**Journal of NeuroVirology (1999) 5,** 319–323

http://www.jneurovirol.com

*Short Communication*

# Detection and quasispecies analysis of hepatitis C virus in the cerebrospinal fluid of infected patients

Fabrizio Maggi[1], Massimo Giorgi[1], Claudia Fornai[1], Antonietta Morrica[1], Maria Linda Vatteroni[1], Mauro Pistello[1], Gabriele Siciliano[2], Andrea Nuccorini[3] and Mauro Bendinelli*[,1]

*[1]Retrovirus Center and Virology Section, Department of Biomedicine, University of Pisa, Via San Zeno, 35–39, 56127 Pisa, Italy [2]Neuroscience Department, Via Roma 55, 55126 Pisa, Italy and [3]U.O. Infectious Diseases, Azienda Ospedaliera Pisana, Via Paradisa 2, 56124 Pisa, Italy*

**Hepatitis C virus (HCV) is a leading cause of liver damage and has also been implicated in extrahepatic pathologies. We examined for HCV RNA paired CSF and plasma samples from 12 viremia positive patients using PCR. The CSF from 5/5 HIV-infected patients and 5/7 HIV-negative patients were HCV RNA positive. Branched DNA analysis showed that HCV loads in CSF were much lower than in plasma. Several HCV-positive CSF showed no evidence of blood contamination, impaired blood-brain barrier, or intrathecal IgG production. Comparison of HCV quasispecies in three sets of samples suggested that the virus in CSF was of plasma origin.**

**Keywords:** hepatitis C virus infection; central nervous system; cerebrospinal fluid; viral quasispecies

Hepatitis C virus (HCV) has an extraordinary ability to persist in infected patients where it produces prolonged plasma viremias of variable magnitude and, after variably long intervals from infection, frequently leads to chronic liver diseases ranging from active hepatitis to cirrhosis and hepatocellular carcinoma. In addition, although the etiologic link is uncertain, HCV has been suggested as a possible cause of several extrahepatic affections including certain forms of cryoglobulinemia and porphyria, membrano-proliferative glomerulonephritis, autoimmune diseases, and lymphoma (reviewed in Czaja, 1997). Sporadic reports have also suggested that HCV might be involved in the genesis of disturbances of the central (CNS) and peripheral nervous system (Kashihara *et al*, 1995; Bolay *et al*, 1996; Caudai *et al*, 1997; Propst *et al*, 1997).

The life cycle of HCV in infected hosts is poorly understood. Also unknown is the full spectrum of cell types that support its replication, due in part to great difficulties encountered in growing the virus in tissue culture (Ito *et al*, 1996; Nakajima *et al*, 1996; Seipp *et al*, 1997). Undoubtedly, HCV is characterised by a marked tropism for the hepatocyte, but it seems likely that it can also invade other cell types. Studies have, for example, demonstrated that HCV is found closely associated with peripheral blood mononuclear cells (PBMC) and there is convincing evidence that these cells support limited viral replication (Cribier *et al*, 1995; Lerat *et al*, 1996).

In a recent study of patients doubly infected with HCV and HIV-1, 5/21 cerebrospinal fluid (CSF) samples were found positive for HCV RNA by polymerase chain reaction (PCR), which led to suggest that, at least under certain conditions, HCV can reach the CNS (Morsica *et al*, 1997). Here, we have investigated, for the presence of HCV, CSF samples of singly infected patients as well as of HIV-1 co-infected patients. To this purpose, the collection of paired CSF and serum samples sent to our laboratory for evaluation of molecular and serological markers of viral infection between 1994 and 1998 was screened for anti-HCV antibodies by third generation ELISA (Ortho Diagnostic System, Raritan, NJ, USA). A total of 12 HCV-positive serum-CSF sets, obtained on the same day by venipuncture and non-traumatic lumbar puncture, respectively, and stored in aliquots at $-20^{\circ}$C, were selected for further studies. Clinical and laboratory data were obtained from the medical records. Nine patients were diagnosed with encephalitis, two with myelitis, and one with aseptic meningitis. Seven (four

*Correspondence: M Bendinelli
Received 23 October 1998; revised 2 December 1998; accepted 14 December 1998

males and three females, aged 44–84 years) were negative for HIV antibodies, while five (males, aged 32–68) were HIV-1 seropositive and, with one exception (patient 2554), had levels of HIV-1 viremia above the threshold of the branched DNA assay (Chiron Corporation, Emeryville, CA, USA): the average viral load was $1.8 \times 10^5$ copies/ml; range: $< 5 \times 10^2 - 5.2 \times 10^5$. None of the patients had received interferon-$\alpha$ or others treatments for HCV infection at the time of specimen collection. Routine microbiological tests and standard physiochemical examinations had been performed on the CSF. All had been found negative for bacteria, fungi, and protozoa. In addition, most CSF specimens had been tested for herpes viruses, enteroviruses, and JC virus by diagnostic PCR and also found negative.

