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Peripheral blood neutrophils from hepatitis C virus-infected patients are replication sites of the virus

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ABSTRACT

***Background and Objectives*. Hepatitis C virus (HCV) is able to cause not only acute and chronic liver disease, but also immunologic and hematologic disorders. In order to clarify the extra-hepatic tropism of HCV, and to understand the pathogenetic mechanisms of HCV infection, we evaluated viral replication in peripheral blood mononuclear cells.**

***Design and Methods*. The presence of genomic and antigenomic (replicative) forms of HCV in B- and T-lymphocytes, monocytes, and polymorphonuclear leukocytes (PML) was determined by reverse transcriptase-polymerase chain reaction in 54 HCV-RNA positive patients and, as control groups, in 10 patients who had recovered from HCV infection without evidence of serum HCV-RNA, and in 10 HCV-negative subjects.**

***Results.* In HCV-RNA positive patients, the genomic RNA was found in 94% of B-cells, in 14% of T-cells, in 40% of monocytes and in 77% of PML, while only 1 of the HCV-RNA negative subjects showed positivity in B-cells. The anti-genomic form of HCV-RNA was found in 52% of B-cells, in 3% of monocytes, and in 31% of PML. By contrast, it was never detected in T-cells and in HCV-RNA negative subjects. Neither genomic nor anti-genomic forms were found in HCV-negative cases.**

***Interpretation and Conclusions.* These data suggest that PML are replication sites of HCV. Whether the infection occurs at the level of the stem cells or subsequently during myeloid cell differentiation is, as yet, unknown. The absence of correlation between the presence of replicative forms and any clinical and/or laboratory data opens the question of the role of HCV replication in extra-hepatic sites.**
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Key words: hepatitis C virus; mixed cryoglobulinemia, lymphocytes, monocytes, polymorphonuclear leukocytes

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It is known that hepatitis C virus (HCV) is able to infect not only hepatocytes, but also peripheral blood monuclear cells (PBMC).[1,2] As early as 1985, Hellings *et al.*[3] revealed that leukocytes from patients affected by post-transfusional non-A non-B hepatitis were able to infect chimpanzees. Positive and negative HCV-RNA strands (genomic and putative replicative forms) were demonstrated in PBMC by retrotranscription-polymerase chain reaction (RT-PCR) or by *in situ* hybridization.[4,5] Although negative strand RNA was present in serum, false positivity in PBMC was excluded by the absence of HCV-RNA in the washing solution of the final step of PBMC separation. Furthermore, some authors found that HCV-RNA could be undetectable in serum, while it was present in PBMC. All these observations support the hypothesis of viral replication in these cells.[6-8] In contrast, the PBMC from patients with HCV acute hepatitis do not seem to be infected by HCV.[7]

The presence of the replicative form of HCV in PBMC suggests that these cells can be a virus reservoir capable of reinfecting the liver in transplanted patients or in subjects treated with interferon (IFN).[8] Though it is undisputed that HCV infects PBMC, its pathogenetic role in the natural history of the disease is still unknown.

In order to gain a better understanding of the cell-specific tropism of HCV, we planned the present study and evaluated the presence of genomic and anti-genomic RNA not only in B- and T-lymphocytes, but also in the monocytes/macrophages and the polymorphonuclear leukocytes (PML) of a large number of patients with HCV infection of different duration and severity.

Design and Methods

Patients

The presence of HCV-RNA (plus and minus strand) was investigated in B- and T-lymphocytes, monocytes and PML from 54 patients affected by HCV-positive chronic liver disease. The diagnosis was based on the following criteria: anti-HCV antibody positivity, presence of HCV-RNA in serum as detected by PCR, elevated alanine-aminotransferase serum levels, which were at least twice as high as the upper threshold levels of normal and which had lasted for more than 6 months before entry to the study, his-

tologic evidence of chronic hepatitis on liver biopsy.

