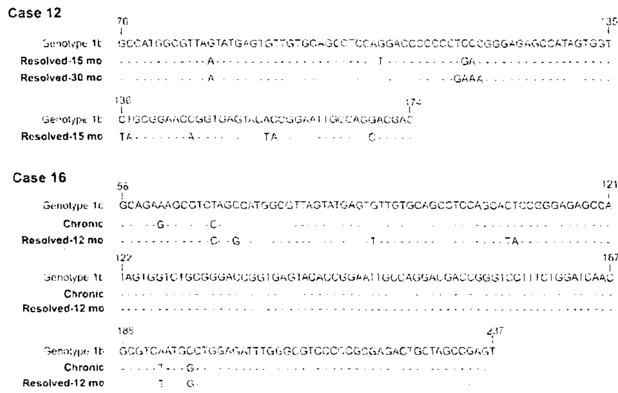

FIG. 4. Nucleotide sequence alignment of the 5′-UTR fragments of HCV RNA amplified from serum or PBMC of individuals with apparently complete serological resolution of chronic hepatitis C following antiviral therapy. Samples from case 12 were obtained 15 (PBMC) or 30 months (serum) after sustained response to treatment. In case 16, the first sample was taken during symptomatic chronic hepatitis (Chronic) and the second 12 months after evidence of sustained clinical resolution of hepatitis. The sequences obtained were aligned with the prototype HCV genotype 1b (10). Nucleotides in the sequences from the patients' samples identical to those in the HCV genotype reference (top line) are shown as dashes, and differences are identified by letters.

negative strand, which is considered to be a reliable indicator of actually progressing replication, was quantified. Since this replication intermediate normally occurs at frequencies lower than those of the positive strand, the replicative strand was examined only in PBMC which were found reactive for the positive strand. Figure 6 illustrates the detection of the HCV RNA negative strand in IL-2- and PHA-treated PBMC isolated from patient 4 with a 12-month follow-up after spontaneous recovery and from four individuals with follow-up between 12 and 60 months after apparently complete SVR due to antiviral treatment (Table 1). Overall, this form of RNA was detected in 9 (75%) out of 12 PBMC samples investigated. The expression of the replicative strand varied between 30 and $3 \times 10^3$ vge/μg of total cellular RNA, as estimated by quantification of hybridization signals. It is of note that culture of PBMC with IL-2 and PHA enhanced expression of the HCV RNA negative strand by approximately twofold (Fig. 6).

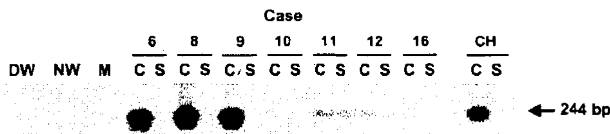


FIG. 5. Expression of the HCV RNA positive strand in monocyte-derived DC from individuals with long-term recovery from chronic hepatitis C. Cultured DC (C) and concentrates of their culture supernatant (S) were tested for HCV RNA by nested RT-PCR–NAH. Three micrograms of DC RNA and all RNA extracted from the pellet obtained after ultracentrifugation of 10 ml of DC culture supernatant were used for analysis. Water (DW and NW) instead of cDNA and mock (M) sample treated as a test RNA sample served as negative controls, while DC and their culture supernatant derived from a patient with chronic hepatitis C (CH) were used as positive controls. Hybridization signals showed 244-bp bands.

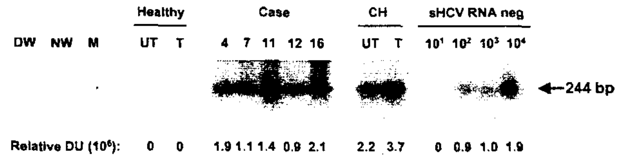


FIG. 6. Detection of the HCV RNA negative strand in PBMC from individuals with clinical and apparently complete virological resolution of hepatitis C. Total RNA was extracted from IL-2- and PHA-stimulated PBMC isolated from individuals with self-limited (case 4) or therapeutically induced (cases 7, 11, 12, and 16) recovery from hepatitis and from control untreated (UT) and IL-2- and PHA-treated (T) PBMC obtained from a healthy donor and a patient with actively progressing chronic hepatitis C (CH). RNA was reverse transcribed and cDNA was amplified by PCR with HCV RNA negative strand-specific 5′-UTR primers. Serial dilutions of a synthetic HCV RNA negative strand (sHCV RNA neg) amplified in parallel were used as semiquantitative standards. Water (DW and NW) and mock (M) samples were used as negative and contamination controls. The positive signals (244-bp fragments) were visualized by hybridization to the recombinant HCV UTR-E2 probe. Values under the panel represent relative densitometric units (DU) given by positive hybridization signals.

