

Detection of neutralizing antibodies to hepatitis C virus using a biliary cell infection model

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The identification and characterization of neutralizing anti-hepatitis C virus (HCV) antibodies may have a major impact on understanding HCV pathogenesis. However, to date, their detection has only been based on the inhibition of either the E2 envelope protein or HCV virions binding to different target cells. The permissivity of primary biliary cells for HCV infection has been demonstrated previously. In the present report, infection of biliary cells was demonstrated further by combining PCR and immunohistochemical detection of the HCV core protein. This study demonstrates, using both serum and purified IgG, the presence of neutralizing anti-HCV antibodies in the serum of patients showing long-term response to antiviral therapy. Overall, the usefulness of the primary biliary cell infection model to investigate anti-HCV neutralization is shown.

Hepatitis C virus (HCV) is a major risk factor for cirrhosis and hepatocellular carcinoma. One of the main features of HCV infection is the extremely high risk of chronicity (60–80%). HCV is a positive-stranded RNA virus whose polyprotein is cleaved into at least ten structural (core and envelope proteins E1 and E2) and non-structural (NS2–NS5B) proteins (reviews by Blight *et al.*, 1998; Major & Feinstone, 1997; Reed & Rice, 2000). The mechanisms implicated in both its persistence and its resistance to antiviral therapy are poorly understood. Variability of the viral genome (Farci *et al.*, 2000) and infection of peripheral mononuclear blood cells (PBMCs) (Azzari *et al.*, 2000; Bréchet, 1996; Lerat *et al.*, 1996) may promote HCV persistence. Modulation of cellular transduction signals controlling lipid metabolism and the cytokine network

by HCV proteins may also affect the outcome of infection (reviews by Kittlesen *et al.*, 2000; Lai & Ware, 2000; Large *et al.*, 1999; Sabile *et al.*, 1999).

The overall profile of the immune response to HCV is now emerging: recovery from acute HCV infection is clearly associated with a strong cellular immune response to the virus, i.e. the proliferation of CD4⁺ and cytotoxic T cells against several HCV proteins (Cerny & Chisari, 1999). The persistence of HCV develops despite humoral and cellular polyclonal and polyspecific immune responses to viral proteins (Baumert *et al.*, 1998; Cerny & Chisari, 1999; Farci *et al.*, 2000; Klennerman *et al.*, 2000). However, the vigorous proliferation of HCV-specific CD4⁺ and CD8⁺ lymphocytes correlates well with the clearance of the virus under therapy. Under these conditions, the mechanisms underlying the ineffectiveness of the immune response in clearing the virus during establishment of a chronic carrier state are largely unknown.

There is some evidence for the appearance of neutralizing antibodies to the virus during the natural course of HCV infection (Shimizu *et al.*, 1994). *In vivo*, infection of a naive chimpanzee can be prevented by preincubating the challenging virus with serum from an infected animal (Farci *et al.*, 1994). In humans, co-infected with HCV and hepatitis B virus (HBV) and transplanted for end-stage cirrhosis, reinfection of liver grafts by HCV can be prevented partially by administering anti-HBV immunoglobulins, this observation being consistent with the presence in such immunoglobulin preparations of neutralizing antibodies to HCV (Feraÿ *et al.*, 1998).

A major problem concerning studies on this issue is the lack of appropriate and reproducible *in vitro* experimental systems. Attempts have been made to identify neutralizing antibodies to HCV by testing for the inhibition of HCV envelope protein E2 binding to target cells such as MOLT-4 cells (Rosa *et al.*, 1996). The relevance of this assay has been established through the correlation of their detection and the resolution of HCV infection in humans (Abrignani & Rosa, 1998), as well as by the partial protection of chimpanzees from a challenge virus after immunization with HCV envelope proteins. Neutralizing antibodies have also been tested for by inhibiting HCV *in vitro*