Ten healthy carriers of HCV infection were used as controls; the diagnosis of healthy carriers of HCV was based of the presence of HCV-RNA in serum associated with normal liver histology (all patients underwent liver biopsy) and normal ALT/AST levels for at least two years. Ten additional patients with anti-HCV antibodies, but no evidence of HCV-RNA in the serum (who had recovered from chronic HCV infection after interferon treatment) were also studied. A small group of patients affected by alcoholic liver disease without anti-HCV antibodies and no evidence of HCV-RNA in the serum were also checked for the presence of HCV-RNA in serum and in PBMC.

In all the groups, the presence of mixed cryoglobulinemia (MC) and of the other HCV-associated immunologic and hematologic disorders was also recorded. The diagnosis of MC was based on the presence of the typical clinical syndrome (arthralgias, weakness and purpura) associated with circulating cryoglobulins (cryocrit >2%).

Methods

Liver function tests as well as hematologic parameters were determined by usual laboratory methods. Anti-HCV antibodies were assayed by the second-generation (four-antigen) immunoenzymatic screening test ORTHO-HCV (Ortho Diagnostic Systems, Raritan, NJ, USA). In all cases an additional confirmatory test (RIBA, Chiron Corp., Emeryville, CA, USA) was carried out.

Hepatitis B virus (HBV) and human immunodeficiency virus (HIV) markers were detected by enzyme-linked immunosorbent assay (ELISA) using available commercial kits. Rheumatoid factor (RF), C3 and C4 fractions of complement were measured by rate nephelometry.

To isolate cryoglobulins, 20 mL of blood were kept at 37°C for 2 hours in a glass tube. The serum was cleared by centrifugation and stored at 4°C for 7 days. The cryoprecipitate was separated by centrifugation at 4,000 rpm for 30 minutes at 4°C. Mixed cryoglobulins were classified as type II on the basis of the presence of monoclonal immunoglobulins with RF activity complexed with polyclonal IgG, and as type III in the presence of polyclonal immunoglobulins.

All the MC patients underwent a bone marrow biopsy with a Jamshidi-like needle (Trapsystem, Kerna, Treviso, Italy). The sample was placed in B5 solution and 2 hours later in ethanol 70%. After decalcification, the samples were stained following standard methods. When indicated, a lymph node biopsy was also performed.

Patients with renal involvement (serum creatinine more than 1.5 mg/dL or daily proteinuria exceeding 165 mg/day) were subjected to kidney biopsy. Immunofluorescence was performed in each case with the following antisera: anti-IgG, –IgA, –IgM, –IgD, –C3c, –C4, –C1q and anti-fibrinogen (Dako, Glostrup, Denmark).

Cell separation was achieved using 20 mL of ACD-anticoagulated blood diluted with 1 volume of physiologic solution, stratified on Ficoll and centrifuged at 550g for 20 min. Following centrifugation, PBMC were recovered, washed three times and centrifuged at 350g for 10 min. The polymorphonuclear cells were recovered from the interface between the erythrocytes and Ficoll and, after the erythrocyte lysis, washed and stored at -80°C. The washed PBMC were grown in RPMI 1640 at 37°C to allow adhesion of the monocytes to the flask bottom. After being washed, the supernatant containing the NK, B- and T-lymphocytes was treated at 4°C for 30 min with monoclonal mouse antibodies specific for the NK and T-lymphocytes (anti-CD2 and anti-CD56). The pellet was then washed, treated with magnetic dynabeads coated with goat anti-mouse antibodies and incubated with moderate shaking. The dynabeads, which at this point contained only B-cells, were separated from the solution by a magnet. The B-lymphocytes recovered from the solution were washed and stored at –80°C. The monocytes were recovered by scraping the flask bottom and, after washing, were frozen at –80°C. The efficiency of the separation was >90% for PML and monocytes, 80-90% for B-lymphocytes and 100% for T-lymphocytes.