## DISCUSSION

In the present study, by applying a highly sensitive HCV RNA detection assay, we show, for the first time, evidence of the long-term persistence of HCV genomes in sera and circulating lymphoid cells in individuals considered to be clinically and serologically cleared of HCV infection. Serum samples of these persons have been repeatedly HCV RNA nonreactive by a standard laboratory assay during follow-up between 12 and 60 months after normalization of liver function tests and apparently complete spontaneous or antiviral treatment-induced sustained virus clearance. However, as the enhanced sensitivity of our assay showed, all of the patients in fact continued to carry low levels of HCV RNA in serum and/or PBMC. The RT-PCR–NAH applied in this study was at least 10-fold more sensitive than the current laboratory tests. The enhanced level of HCV RNA detection was achieved, among other factors, by using a greater amount of RNA for testing, following a classical RNA extraction method, the employment of two rounds of PCR amplification, and the use of hybridization analysis to further increase the sensitivity and specificity of virus identification. The nucleotide sequence analysis of the HCV 5′-UTR confirmed the specificity of the HCV genome detection and revealed that different virus genotypes were associated with the occult long-term carriage of HCV genomes.

Analysis of PBMC, which were collected in parallel with test sera and routinely treated with IL-2 and PHA prior to RNA extraction, gave a significant diagnostic advantage by allowing the identification of HCV genomes in the sera of recovered persons who were HCV RNA nonreactive (i.e., cases 4 and 12; Table 1) and by confirming positive results obtained by serum testing. However, most importantly, the availability of these cells permitted examination of the negative strand of virus RNA, providing an insight into the replication capability of the HCV genome carried by lymphoid cells in the recovered individuals. This analysis showed that trace virus replication persisted in the lymphatic system in the majority (75%) of persons from whom sufficient numbers of PBMC were available for

investigation. This finding is not surprising, considering that several laboratories have shown the presence of the HCV RNA replicative strand in peripheral and organ lymphoid cells of patients with chronic hepatitis C (7, 17, 23, 37), as we also found in this study (Fig. 6). The present data clearly indicate that carriage of HCV in peripheral lymphoid cells is not terminated at the time of clinical resolution of chronic hepatitis C, but rather it subsides to a level that is not readily detectable by the currently used laboratory assays, and the virus genome persists at these low levels for a long time after apparently complete recovery.

In the present study, culture of lymphoid cells with IL-2 and PHA markedly enhanced detection of both the HCV RNA positive and replicative strands. PHA is a potent nonspecific inducer of T-cell proliferation, whereas IL-2 is a cytokine important for T-cell growth and in vitro survival (12). In this context, it has been shown that T-cell (11, 25, 33) as well as B-cell (23) lines can be infected in culture by HCV, although the infection was usually inefficient and transient. Furthermore, the most recent and unambiguous study documented that HCV infects B cells and that these cells are capable of producing infectious HCV virions (35). Although the molecular mechanism of mitogen-induced upregulation of HCV replication and genome expression in lymphoid cells is unknown and will require separate studies, this finding is not unique. Similar observations have been made in infections with other viruses which display a tropism for lymphoid cells, including serologically occult infection caused by woodchuck hepatitis virus that invariably involves the host's lymphatic system (3, 20). The strategy of enhancing HCV genome detection in PBMC established in this study provides a valuable aid to the diagnosis of serologically silent HCV persistence. Subsequent work should include a definition of culture conditions under which an increased HCV genome expression can be achieved in both T and B cells. This might further enhance the identification of HCV genomes in situations where low virus loads are expected and where sera and resting lymphoid cells remain seemingly virus nonreactive.

In light of recent findings demonstrating that monocyte-derived DC from patients with chronic hepatitis C might be a reservoir of replicating HCV (2, 7), we assessed whether DC obtained from the recovered individuals by a similar procedure also carry the virus. Here, we provide molecular evidence for the presence of HCV RNA in DC from six of the seven cases examined (Table 1 and Fig. 3). Although the amount of the genomic material was not sufficient to determine the presence of the HCV replicative strand, the absence of a viral RNA signal in the concentrated DC culture supernatant, except for that of a single specimen (case 11), strongly suggests that the HCV RNA detected originated from the cells but not from a culture aliquot. The allostimulatory function and maturation of DC in individuals with resolved hepatitis C do not seem to be noticeably affected (1, 2). However, the persistence of trace amounts of the virus in DC, which are essential for T-cell activation, may play an important role by providing a sustained stimulus to HCV-specific T cells. Consistent with this interpretation is the existence of strong HCV-specific cytotoxic T- and Th-lymphocyte responses for many years after resolution of hepatitis C (5, 14, 36).