HCV detection was carried out by nested RT–PCR using primers covering the highly conserved 5′ untranslated region of the viral genome as previously described (Vatteroni *et al*, 1994) to obtain a 250 bp fragment spanning from nucleotide −279 to −29 of the HCV genome. Each sample was tested in duplicate and scored as positive when the two results were concordant. In the case of discordant results, a new aliquot of the sample was re-examined in duplicate and the sample was scored as positive if at least one of the reactions was positive. In each assay, appropriate negative controls were included during the steps of RNA extraction and PCR amplification to rule out carry-over contamination. The specificity of the reaction was confirmed by examining 15 CSF samples from HCV-negative patients, which tested uniformly PCR negative. When used to detect HCV in serum, the lower limit of sensitivity of the assay was approximately $10^3$ genomes per ml, and this limit was not appreciably affected by the addition of selected CSF samples to test sera (results not shown).

As shown in Table 1, HCV RNA was detected in all the 12 sera and in ten CSF samples, of which five were from HIV-positive patients and five from HIV-negative patients. The genotype of HCV was determined by LiPA (Innogenetics, Zwijnaarde, Belgium), according to manufacturers' instructions. As expected, the viral genotype found in the CSF of all patients was the same as in serum; nine were type 1, two type 2, and one type 3, thus reflecting the distribution of HCV genotypes in sporadic cases of infection in Italy (Maggi *et al*, 1997a). The 12 sera and seven CSF samples, for which the amount was sufficient, were also quantitatively evaluated for HCV load by branched DNA assay (Quantiplex™ HCV 2.0, Chiron). As detailed in Table 1, with one exception, all the sera had measurable HCV RNA levels, with values that ranged between 0.28 and 13.1 MEq per ml and a mean value of 3.5 MEq per ml. In contrast, all of the CSF were below the lower limit of detection of the assay used (0.2 MEq/ml), thus showing that their HCV contents were markedly lower than in the respective sera. Thus, our results confirmed that HCV can be detected in CSF samples obtained from patients co-infected with HIV-1 and demonstrated that the virus is detectable also in CSF samples from patients singly infected with HCV.

In theory, possible sources for the detection of small copy numbers of HCV genomes in the CSF are specimen contamination by blood during collection, passive spill-over of virus from plasma into the CNS through the blood-brain barrier, transport into the CNS by infected PBMC, and local virus replication within the CNS (Morsica *et al*, 1997; McMinn, 1997). The only two HCV-negative CSF specimens were both from patients with low viremia levels, which may indicate that a high systemic viral burden is required for HCV to reach

**Table 1** HCV parameters in paired CSF-serum samples from 12 infected patients

| | | *Serum* | | *CSF* | |
|---|---|---|---|---|---|
| *Patients* | *Clinical diagnosis* | *Viral load (MEq/ml)* | *Genotype* | *HCV RNA* | *Viral load (MEq/ml)* |
| *HIV negative* | | | | | |
| 1313 | Myelitis | 0.35 | 1b | – | N.D. |
| 2100 | Myelitis | 1.08 | 2a/2c | + | <0.2 |
| 2675 | Encephalitis | 0.41 | 1b | + | <0.2 |
| 4923 | Encephalitis | 0.28 | 1 | – | N.D. |
| 5141 | Acute encephalitis | 1.73 | 1b | + | <0.2 |
| 6512 | Encephalitis | 6.68 | 2 | + | N.D. |
| 8852 | Encephalitis | 10.4 | 1b | + | <0.2 |
| *HIV positive* | | | | | |
| 101 | Encephalitis | 3.52 | 1b | + | <0.2 |
| 338 | Encephalitis<br>Neurotoxoplasmosis | <0.2 | 1b | + | N.D. |
| 2554 | Meningitis | 0.81 | 1b | + | <0.2 |
| 4845 | Encephalitis | 0.42 | 1b | + | <0.2 |
| 5968 | Encephalitis | 13.1 | 3a | + | N.D. |

N.D., not determined.

