791)	0.19
Pretherapy HCV-Ag-positive hepatocytes ≥50%	4	13	0.011
Median % of pretherapy HCV-Ag-positive hepatocytes (range)	30 (3-80)	65 (3-95)	0.05
HCV-RNA pretherapy >4 Meq/mL	7	15	0.022
HCV-RNA pretherapy (Meq/mL): median (range)	4.64 (0.41-36.22)	15.29 (3.40->40)	0.004
Genotype 1	5	13	0.038
Pegylated/standard interferon	7/4	7/8	0.32
Immunosuppressive therapy: tacrolimus/cyclosporine	7/4	11/4	0.46

cases.[1-9] Usually, the presence of reinfection is suspected mostly on the basis of histologic features (i.e., focal lobular necrosis)[15] and it is considered infrequent during the first months after LT, when rejection seems to represent the main clinical problem. Qualitative determination of HCV-RNA is of little help in the diagnosis of reinfection, since patients become RNA-negative after LT only very rarely. Quantitative viremia, on the other hand, has so far produced conflicting results.[3,5,7,22-24] It is almost impossible to diagnose reinfection, when it is not associated to histologic liver damage, unless in situ HCV-RNA[30-32] or HCV-Ag[32-36] are determined.

We have previously demonstrated that the immunohistologic detection of liver HCV-Ag is sensitive and specific enough to be used in clinical practice as a diagnostic tool to detect reinfection of the transplanted liver.[37]

With the limitations intrinsic to a retrospective study performed in patients with altered serum parameters, we now have shown that liver reinfection does occur early after LT, as liver HCV-Ag expression is detectable as early as 8 days after LT and 57% of biopsies obtained within 30 days after LT are already HCV-Ag-positive. Reinfection appears to reach its peak (92% of cases) during the "acute" phase and decreases to 74% in the "chronic" phase. This trend in post-LT HCV reinfection has been confirmed by evaluating 38 patients with serial biopsies.

We confirm in a larger series our previous data,[37] showing that HCV-Ag are more frequently found in patients with histologic hepatitis with respect to rejection.

We previously made the hypothesis of a possible direct viral cytotoxic mechanism in the presence of early massive reinfection, but this does not explain why massive infection was never found in a patient with severe rejection.

Nevertheless, our data appear to be in agreement with data reporting higher hepatic HCV-RNA levels in patients with recurrent HCV hepatitis with respect to patients with rejection,[41] and a low serum HCV-RNA during moderate or severe acute and chronic rejection.[39] From a pathogenic point of view, a significant

acute and chronic rejection in liver allografts have been associated with a strong T-helper-1-type microenvironment and cytotoxic T-lymphocyte response, which cause rejection and, simultaneously, enhance HCV clearance,[42] thus giving a possible physiopathological explanation to this finding. This hypothesis is in keeping with the observation of a detrimental role of steroid pulses and excessive immunosuppression on HCV recurrence.[43]

As a consequence, the impact of liver HCV-Ag evaluation in the clinical management of patients appears particularly important in the first 6 months after LT. In fact, in 15 out of 118 (13%) biopsies performed in this period, a final diagnosis of recurrent hepatitis was made during the follow-up despite previous inconclusive or discordant histopathologic diagnosis. In all these patients, a high number of infected hepatocytes was detected. Moreover, we showed that a number of HCV-Ag-positive hepatocytes greater than 30% "sensu stricto" confirmed the presence of recurrent hepatitis and "absolutely" excluded the presence of significant rejection. Urgent therapeutic decisions are usually not necessary in this phase, when mild rejection is found. However, we underline that immunohistologic evaluation proved to be useful to promptly confirm the presence of recurrent hepatitis and to exclude significant rejection, thus allowing to assume informed therapeutic decisions and possibly avoiding further biopsies to the patient.