The present study shows that HCV RNA can persist, albeit at very low levels, in the serum and circulating lymphoid cells for years after apparently complete clinical and virological resolution of chronic hepatitis C. If the present findings reflect the existence of the biologically competent, infectious virus, this silent persistence may have important epidemiological and pathogenic implications. Among other factors, these trace amounts of the virus may lead to reactivation of hepatitis C after termination of antiviral therapy or due to severe immunosuppression and may support perpetuation on the subclinical level of liver disease which becomes symptomatic years after exposure to the virus. They may also constitute a source from which HCV may spread through blood and organ donations to susceptible individuals.

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# HIV and hepatitis C virus RNA in seronegative organ and tissue donors

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**The objective of our study was to determine whether nucleic acid testing could detect HIV RNA or hepatitis C virus (HCV) RNA in a large series of seronegative organ and tissue donors, and whether this technique should be routinely used to improve viral safety of grafts. We studied 2236 organ donors, 636 tissue donors, and 177 cornea donors. We identified five HCV RNA-positive donors in 2119 HCV-seronegative organ donors, and one HCV RNA-positive donor in 631 HCV-seronegative tissue donors. No HIV-seronegative, HIV RNA-positive donor was identified. Our data suggest that routine nucleic acid testing of organ and tissue donors might increase viral safety in transplantation.**

Modern societies regard the viral safety of medical procedures as a requisite. Nucleic acid testing (NAT) has been introduced for the detection of HIV RNA and hepatitis C virus (HCV) RNA in pooled blood donations. Before its implementation, the procedure was criticised for not being cost-effective; however, a substantial number of viral transmissions have been avoided.[1] Transplantation of an organ or tissue from a dead or living donor exposes the recipient to viral transmission.[2] Organ donors are at a heightened risk of HIV and HCV infection compared with the general population.[3] A report describes an HCV-seronegative, brain-dead, heart-beating donor who infected at least eight of 40 recipients (including three organ and five tissue recipients) with HCV.[4] On retesting, the donor was confirmed to be anti-HCV-antibody negative, but was HCV RNA-positive.[4] This report lends support to the potential use of NAT in the selection of organ and tissue donors.

We studied serum samples from 3049 consecutive organ and tissue donors, including 2236 brain-dead, heart-beating organ donors, 636 living tissue-donors, and 177 dead cornea-donors. The samples were prospectively obtained, frozen, and stored at –80°C, and were retrospectively tested for HIV and HCV antibodies and RNA. HIV and HCV RNA were initially tested for by a duplex transcription-mediated amplification (TMA)-based assay (Chiron Procleix HIV-1/HCV Assay System, Chiron-Blood Testing, Emeryville, CA), with lower limits of detection (LLD) of 50 IU HCV RNA per mL and 100 HIV RNA copies per mL. All samples that were positive in the duplex TMA assay were retested to confirm the presence of HIV-1 or HCV RNA with a qualitative in-house HIV RNA real-time PCR assay (LLD 200 copies per mL) and a standardised qualitative HCV RNA PCR assay (Amplicor HCV version 2·0, Roche Molecular Systems, Pleasonton, CA; LLD 50 IU per mL). All samples were also retested with the most recent generations of anti-HIV-1, anti-HIV-2, HIV p24, and anti-HCV assays. The donors (or their families in the case of dead donors) gave their informed oral consent to organ or tissue sampling, and to blood sampling for complete virological testing. Under French law, ethics committee approval was not necessary.

Table 1 shows the prevalence of anti-HIV and anti-HCV antibodies in the 3049 organ and tissue donors. In total, 129 samples were reactive in the duplex TMA assay, including 121 of 2236 organ donors (5·4%), four of 636 tissue donors (0·6%), and four of 177 cornea donors (2·3%). 77 TMA results (2·5%) were invalid: 65 could be retested by TMA, of which 62 were non-reactive, one was reactive, and two were again invalid.

25 seronegative organ donors were initially TMA-reactive (table 2), of which five were confirmed to be HCV RNA-positive by repeat PCR. The organs of three of the five were not used for non-virological reasons; the liver and a kidney of one donor and both kidneys of the other donor were transplanted. No information on possible HCV transmission to the recipients was available. None of the 18 seronegative organ donors with enough serum available to be retested were confirmed as positive for HIV RNA or p24 antigen. Four seronegative tissue donors were TMA-reactive (table 2). The presence of HCV RNA was confirmed by repeat PCR in one of them, whereas the presence of HIV RNA was not confirmed in any. The HCV RNA-positive tissue donor was retested a year after donation and found to be anti-HCV antibody-negative and HCV RNA-negative in the same assays, suggesting transient viraemia without seroconversion. The tissue recipient refused to be tested. Finally, one seronegative cornea donor was TMA-reactive (table 2), but the presence of HIV RNA and HCV RNA could not be confirmed by PCR because of the presence of inhibitors in the sample. Overall, we identified five HCV RNA-positive donors among