**Table 2** Relevant parameters in the 12 patients examined, grouped by detection of HCV RNA in the CSF

| Patients | WBC /mm$^3$ | RBC /mm$^3$ | CSF Brain-blood barrier impaired | CSF Intrathecal IgG production |
|---|---|---|---|---|
| *HCV-RNA negative* | | | | |
| 1313 | 6 | 320 | No | No |
| 4923 | 11 | 260 | Yes | No |
| *HCV-RNA positive* | | | | |
| 101* | 2 | Absent | Yes | Yes |
| 338 | 2 | 160 | No | Yes |
| 2100 | 54 | Absent | No | No |
| 2554 | 30 | 650 | Yes | Yes |
| 2675 | 17 | Absent | Yes | No |
| 4845 | 4 | 480 | Yes | No |
| 5141 | 2 | Absent | No | No |
| 5968 | 1 | Absent | No | Yes |
| 6512 | 2 | 21 | No | Yes |
| 8852 | 1 | Absent | No | No |

*Underlined, HIV positive patients.

the CNS but also that high viremia levels facilitate the contamination of CSF during lumbar puncture. RBC, an indicator of CSF contamination with blood during collection, were detected in 4/10 HCV RNA positive and in 2/2 HCV RNA negative CSF samples, at numbers ranging between 21 and 650 per $\mu$l (Table 2). This finding does not completely exclude that a slight contamination with HCV-circulating in blood had occurred during lumbar puncture but makes the possibility unlikely. That contamination by blood represents the only or major cause for the presence of HCV in CSF samples was also deemed unlikely in a previous study (Morsica *et al*, 1997). Among the ten HCV positive CSF, most had very low WBC counts, and only four and five showed changes indicative of impaired brain-blood barrier and intrathecal IgG production, respectively (Table 2). Although not an absolute criterion (Epstein, 1988), the absence of intrathecal antibody production argues against a significant replication of HCV within the CNS. Incidentally, intrathecal IgG production was present in 4/5 HIV-positive individuals *versus* 1/7 HIV-negative subjects, confirming that it is a frequent finding in HIV-associated neurological conditions (Conrad *et al*, 1995; Cinque *et al*, 1997; Gisslen *et al*, 1998).

Extensive data have shown that in individual hosts the quasispecies composition of viruses may vary significantly depending on the body district where it is analysed, and this is considered to stem from independent virus evolution in different cell types or body compartments (Sankale *et al*, 1995; Domingo and Holland, 1997; Wong *et al*, 1997). For example, we and others have recently shown that the spectrum of genetic variants of HCV found in the plasma, PBMC and liver of individual patients differs significantly and have also obtained evidence suggesting that this reflects a partial divergence of virus evolution in distinct host cells (Shimizu *et al*, 1997; Navas *et al*, 1998; Maggi *et al*, 1997b, 1999). In an attempt to shed light on the source of the virus present in CSF samples, we compared the quasispecies composition of the HCV found in the CSF and serum. The analysis was carried out in three patients (one of which HIV-1-positive) by investigating a region of the viral genome known to lend itself to intrapatient quasispecies analysis due to its high variability (Clarke, 1997). Briefly, a 307 bp fragment of the E2/NS1 region of the HCV genome, spanning nucleotides 956 to 1262 and covering the hypervariable domain-1, was amplified by nested RT–PCR and examined by single strand conformation polymorphism (SSCP) analysis using a recently published procedure (Maggi *et al*, 1997b) with slight modifications. The product of the second PCR was purified and submitted to SSCP analysis. To this purpose, a volume of the single-strand DNA products obtained by asymmetric PCR was mixed with two volumes of formamide loading dye and electrophoresed in 10% non-denaturing polyacrylamide gel at 23°C at 40 mA for 5–6 h. Finally, the gel was visualised using silver staining and SSCP bands were analysed with the GelBase Pro software program (UVP, Cambridge, England). As detailed elsewhere (Maggi *et al*, 1999), the method is highly reproducible and capable of detecting HCV se-