After a variable time (not exceeding two weeks), the RNA was extracted from each different cell sample using standard methods.[9] Subsequently, RNA was retrotranscribed with primers specific for the 5' UTR region of HCV (antisense primer for the plus RNA strand: 1A 5'-GAT GCA CGG TCT ACG AGA CCT-3' and sense primer for the minus RNA strand: 1B 5'- AAC TAC TGT CTT CAC GCA GAA-3').

A nested PCR was then performed using two different primer sets: 1A and 1B in the first step , 2A (anti sense 5'-GCG ACC CAA CAC TAC TCG GCT-3') and 2B (sense: 5'-ATG GCG TTA GTA TGA GTG) in the second step.

The amplified product (188 bp) was visualized on ethidium bromide-stained agarose gel (Figure 1).

The HCV genotype was determined by PCR amplification of the core region with type-specific primers, according to Okamoto *et al*.[10,11]

HCV was quantified by Amplicor HCV Monitor (Roche Molecular Systems, Roche). The test was performed according the instructions provided by the manufacturer.

Statistical analysis

The data are expressed as mean ± standard deviation. Statistical analyses were performed using the statistics package "SPSS" for Windows. For continuous variables, one-way analysis of variance between the groups was calculated yielding Snedecor's F factor. Categorical variables were analyzed with Pearson's chi-square test. Associations were studied by using the hierarchical log-linear analysis in a multiway cross tabulation.

Results

The main clinical and laboratory characteristics of the patients are indicated in Table 1. Of the 54 patients with histologic evidence of chronic liver disease, measurable levels of cryoglobulins were found in 35 cases (65%), but only a fraction of them (17 cases, 31%) revealed clinical symptoms and could be considered as MC carriers. The bone marrow biopsy was normal in only 5 of these patients (29%); in the others, paratrabecular foci of infiltration by small

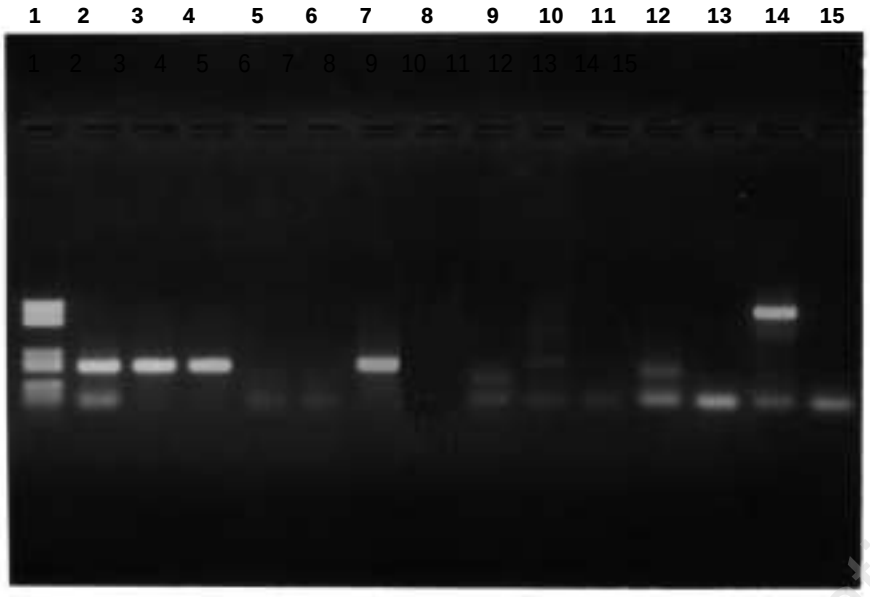

Figure 1. Ethidium bromide stained agarose gel of minus HCV-RNA from polymorphonuclear cells. Lane 1: molecular weights; lane 2: HCV-RNA minus strand positive control; lanes 3, 4 and 7: HCV-RNA minus strand positive samples; lanes 5, 6, 8, 9, 10, 11 and 12: minus strand nergative samples; lanes 14 and 15: positive and negative Taq controls.