On the contrary, in the "chronic" phase (after more than 180 days after LT) diagnosis was changed in only 1 out of 97 cases (1%) and all the patients have been treated either for rejection or for hepatitis independently of liver HCV-Ag expression. This is in keeping with our previous data in chronic HCV hepatitis, in which we found no relation between histologic activity and the number of infected cells.[35]

The number of HCV-Ag-positive hepatocytes significantly correlated with serum quantitative HCV-RNA, and this correlation was particularly high during the "reinfection" period, but not during the "acute" phase, when the need to differentiate between recurrent hep-

atitis and rejection is more frequently perceived. Moreover, only the number of HCV-Ag-positive hepatocytes was found to be an independent predictor for recurrent hepatitis at multivariate analysis. Thus, although quantitative HCV-RNA (a more standardized and readily-available technique than immunohistochemistry) can be used to suspect recurrent hepatitis in the very early phase post-LT, it represents only an indirect parameter. In fact, liver HCV antigens and not HCV-RNA play a role in inducing liver damage, either directly or as a target of immunologic reactions. Moreover, from a technical point of view, immunohistochemical evaluation of liver HCV-Ag expression can be available as early as 6 hours from the liver biopsy, thus allowing an earlier diagnosis and, eventually, an earlier treatment, whereas serum HVC-RNA determination takes at least 48 hours.

Multivariate analysis identified the number of HCV-Ag-positive hepatocytes as the only significant independent predictor for end-of-treatment virological nonresponse. Therefore, even if in clinical practice genotype and viremia represent appropriate tools to evaluate the "a priori" chance of response to treatment, our data suggest that a low chance of response should not be directly related to genotype 1 and/or high viremia, but rather to the number of infected hepatocytes (possibly resulting from complex virus and host interaction), usually higher in genotype 1, as previously reported in chronic hepatitis.[35] The fact that response to treatment is more frequent in genotype 1 patients with a low number of infected hepatocytes supports this hypothesis.

In conclusion, we have demonstrated that HCV reinfection occurs early post-LT, reaching its acme within 6 months. During this period the inevitable uncertainty in distinguishing acute cellular rejection from recurrence of HCV has prompted the search for tools able to corroborate histological and clinical information. Immunohistochemical detection of HCV-Ag, demonstrated to be easy to handle and sensitive enough to be used in the clinical setting, might be relevant for the management of transplanted patients, as it supports a clinical diagnosis of hepatitis when histologic features are not concordant or inconclusive and allows us to undertake the adequate treatment earlier. Moreover, in the presence of recurrent hepatitis, a high number of infected hepatocytes represents an independent predictive factor of nonresponse to antiviral therapy.

REFERENCES

1. Boker KH, Dalley G, Bahr MJ, Maschek H, Tillmann HL, Trautwein C, et al. Long-term outcome of hepatitis C virus infection after liver transplantation. Hepatology 1997;25: 203-210.
2. Negro F, Giostra E, Krawczynski K, Quadri R, Rubbia-Brandt L, Mentha G, et al. Detection of intrahepatic hepatitis C virus replication by strand-specific semi-quantitative RT-PCR: preliminary application to the liver transplantation model. J Hepatol 1998;29:1-11.
3. Di Martino V, Saurini F, Samuel D, Gigou M, Dussaix E, Reynes M, et al. Long-term longitudinal study of intrahepatic hepatitis C virus replication after liver transplantation. Hepatology 1997;26:1343-1350.
4. Petrovic LM, Villamil FG, Vierling JM, Makowka L, Geller SA. Comparison of histopathology in acute allograft rejection and recurrent hepatitis C infection after liver transplantation. Liver Transpl Surg 1997;3:398-406.
5. Gane EJ, Naomov NV, Qian KP, Mondelli MU, Maertens G, Portman BC, et al. A longitudinal analysis of hepatitis C virus replication following liver transplantation. Gastroenterology 1996;110:167-177.
6. Gretch DR, Bacchi CE, Corey L, Dela Rosa C, Lesniewsky RR, Kowdley K, et al. Persistent hepatitis C virus infection after liver transplantation: clinical and virological features. Hepatology 1995;22:1-9.
7. Vargas V, Krawczynski K, Castells L, Martinez N, Esteban J, Allende H, et al. Recurrent hepatitis C virus infection after liver transplantation: immunohistochemical assessment of the viral antigen. Liver Transpl Surg 1998;4:320-327.
8. Fukumoto T, Berg T, Ku Y, Bechstein WO, Knoop M, Lemmens HP, et al. Viral dynamics of hepatitis C early after orthotopic liver transplantation: evidence for rapid turnover of serum virions. Hepatology 1996;24:1351-1354.
9. Gane EJ, Portmann BC, Naoumov NV, Smith HM, Underhill JA, Donaldson PT, et al. Long-term outcome of hepatitis C infection after liver transplantation. N Engl J Med 1996;334:815-820.
10. Forman LM, Lewis JD, Berlin JA, Feldman HI, Lucey MR. The association between hepatitis C infection and survival after orthotopic liver transplantation. Gastroenterology 2002;122:889-896.
11. Prieto M, Berenguer M, Rayon JM, Cordoba J, Arguello L, Carrasco D, et al. High incidence of allograft cirrhosis in hepatitis C virus genotype 1b infection following transplantation: relationship with rejection episodes. Hepatology 1999;29:250-256.
12. Feray C, Caccamo L, Alexander GJ, Ducot B, Gugenheim J, Casanovas T, et al. European collaborative study on factors influencing outcome after liver transplantation for hepatitis C. European Concerted Action on Viral Hepatitis (EUROHEP) Group. Gastroenterology 1999;117:619-625.
13. Sanchez-Fueyo A, Restrepo JC, Quinto L, Bruguera M, Grande L, Sanchez-Tapias JM, et al. Impact of the recurrence of hepatitis C virus infection after liver transplantation on the long-term viability of the graft. Transplantation 2002;73:56-63.
14. Ben-Ari Z, Dhillon AP, Moqbel R, Garwood L, Booth D, Rolles K, et al. Monoclonal antibodies against eosinophils in liver allograft rejection. Liver Transpl Surg 1996;2:46-51.
15. Lautenschlager I, Nashan B, Schlitt HJ, Hoshino K, Ringe B, Tilmann HL, et al. Different cellular patterns associated with hepatitis C virus reactivation, cytomegalovirus infection and acute rejection in liver transplantation patients monitored with transplant aspiration cytology. Transplantation 1994;58:1339-1345.
16. Ferrell L, Wright T, Roberts J, Ascher N, Lake J. Hepatitis C viral infection in liver transplant recipients. Hepatology 1992;16:865-876.
17. Rosen H, Shackleton C, Higa L, Gralnek I, Farmer D, McDiarmid SV, et al. Use of OKT3 is associated with early and severe recurrence of hepatitis C after liver transplantation. Am J Gastroenterol 1997;92:1453-1457.
18. Sheiner P, Schwartz M, Mor E, Schluger L, Theise N, Kishikawa K, et al. Severe or multiple rejection episodes are associated with early recurrence of hepatitis C after orthotopic liver transplantation. Hepatology 1995;21:30-34.
19. Younossi Z, Boparai N, Gramlich T, Goldblum J, George P, Mayes J. Agreement in pathologic interpretation of liver