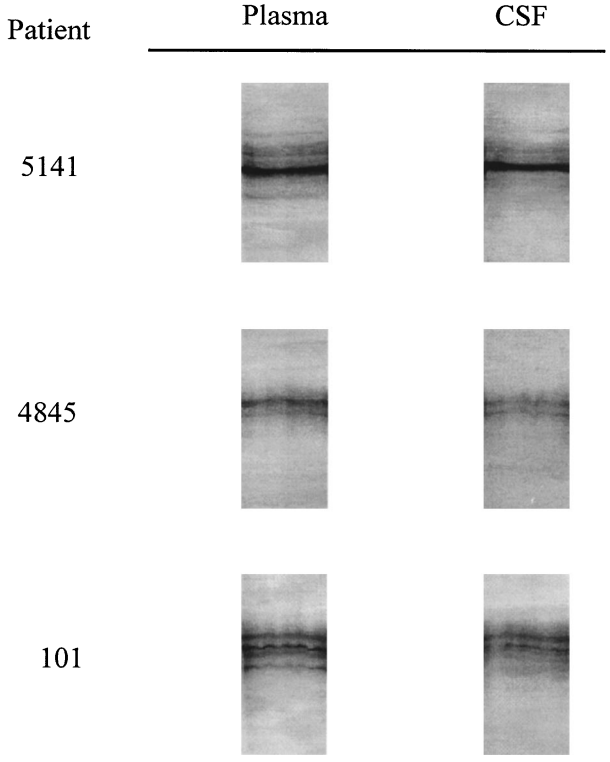


**Figure 1** SSCP analysis of the HCV genomes found in paired CSF-serum samples from three infected patients.

quences representing approximately 5% of the entire population of genetic variants.

As shown by Figure 1, multiple SSCP bands were observed in the CSF samples studied, thus demonstrating that HCV was present as a relatively heterogeneous population of genetic variants in the CSF as well as in serum samples (Martell *et al*, 1992; Maggi *et al*, 1997b). Moreover, in each of the patients examined the number, appearance and positions of the SSCP bands produced by the CSF were identical to those derived from the corresponding serum samples, thus showing no evidence of significant genetic diversity between the viral populations present in the two fluids and implying that most if not all of the HCV found in the CSF was of plasma origin and was not produced locally within the CNS.

In conclusion, we have shown that HCV can be present at small copy numbers in the CSF of infected individuals, independently on concomitant infection with HIV-1. Based on available evidence the most plausible explanation is that, at least under conditions of CNS stress, some of the HCV circulating in plasma can reach the CNS where, however, the virus does not seem to undergo significant local replication. Additional studies are needed to investigate how HCV may enter the CNS and what the clinical implications may be, if any (Dix *et al*, 1994).

## Acknowledgements

This work was supported in part by grants from the Ministero della Sanità and Ministero della Università e Ricerca Scientifica, Rome, Italy.

## References

Bolay H, Soylemezoglu F, Nurlu G, Tuncer S, Varli K (1996). PCR detected hepatitis C virus genome in the brain of a case with progressive encephalomyelitis with rigidity. *Clin Neurol Neurosurg* **98:** 305–308.

Caudai C, Maimone D, Almi P, Annunziata P, Bastianoni I, Boggiano CA, Guazzi GC, Padula M, Valensin PE (1997). The potential role of hepatitis C virus in the pathogenesis of the neurological syndrome in chronic hepatitis C. *Gut* **41:** 411–412.

Cinque P, Scarpellini P, Vago L, Linde A, Lazzarin A (1997). Diagnosis of central nervous system complications in HIV-infected patients: cerebrospinal fluid analysis by the polymerase chain reaction. *AIDS* **11:** 1–17.

Clarke B (1997). Molecular virology of hepatitis C virus. *J Gen Virol* **78:** 2397–2410.

Conrad AJ, Schmid P, Syndulko K, Singer EJ, Nagra RM, Russell JJ, Tourtellotte WW (1995). Quantifying HIV-1 RNA using the polymerase chain reaction on cerebrospinal fluid and serum of seropositive individuals with and without neurologic abnormalities. *J Acquir Immune Defic Syndr Hum Retrovirol* **10:** 425–435.

Cribier B, Schmitt C, Bingen A, Kirn A, Keller F (1995). In vitro infection of peripheral blood mononuclear cells by hepatitis C virus. *J Gen Virol* **76:** 2485–2491.

Czaja AJ (1997). Extrahepatic immunologic features of chronic viral hepatitis. *Dig Dis* **15:** 125–144.

Dix RD, McCarthy M, Berger JR (1994). Diagnostic value for culture of cerebrospinal fluid from HIV-1 infected individuals for opportunistic viruses: a prospective study. *AIDS* **8:** 307–312.

Domingo E, Holland JJ (1997). RNA virus mutations and fitness for survival. *Annu Rev Microbiol* **51:** 151–178.

Epstein LG (1988). Human immunodeficiency virus (HIV) brain infection in infants and children. In: *Virus infections and the developing nervous system.* Johnson RT, Lyon G, (eds). Kluwer Academic Publishers: Boston. pp 57–67.

