Detection of Hepatitis C Virus RNA in Peripheral Blood Mononuclear Cells of Infected Patients by In Situ Hybridization

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We used in situ hybridization to detect hepatitis C virus (HCV) infection of peripheral blood mononuclear cells (PBMC) from 11 patients with chronic active hepatitis. Using 35S-labeled HCV-RNA probe, HCV-RNA-positive and -negative strands were observed in unstimulated PBMC from three patients, all of whom were receiving immunosuppressive drugs after orthotopic liver transplantation (OLT). HCV-RNA sequences were also identified in PBMC from three patients who were not undergoing immunosuppression, after stimulation with either phytohemagglutinin (PHA) or pokeweed mitogen (PWM). In contrast, HCV-RNA was not found in the remaining five patients, who had not undergone OLT and whose cells were not stimulated with mitogens. These results show that mononuclear cells can be infected by HCV and that mitogenic stimulation of infected cells increases HCV-RNA replication.

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HEPATITIS C VIRUS (HCV) is a small RNA virus responsible for the vast majority of posttransfusional and sporadic cases of non-A, non-B hepatitis. Its structure and pathogenicity are still under investigation. Infection becomes chronic in more than 50% of cases, but the mechanism of viral persistence and the cellular tropism of HCV are still unclear and there is no cell culture system available.

There is also little information on the involvement of peripheral blood mononuclear cells (PBMC) during HCV infection, but it has recently been shown that HCV-RNA can be detected by polymerase chain reaction (PCR) in PBMC, although contamination by serum particles during cell isolation cannot be completely ruled out.

Studies by our group and others clearly indicate, that in the infected liver tissue, HCV-RNA-positive and -negative strands can be detected by in situ hybridization in scattered hepatocytes. We have also detected HCV-RNA in cells present in inflammatory infiltrates, strongly suggesting the involvement of mononuclear cells.

To assess definitely whether HCV indeed infects PBMC, we used in situ hybridization with 35S-labeled HCV-RNA probes to test blood samples from 11 patients with chronic active hepatitis C.

MATERIALS AND METHODS

Patients. Blood samples were obtained from 11 patients (six women and five men; mean age, 46 years) with histologically confirmed chronic active hepatitis (Table 1). All patients were anti-HCV-positive (recombinant immunoassay 2; Ortho Diagnostic Systems, California), hepatitis B surface antigen (HBsAg)- and human immunodeficiency virus (HIV)-negative, and had elevated ALT values (1.5 to five times upper limit of normal). Three patients (no. 6, 7, and 8) had undergone liver transplantation (21, 15, and 17 months previously).

Controls. PBMC from four healthy, anti-HCV-negative blood donors served as negative controls. To check the specificity of the HCV probes, we used HCV-PCR-positive and -negative liver tissue sections sections placed on the same slides.

Preparation of cells. PBMC were prepared from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (Ficoll-Hypaque, Pharmacia, Uppsala, Sweden). The PBMC pellet was washed three times by centrifugation in RPMI 1640 medium (Seromed, Berlin, Germany) and the cells were counted in a hemacytometer.

In cases no. 1 through 5 (Table 1), we separated T lymphocytes from other white blood cells (B lymphocytes, monocytes, and polymorphonuclear cells) by rosette formation. PBMC at a density of 10 × 10^6/mL were incubated overnight with neuraminidase-treated sheep red blood cells and complement-free fetal calf serum. The cells were then separated by Ficoll-Hypaque density gradient centrifugation, washed three times, and resuspended in RPMI. The degree of separation was determined by fluorescence activated cell sorter analysis (FACScan, Becton Dickinson) using monoclonal antibodies (CD3, CD16, CD19; Immunotech SA, France). The cells (0.5 to 0.7 × 10^6/mL) were then attached to gelatin-prepared slides by cytocentrifugation. After air-drying, the cells (~10,000 per slide) were fixed with 4% paraformaldehyde (PFA) for 10 minutes, washed three times in phosphate-buffered saline (PBS), and dehydrated through a graded series of ethanol solutions (30% to 100%). The slides were stored at ~20°C.