biopsy specimens in posttransplant hepatitis C infection. Arch Pathol Lab Med 1999;123:143-145.

20. Regev A, Molina E, Moura R, Bejarano PA, Khaled A, Ruiz P, et al. Reliability of histopathologic assessment for the differentiation of recurrent hepatitis C from acute rejection after liver transplantation. Liver Transpl 2004;10:1233-1239.

21. McTaggart RA, Ferrel L, Lassman CR, Roberts JP, Terrault NA, Feng S. The reliability of liver biopsy to diagnose acute rejection? The impact of recurrence hepatitis C. Hepatology 2004;40:356A.

22. Sreekumar R, Gonzalez-Koch A, Mao-Kendler Y, Batts K, Moreno-Luna L, Poterucha J, et al. Early identification of recipients with progressive histologic recurrence of hepatitis C after liver transplantation. Hepatology 2000;32:1125-1130.

23. Asanza CG, Garcia-Monzon C, Clemente G, Salcedo M, Garcia-Buey L, Garcia-Iglesias C, et al. Immunohistochemical evidence of immunopathogenetic mechanisms in chronic hepatitis C recurrence after liver transplantation. Hepatology 1997;26:755-763.

24. Freeman RM, Tran S, Lee YM, Rohrer RJ, Kaplan MM. Serum hepatitis C RNA titres after liver transplantation are not correlated with immunosuppression or hepatitis. Transplantation 1996;61:542-546.

25. Pageaux GP, Ducos J, Mondain AM, Costes V, Picot MC, Perrigault PF, et al. Hepatitis C virus genotypes and quantitation of serum hepatitis C virus RNA in liver transplant recipients: relationship with severity of histological recurrence and implications in the pathogenesis of HCV infection. Liver Transpl Surg 1997;3:501-505.

26. Gayowski T, Singh N, Marino IR, Vargas H, Wagener M, Wannstedt C, et al. Hepatitis C virus genotypes in liver transplant recipients: impact on posttransplant recurrence, infections, response to interferon-alpha therapy and outcome. Transplantation 1997;64:422-426.