Gisslen M, Hagberg L, Fuchs D, Norkrans G, Svennerholm B (1998). Cerebrospinal fluid viral load in HIV-1-infected patients without antiretroviral treatment. *J Acquir Immune Defic Syndr Hum Retrovirol* **17:** 291–295.

Ito T, Mukaigawa J, Zuo J, Hirabayashi Y, Mitamura K, Yasui K (1996). Cultivation of hepatitis C virus in primary hepatocyte culture from patients with chronic hepatitis C results in release of high titer infectious virus. *J Gen Virol* **77:** 1043–1054.

Kashihara K, Terai T, Shomori T (1995). Relapsing neuropathy associated with hepatitis C virus infection. *Intern Med* **34:** 265–266.

Lerat H, Berby F, Trabaud MA, Vidalin O, Major M, Trepo C, Inchauspe G (1996). Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. *J Clin Invest* **97:** 845–851.

Maggi F, Vatteroni ML, Fornai C, Morrica A, Giorgi M, Bendinelli M, Pistello M (1997a). Hepatitis C virus subtype C is highly prevalent in Italy and is heterogeneous in the NS5A region. *J Clin Microbiol* **35:** 161–164.

Maggi F, Fornai C, Vatteroni ML, Giorgi M, Morrica A, Pistello M, Cammarota G, Marchi S, Ciccorossi P, Bionda A, Bendinelli M (1997b). Differences in hepatitis C virus quasispecies composition between liver, peripheral blood mononuclear cells and plasma. *J Gen Virol* **78:** 1521–1525.

Maggi F, Fornai C, Morrica A, Vatteroni ML, Giorgi M, Marchi S, Ciccorossi P, Bendinelli M, Pistello M (1999). Divergent evolution of hepatitis C virus in the liver and peripheral blood mononuclear cells of infected patients. *J Med Virol* **57:** 57–63.

Martell M, Seteban JI, Genesca J, Weiner A, Esteban R, Guardia J, Gomez J (1992). Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. *J Virol* **66:** 3225–3229.

McMinn PC (1997). The molecular basis of virulence of the encephalitogenic flaviviruses. *J Gen Virol* **78:** 2711–2722.

Morsica G, Berardi MT, Novati R, Uberti Foppa C, Castagna A, Lazzarin A (1997). Detection of hepatitis C virus genomic sequences in the cerebrospinal fluid of HIV-infected patients. *J Med Virol* **53:** 252–254.

Nakajima N, Hijikata M, Yoshikura H, Shimizu YK (1996). Characterization of long-term cultures of hepatitis C virus. *J Virol* **70:** 3325–3329.

Navas S, Martin J, Quiroga JA, Castillo I, Carreno V (1998). Genetic diversity and tissue compartmentalisation of the hepatitis C virus genome in blood mononuclear cells, liver and serum from chronic hepatitis C patients. *J Virol* **72:** 1640–1646.

Propst T, Propst A, Nachbauer K, Graziadei I, Willeit H, Margreiter R, Votel W (1997). Papillitis and vasculitis of the arteria spinalis anterior as complications of hepatitis C reinfection after liver transplantation. *Transpl Int* **10:** 234–237.

Sankale JL, Sallier de La Tour R, Marlink RG, Scheib R, Mboup S, Essex ME, Kanki PJ (1995). Distinct quasispecies in the blood and the brain of an HIV-2-infected individual. *Virology* **228:** 418–423.

Seipp S, Mueller HM, Pfaff E, Stremmel W, Theilmann L, Goeser T (1997). Establishment of persistent hepatitis C virus infection and replication in vitro. *J Gen Virol* **78:** 2467–2476.

Shimizu YK, Igarashi H, Kanematu T, Fujiwara K, Wong DC, Purcell RH, Yoshikira H (1997). Sequence analysis of the hepatitis C virus genome recovered from serum, liver, and peripheral blood mononuclear cells of infected chimpanzees. *J Virol* **71:** 5769–5773.

Vatteroni ML, Pistello M, Maggi F, Cecconi N, Panicucci F, Bendinelli M (1994). Hepatitis C virus serological and polymerase chain reactions in human immunodeficiency virus-positive and negative patients. *Clin Diag Virol* **2:** 7–16.

Wong JK, Ignacio CC, Torriani F, Havlir D, Fitch NJ, Richman DD (1997). In vivo compartmentalization of human immunodeficiency virus: evidence from the examination of *pol* sequences from autopsy tissues. *J Virol* **71:** 2059–2071.