As patients no. 6 through 8 (Table 1) had marked lymphocytopenia, total PBMC were resuspended at a density of 0.5 to 0.7 × 10^6/mL in RPMI for cytocentrifugation and fixation as described earlier. In cases no. 9 through 11 (Table 1) total PBMC were cultured with phytohemagglutinin (PHA; 4 μg/10^6 cells/mL) and pokeweed mitogen (PWM; 50 μL/10^6 cells/mL; Gibco, Grand Island, NY). PHA essentially stimulates T lymphocytes, while PWM essentially stimulates B lymphocytes. After 48 hours of incubation, the cells were pelleted, washed, and resuspended at a density 0.5 to 0.7 × 10^6/mL in RPMI for cytocentrifugation and fixation.

Preparation of probes. We used cDNA corresponding to the 5′ noncoding region of HCV-RNA (259 bp), which is highly conserved among different viral isolates. This cDNA was subcloned into the Bluescript SK plasmid (Stratagene, San Diego, CA). Antisense RNA was transcribed by T7 RNA polymerase (Pharmacia) after plasmid linearization with BamHI (Pharmacia). To prepare the sense probe, we used T7 RNA polymerase (Pharmacia) after linearization with EcoRI (Boehringer, Mannheim, Germany). The Bluescript vector without cDNA was used as a negative control. High-specific activity RNA probes were synthesized using 35S uridine triphosphate (U-370, CHU Necker, Paris; the Laboratoire de Bacteriologie Virologie, Hopital Avicenne, Bobigny, France; the Unité d'Hépatologie, Hopital Laennec, Paris; and Hybridotest, Institut Pasteur, Paris, France)

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269

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phosphate (UTP; specific activity, >1,000 Ci/mmol; Amersham, UK) according to the supplier’s recommendations. Probe length was reduced to a mean size of approximately 50 nucleotides by alka- lali hydrolysis, and unincorporated nucleotides were removed by gel filtration on G75 sephadex. After precipitation, the probe was redissolved in a hybridization buffer (50% deionized formamide, 0.3 mol/L NaCl, 20 mmol/L Tris-HCl, pH 7.4, 5 mmol/L EDTA, 10 mmol/L NaPO₄, pH 8.10% dextran sulfate, 1× Denhardt’s solution, and 50 μg/mL total yeast RNA) at a final concentration of 500,000 cpm/20 μL and stored at -20°C.

**In situ hybridization.** The slides were rinsed in a 0.85% saline solution for 5 minutes and postfixed in freshly prepared 4% PFA for 20 minutes. They were then rinsed twice in PBS for 5 minutes, and the cells digested with proteinase K (Merck, France; 10 μg/mL for 7 minutes). The slides were rinsed in PBS for 5 minutes, refixed in 4% PFA for 5 minutes, dipped in distilled water, and acetylated in a 0.09-mol/L solution of triethanolamine with acetic anhydride (1/40 vol/vol, 10 minutes). They were then rinsed in PBS and 0.85% saline for 5 minutes each, dehydrated through a series of ethanol (30% to 100%), and allowed to dry for at least 2 hours before hy- bridization.

The probe was denatured for 2 minutes at 80°C, then applied to the slides, which were covered with a siliconized coverslip. Hybrid- ization was performed at 52°C for 16 hours in a humid chamber. The coverslips were gently floated off in a 5× SSC buffer (1× SSC = 0.15 mol/L NaCl, 0.015 mol/L sodium citrate) and 10 mmol/L dithi- otreitol at 42°C followed by a stringent wash at 42°C, 37°C, and 30°C. After washes at 42°C in 2× SSC, 0.5× SSC, and 0.5× SSC (15 minutes each), the slides were dehydrated in a series of ethanol containing 0.3 mol/L of ammonium acetate. The slides were exposed for 10 to 15 days at 4°C using Kodak NTB2 emulsion (Eastman Kodak, Rochester, NY). The slides were counterstained with Harris hematoxylin.

**RESULTS**

We have examined PBMCN of 11 patients with chronic active hepatitis C (Table 1). For three patients, the samples were obtained during the follow-up after orthotopic liver transplantation (OLT).

All 11 PBMCN samples were first examined without mi- togenic cell stimulation. HCV-RNA molecules were found in three samples (Table 2), corresponding to the OLT recipients. An antisense probe that detects positive HCV-RNA strands gave signals in all three of these cases (Fig 1A and B), while an HCV-RNA sense probe that detects negative strands gave signals in two cases (Fig 1C and D). PBMCN from three of the negative patients were also examined after stimulation with PHA and PWM; positive HCV-RNA strands were found in all three, and negative HCV-RNA strands in two, regardless of the mitogen used. No signals were detected in PBMCN from the remaining five patients (Table 1, patients no. 1 through 5), who had not undergone liver transplantation and whose cells were not exposed to mitogen.