27. Gordon FD, Poterucha JJ, Germer J, Zein NN, Batts KP, Gross JB Jr, et al. Relationship between hepatitis C genotype and severity of recurrent hepatitis C after liver transplantation. Transplantation 1997;63:1419-1423.

28. Zhou S, Terrault NA, Ferrell L, Hahn JA, Lau JY, Simmonds P, et al. Severity of liver disease in liver transplantation recipients with hepatitis C virus infection: relationship to genotype and level of viremia. Hepatology 1996;24:1041-1046.

29. Vargas HE, Laskus T, Wang LF, Radkowski M, Poutous A, Lee R, et al. The influence of hepatitis C virus genotypes on the outcome of liver transplantation. Liver Transpl Surg 1998;4:22-27.

30. Agnello V, Abel G, Knight GB, Muchmore E. Detection of widespread hepatocyte infection in chronic hepatitis C. Hepatology 1998;28:573-584.

31. Fragulidis GP, Cirocco RE, Weppler D, Berho M, Gillian G, Markou M, et al. In situ enzymatic oligonucleotide amplification of hepatitis C virus-RNA in liver biopsy specimens (reverse transcriptase in situ polymerase chain reaction) after orthotopic liver transplantation for hepatitis C-related liver disease. Transplantation 1998;66:1472-1476.

32. Vona G, Tuveri R, Delpuech O, Vallet A, Canioni D, Ballardini G, et al. Intrahepatic hepatitis C virus RNA quantification in microdissected hepatocytes. J Hepatol. 2004;40:682-688.

33. Krawczynski K, Beach MJ, Bradley DW, Kuo G, Di Bisceglie A, Houghton M, et al. Hepatitis C Virus antigen in hepatocytes: immunomorphological detection and identification. Gastroenterology 1992;103:622-629.

34. Di Bisceglie AM, Hoofnagle H, Krawczynski K. Changes in hepatitis C virus antigen in liver with antiviral therapy. Gastroenterology 1993;105:858-862.

35. Ballardini G, Groff P, Pontisso P, Giostra F, Francesconi R, Lenzi M, et al. Hepatitis C virus genotype, tissue HCV antigens, hepatocellular expression of HLA-A,B,C, and intercellular adhesion-1 molecules. Clues to pathogenesis of hepatocellular damage and response to interferon treatment in patients with chronic hepatitis C. J Clin Invest 1995;95:2067-2075.

36. Ballardini G, Manzin A, Giostra F, Francesconi R, Groff P, Grassi A, et al. Quantitative liver parameters of HCV infection: relation to HCV genotypes, viremia and response to interferon treatment. J Hepatol 1997;26:779-786.

37. Ballardini G, De Raffele E, Groff P, Bioulac-Sage P, Grassi A, Ghetti S, et al. Timing of reinfection and mechanism of hepatocellular damage in transplanted hepatitis C virus-reinfected liver. Liver Transpl 2002;8:10-20.

38. Demetris AJ, Batts KP, Dhillon AP, Ferrell L, Fung J, Geller SA, et al. Banff schema for grading liver allograft rejection: an International Consensus Document. Hepatology 1997;25:658-663.

39. Demetris AJ, Eghtesad B, Marcos A, Ruppert K, Nalesnik MA, Randhawa P, et al. Recurrent hepatitis C il liver allografts. Prospective assessment of diagnostic accuracy, identification of pitfalls, and observations about pathogenesis. Am J Surg Pathol 2004;28:658-669.

40. Ballardini G, Groff P, Giostra F, Francesconi R, Miniero R, Ghetti S, et al. Hepatocellular codistribution of c100, c33, c22, and NS5 hepatitis C virus antigens detected by using immunopurified polyclonal spontaneous human antibodies. Hepatology 1995;21:729-734.

41. Gottschlich MJ, Aardema KL, Burd EM, Nakhleh RE, Brown KA, Abouljoud MS, et al. The use of hepatitis C viral RNA levels in liver tissue to distinguish rejection from recurrent hepatitis C. Liver Transpl 2001;7:436-441.

42. Zekry A, Bishop GA, Bowen DG, Gleeson MM, Guney S, Painter DM, McCaughan GW. Intrahepatic cytokine profiles associated with posttransplantation hepatitis C virus-related liver injury. Liver Transpl 2002;8:292-391.

43. McCaughan GW, Zekry A. Mechanisms of HCV reinfection and allograft damage after liver transplantation. J Hepatol 2004;40:368-374.