| | Total number | Anti-HIV antibody-positive | Anti-HCV antibody-positive | Anti-HCV and anti-HIV antibody-negative |
|---|---|---|---|---|
| Organ donors | 2236 | 21 (0·9%) | 99 (4·4%) | 2140 (95·7%) |
| Tissue donors | 636 | 2 (0·3%) | 3 (0·5%) | 631 (99·2%) |
| Cornea donors | 177 | 22 (12·4%)* | 6 (3·4%)* | 150 (84·7%) |

*The high number of positive antibody results in cornea donors was partly due to false-positives resulting from poor-quality post-mortem samples (haemolysed or icteric).

**Table 1: Prevalence of anti-HIV and anti-HCV antibodies in the 3049 donors of organs, tissues, or corneas**

| | Total number | No detectable HIV or HCV antibodies | HIV antibody-positive | HCV antibody-positive | HIV and HCV antibody-positive |
|---|---|---|---|---|---|
| **Organ donors** | | | | | |
| TMA-reactive | 121 | 25 (1·1%) | 15 (0·7%) | 78 (3·5%) | 3 (0·1%) |
| TMA-negative | 2215 | 2094 (93·7%) | 3 (0·1%) | 18 (0·8%) | 0 |
| Total | 2236 | 2219 (94·8%) | 18 (0·8%) | 96 (4·3%) | 3 (0·1%) |
| **Tissue donors** | | | | | |
| TMA-reactive | 4 | 4 (0·6%) | 0 | 0 | 0 |
| TMA-negative | 632 | 627 (98·6%) | 2 (0·3%) | 3 (0·5%) | 0 |
| Total | 636 | 631 (99·2%) | 2 (0·3%) | 3 (0·5%) | 0 |
| **Cornea donors** | | | | | |
| TMA-reactive | 4 | 1 (0·6%) | 0 | 3 (1·7%) | 0 |
| TMA-negative | 173 | 149 (84·2%) | 21 (11·9%) | 2 (1·1%) | 1 (0·6%) |
| Total | 177 | 150 (84·8%) | 21 (11·9%) | 5 (2·8%) | 1 (0·6%) |

**Table 2: Prevalence of anti-HIV and anti-HCV antibodies in TMA-reactive and TMA-negative donors of organs, tissues, or corneas**

2119 HCV-seronegative organ donors, and one HCV RNA-positive donor among 631 HCV-seronegative tissue donors. We identified no HIV-seronegative donors who were HIV RNA-positive. However, the 95% CI of prevalence in the organ-donor population was 0·00–0·17%.

The clinical significance of seronegative HCV viraemia and the infectivity of the corresponding grafts need to be determined. Nevertheless, our data, together with the reported cases of HCV transmission to recipients from a seronegative HCV RNA-positive donor,[4] suggest that routine NAT screening of organ and tissue donors might increase viral safety in the transplantation setting. Cost-effectiveness could be even higher than in the blood transfusion setting, with the high exposure rate of organ donors[3] and the poor outcome of transmitted viral disease in immunodepressed patients. Implementation of systematic NAT screening of tissue (and cell) donors is highly feasible because viral testing can be done every day and can be based on standardised, partly automated, commercial techniques and procedures.[5] The principal issue here is cost. By contrast, systematic NAT screening in the organ transplantation setting faces major technical challenges, although it is here that this measure would probably be most effective in prevention of viral transmission. Indeed, no current standardised molecular biology technique can be done in an emergency—ie, within 3–4 h after sampling—on an individual basis. Additionally, the high rate of retrospective non-confirmation of initial RNA reactivity noted in this study makes routine use difficult, with the current organ shortage.

We have shown that routine NAT screening of organ and tissue donors can identify potentially infectious seronegative donors. Technical flaws currently hinder routine NAT screening of organ donors, but not of tissue and cell donors. Thus NAT techniques should be rapidly adapted to organ donor screening, and implementation should be discussed.

#### Contributors
D Challine designed the study, analysed the results, and wrote the article. B Pellegrin developed the HIV RNA real-time PCR assay and ran the HCV and HIV RNA PCR confirmatory assays. M Bouvier-Alias was responsible for serological testing and analysis of their results. P Rigot and L Laperche ran the TMA assays. J-M Pawlotsky designed the study, participated in data analysis, and oversaw the writing of the article.

#### Conflict of interest statement
None of the authors has a conflict of interest that could inappropriately bias the work.

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