The proportion of positively labeled cells approximated 0.01% to 0.03%, although it reached 0.3% in one case with the antisense probe after stimulation with PHA (Table 2, patient no. 11; Fig 2A through E). There was also a slightly but not significantly higher proportion of labeled cells after mitogenic stimulation with PHA than with PWM. As labeled cells were usually entirely covered by silver grains, it was impossible to distinguish between nuclear and/or cytoplasmic labeling (Fig 2E), or to compare the labeling intensity with the antisense and sense probes; however, there was an apparent difference in favor of the antisense probe.

To check the specificity of the signals obtained in our experimental conditions, several controls were used. PBMCN from four healthy anti-HCV–negative blood donors showed no labeling when hybridized with the HCV-RNA antisense or sense probes. To two of them were also studied after stimulation with PHA and with PWM. These two samples showed no labeling with HCV-RNA antisense or sense probes (Fig 2F).

Similarly, a cDNA-free 35S-labeled Bluescript SK plasmid probe, also used as a negative control, showed no hy- bridization with any of the 11 PBMCN samples (Fig 1E and F). Liver tissue sections from HCV-positive patients containing HCV-RNAs detected by PCR were used as positive controls. Using both HCV antisense and sense probes, spe-
cific signals were observed in scattered hepatocytes and in cells of the inflammatory infiltrates, whereas negative control tissue was unlabeled. We only detected positive signals with unstimulated PBMNC from the three OLT recipients (Table 2), probably because of the drug-induced immunosuppression in these patients. Interestingly, HCV-RNAs were previously found either in PBMNC by means of PCR, or in inflammatory infiltrates of the liver by in situ hybridization, only in HCV-positive patients with immunodeficiency caused by HIV coinfection, a clinical situation with increased HIV viremia.

We have clearly demonstrated that HCV-RNA sequences can be detected in PBMNC from patients on immunosuppressive therapy (ie, after OLT), and after in vitro mitogenic stimulation. Based on PCR results, it has been previously suggested that HCV infects PBMNC, but in these experiments, contamination by infected serum particles during cell preparation could not be ruled out completely. Although one sample contained 0.3% of labeled cells after PHA stimulation, with the HCV antisense probe, labeled cells were generally present at very low frequency (0.01% to 0.03%). This range of values is in keeping with that reported in PBMNC from HIV-infected patients using HIV-RNA probe (0.001% to 0.01%). Similarly, less than 0.03% of PBMNC from calves infected with bovine immunodeficiency-like virus contain the viral RNA. In contrast, using in situ hybridization, 1% to 10% of mononuclear cells from hepatitis B virus (HBV)-infected patients were found to contain HBV-DNA sequences. Using PCR, it has been shown in the liver and in the serum that the number of negative strands is usu-
ally 10 to 100 times lower than the number of positive strands. In the present study, we found only a slight difference in the density of silver grains with the sense and antisense probes, but it was in favor of the antisense probe (which detects positively stranded HCV-RNA molecules).

We also found a slightly higher proportion of labeled cells after stimulation with PHA than with PWM (0.03% and 0.01%, respectively), but were unable to identify clearly which subpopulations of PBMC were infected by HCV. This was due to the low number of cells disposed to perform the experiments. This question could be investigated further by means of in situ PCR combined with FACS selection after antibody labeling. However, the fact that cells were labeled after stimulation with PHA and PWM suggests that T and B lymphocytes are both infected. This can be correlated with data presented recently by Zignego et al, who found that HCV-RNAs were detected only in T and B lymphocytes in some cases by means of PCR, but not in the monocyte macrophage fraction. Our results are also consistent with the recent demonstration that the human lymphocytic cell line MOLT4 can be infected by HCV. However, it is noteworthy that a recent report suggested that the HCV capsid antigen might be detected in some HCV-positive patients in the circulating monocytes.

In conclusion, we provide morphological evidence of infection by HCV of circulating mononuclear cells. Similar observations have been made with related viruses such as the Flaviviruses and the Pestiviruses (Thiel Heinz-Jürgen, personal communication, 1993). HCV infection of PBMC might lead to the selection of variants, as is the case with lymphocytic choriomeningitis virus. Moreover, HCV-infected cells may become functionally defective and thus lead to viral persistence and chronic infection. Finally, infected PBMC may serve as an HCV reservoir, causing reinfection of liver graft after OLT.

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