lymphocytes with lymphoplasmacytoid features were present. In 6 subjects (35%), the marrow histology (massive B-lymphocyte infiltrate) suggested the diagnosis of low-grade non-Hodgkin's lymphoma (NHL) (group A according to the Working Formulation). This diagnosis was supported by more than 50% marrow infiltrate with the presence of lymphoplasmacytoid lymphocytes (CD19 and CD20 positive). Chronic lymphocytic leukemia was ruled out by the low number of CD5-positive cells (less than 15%) and by the high concentration of light chains on the cell surface. The diagnosis, in two of the patients, of NHL was also supported by lymph node histology.

HCV-RNA was detected in serum of all patients with chronic liver disease (CLD). The HCV genotyping showed the presence of type 1a in 11 cases (21%), type 1b in 19 cases (35%), type 2a in 13 cases (24%), type 2b in 2 cases (3%), type 2c in 2 cases (3%), type 3a in 2 cases (3%) and 5 cases were considered not possible to type (9%). The 10 HCV-RNA negative subjects, previously affected by CLD and successfully treated with interferons, were former carriers (before therapy) of type 1a (1 case, 10%), type 2a (6 cases, 60%), and type 3a (3 cases 30%).

HCV-RNA quantification was available for 51 of the 54 cases (94%). The HCV-RNA serum concentration ranged from 2×10^3 to 1×10^6 with a mean of 442,812±264,455 copies/mL. No correlation was found between the HCV-RNA serum concentration and the severity of liver disease, judged by liver histology, or with any liver function test, cryoglobulin concentration, clinical symptoms, and presence of minus-strand in lymphocytes or polymorphonuclear leukocytes. Patients carrying genotype 1b showed a higher viral load than those patients infected by the other genotypes, but the difference (given the high standard deviation) was not statistically significant (482,111±288,374 vs. 392,285± 219,654 copies/mL; t: 0.356, *p*: ns).

No HCV-RNA (plus or minus strands) was present in cells from the 10 patients without anti-HCV antibodies and no evidence of HCV-RNA in serum.

Among the patients positive for anti-HCV antibodies but negative for HCV-RNA in the serum, viral RNA plus strand was detected in B-lymphocytes and in PML in only one patient.

The results for cell sub-populations from the 54 patients with HCV-RNA in the serum are reported in Table 2. The HCV-RNA plus strand was present in B lymphocytes of 51 patients (94%), in T-lymphocytes

Table 1. Main clinical, histologic and laboratory data of the patients enrolled in the study.

Patients (N°cases)	*M/F ratio*	*Age (yrs)*	*Liver histology*			*AST U/L (NV<40)*	*ALT U/L (NV<40)*	*MC (no.%)*	*Disease length (years)*
			CPH	*CAH*	*C or HCC*				
CLD anti-HCV pos serum HCV-RNA pos (48 cases)	3/1	57±17	17	20	11	82±76	91±73	14 (29%)	10±5
NHL±CLD anti-HCV pos serum HCV-RNA pos (6 cases)	2/1	61±18	2	3	1	69±81	101±89	3 (50%)	12±7
Recovered CLD anti-HCV pos serum HCV-RNA neg (10 cases)	4/1	40±14	6	4	0	20±17	29±14	3 (30%)	10±4
Control CLD anti-HCV neg serum HCV-RNA neg (10 cases)	1/1	59±11	5	5	0	124±203	79±73	1 (10%)	9±4

CLD: chronic liver disease; CPH: chronic persistent hepatitis; CAH: chronic active hepatitis; C: cirrhosis; HCC: hepatocellular carcinoma; AST: aspartate-aminotransferase; ALT: alanin-aminotransferase; MC: mixed cryoglobulinemia.

Table 2. Positive and negative strand HCV-RNA distribution in peripheral blood mononuclear cells.

Patients	*HCV-RNA positivity*							
	B-lymphocytes		*T-lymphocytes*		*Monocytes*		*Polymorphonuclear cells*	
	Pos	*Neg*	*Pos*	*Neg*	*Pos*	*Neg*	*Pos*	*Neg*
CLD anti-HCV pos serum HCV-RNA pos (48 cases)	44 (92%)	24 (50%)	13 (27%)	0	21 (44%)	1 (2%)	43 (90%)	18 (37%)
NHL ± CLD anti-HCV pos serum HCV-RNA pos (6 cases)	6 (100%)	4 (67%)	1 (16%)	0	2 (33%)	0	3 (50%)	1 (16%)
Recovered CLD anti-HCV pos serum HCV-RNA neg (10 cases)	1 (10%)	0	0	0	0	0	1 (10%)	0
Control CLD anti-HCV neg serum HCV-RNA neg (10 cases)	0	0	0	0	0	0	0	0

CLD: chronic liver disease; NHL: non-Hodgkin's lymphoma.

of 14 cases (31%), in monocytes of 23 cases (40%) and in PML of 47 patients (77%).

Replicative HCV form (minus strand) was present in B-lymphocytes from 28 patients (52%), in monocytes from only 1 case (2%), and in polymorphonuclear cells from 19 patients (31%). Minus strand was never detected in T-lymphocytes. Univariate analysis of these data did not show any correlation between the presence or absence of positive or negative HCV-RNA in B- T-lymphocytes or PML and any clinical, histologic or biochemical parameter. A significant ($p<0.004$) association was also found between the presence of positive-strand HCV-RNA in PML and liver cirrhosis. The multivariate analysis confirmed the well-known associations between disease duration and poor response to antiviral therapy, or the presence of cirrhosis, or between cirrhosis (and HCC) and the duration of the disease, but did not find any significant correlation between the presence of positive or negative strand HCV-RNA in PBMC and the other parameters.

Discussion

Our data confirm the marked tropism of HCV for B-lymphocytes, in which the virus is constantly present in both genomic and replicative forms.[12] HCV seems to be less frequent in T-lymphocytes (and never in replicative forms) and in monocytes. However, negative-stranded HCV-RNA, core, and NS4 proteins were detected in cultured murine retrovirus infected T-cells (MOLT-4 and MOLT-4 Ma)[13] suggesting possible HCV replication and expression also in T-cells, at least *in vitro*. Some authors[14] found HCV-RNA in CD19-positive lymphocytes and in monocytes in patients with chronic hepatitis, while HCV-RNA was absent from CD4-positive lymphocytes. Unfortunately, the replicative HCV-RNA was not considered in that paper.

Our data confirm that blood cells are a possible reservoir for HCV, at least in a small fraction of cases. In fact, one patient with anti-HCV antibodies, but with HCV-RNA undetectable in serum, showed HCV-RNA in the B-lymphocytes and the PML. In this case, the possibility that serum HCV concentration was below the detection limits of PCR cannot be ruled out. However, on the basis of our data, HCV-RNA concentration does not seem to give important information on the severity of liver disease or on the outcome of the antiviral therapy. This may be due to several factors, such as spontaneously fluctuating HCV-RNA levels, or not-standardized sample storage procedures, but the main problem is the presence of measurable levels of cryoglobulins. These immunocomplexes are constituted by IgG-IgM-HCV-RNA which precipitate at room temperature, therefore, the HCV-RNA is cleared from serum and its recorded concentration misleadingly decreases. Since cryoglobulin production varies over time, the fraction of HCV-RNA removed by precipitation is not predictable, and, therefore, its serum level determination is not reliable. In our geographical area the frequency of measurable cryoglobulin levels is very high, ranging from 30 to 50% of HCV-infected subjects, so HCV-RNA quantification is often unreliable.

The most surprising finding of this work is the elevated frequency of HCV-RNA in PML both in genomic and replicative forms (80% and 36% respectively). Though the PMN separation procedure is not 100%, contamination by other PBMCs cannot be claimed as a source of false positivity because the potentially contaminating cells are T-lymphocytes (negative for viral replicative forms) and never B-lymphocytes. It is known that few viruses are able to infect PML:[15] cytomegalovirus (CMV)[16,17] and HIV[18,19] are the most common. In the course of HIV infection, the mean percentage of infected PML is relatively low (30%), ranging from 18.2% in asymptomatic carriers to 46.7% in patients with opportunistic infections. PML should not be considered as only anti-bacterial cells, but also as anti-viral. Indeed, a virus may be inactivated *in vitro* by reaction oxygen intermediate (ROI) production, or by defensins, but the significance of this virucidal activity *in vivo* is not well understood.[20]

The presence of replicative forms of HCV in PML opens a new question: when does HCV infect the PML or rather, at what level of myeloid cell differention does the infection occur? This event could occur at two points: 1) at the level of the stem cells, when the differentiation between granulocytes and monocytes has not yet occurred, 2) when the PML have already differentiated. The only available *in vivo* model of bone marrow cell infection is that of CMV; in infected PBMC, the expression of typical mRNA of the final phase of the viral replicative cycle (late mRNA) occurs 48 hours after infection, indicating a possible infection of the bone marrow hematopoietic precursor cells. This hypothesis is supported by several studies showing that infection of hematopoietic stem cells by CMV can occur easily.[21] The higher frequency of HCV replicative forms in PML than in

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monocytes (both derived from a common precursor) may point to a different susceptibility of the cells, or indicate that the infection does not occur in the common precursors. Infection of mature PML could occur as a non-opsonized virus particle through specific receptors or through immune complexes. The latter hypothesis is supported by several reports of the presence of HCV-RNA in cryoprecipitates. Since it is known that PML have a moderately short life span (they circulate for only eight hours), the presence of negative-stranded RNA might imply a higher than expected HCV replicative rate. The potential consequences of HCV infection on PML functional properties have never been studied. In fact, several viral infections[22] are able to generate some functional alterations of PML (e.g., burst activation, inhibition of phagosome/lysosome fusion, reduced chemotactic, oxidative and secretory functions). The potential modification of some functions of infected PML could explain the multiple aspects of HCV infection. For instance, the frequent presence of purpura in MC might be associated with altered PML-endothelial cell interactions in the capillary tree, and the high frequence of fatal bacterial or mycotic infections in HCV-associated membrano-proliferative glomerulonephritis (*Pozzato et al., J Intern Med 2000; in press*) could also be explained by acquired defects of PMN functions. However, the lack of any association between the presence or absence of MC, the clinical severity of MC or other clinical parameters and HCV replication in PML is in contrast with this hypothesis.

Alhough in this study the *ultimate* proof of viral replication in PBMC, i.e. the detection of viral proteins in their cytoplasm is lacking, it is likely that HCV infects several cellular systems. In fact, the putative HCV receptor, CD81,[23,24] is widespread in human cells and the highest concentrations of this receptor are expressed in B- and T-cells, in which it usually determines cellular differentiation;[25] it is also involved in several other functions such as cell adhesion, motility and signal transductions.[26] For a better understanding of the complex pathways involving the immune and non-immune systems in HCV infection, it might be useful to investigate the release of cytokines by B- and T-lymphocytes in infected individuals. Further help could be obtained through the nucleotide sequence of the different viral strains (quasispecies) which are simultaneously present in the same individual, with potentially different tissue tropisms and specificities.

Contributions and Acknowledgments

MC was responsible for data collection. GP contributed to data analysis, literature revision and writing the manuscript. FZ, EP, FN, SB, CM were the clinicians responsible for the patients' clinical management. MGG, MR, MLM, PM, AS carried out the PCR and other laboratory analyses. GFS supervised the entire study and revised the final version of the paper.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential Implications for clinical practice

- HCV replication can occur in PBNC in absence of detectable serum HCV-RNA levels.
- The infection of PBMC is not responsible for the progression of hematologic manifestations of HCV chronic infection.

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