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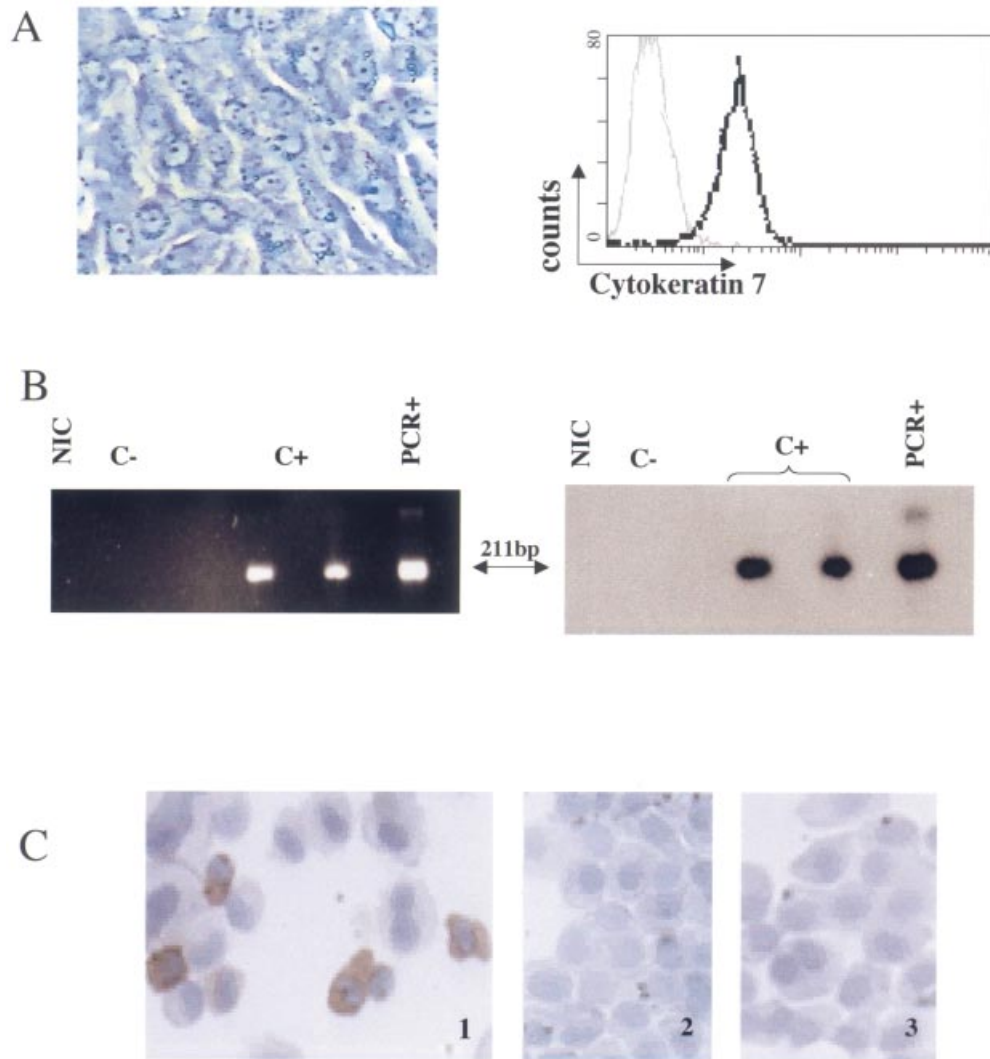


Fig. 1. Morphology of primary biliary cells and detection of HCV RNA and HCV proteins in these *in vitro*-infected cells. (A) Left panel, light micrograph of biliary cells after 7 days of culture; right panel, detection of cytokeratin 7 by flow cytometry using anti-cytokeratin 7 monoclonal antibody. Grey histogram, biliary cells treated with only the secondary antibody (negative control). Black histogram, biliary cells treated with an anti-cytokeratin 7 antibody. (B) Detection of HCV RNA and HCV proteins in biliary cells after *in vitro* infection. Left panel, detection by RT-PCR of intracellular HCV positive-stranded RNA in *in vitro*-infected biliary cells 5 days after infection with HCV⁺1b reference serum (10^5 copies/ml). Right panel, confirmation of RT-PCR specificity by hybridization with a 5' non-coding region HCV-specific probe. NIC, non-infected cells (negative control); C-, cells inoculated with heat-inactivated serum (negative control); C+, cells infected with non-heat-treated serum; PCR+, positive control for PCR (amplification of HCV RNA from infectious serum used for cell infection). (C) Detection of core protein in infected biliary cells by staining with a monoclonal anti-core antibody directed against the HCV sequence of aa 1–120. Panel 1, infected biliary cells stained with a monoclonal anti-core antibody; panel 2, negative control of uninfected biliary cells; panel 3, negative control of infected cells treated for HCV core staining without the primary antibody.

infection of mononuclear cell lines (Azzari *et al.*, 2000; Shimizu *et al.*, 1994) or HCV binding to fibroblasts (Zibert *et al.*, 1995). These experimental systems have provided significant information, demonstrating both the appearance of neutralizing anti-HCV antibodies during the course of chronic HCV infection in chimpanzees and humans (Ishii *et al.*, 1998; van Doorn *et al.*, 1995) and the importance of antibodies directed against epitopes encoded by the hypervariable region of the envelope protein E2 for virus clearance. However, it is difficult

to achieve reproducible infection of these cells and fibroblasts are not infected *in vivo*. Thus, it is still necessary to develop reproducible assays to precisely define the patterns of neutralizing anti-HCV antibodies and their actual relevance to HCV pathogenesis.

We reported previously the permissivity of human biliary cells for *in vitro* HCV infection (Loriot *et al.*, 1999). Although HCV replicates at a very low level in biliary cells, we were able to show that *in vitro* HCV infection is highly reproducible. In

Table 1. Patient details and results of neutralization assays and serological reactivity

All patients were shown by histology to have chronic active hepatitis (CAH). Sampling times are indicated as the interval (months) between the end of IFN therapy and serum sampling. Reactivity was as determined by LIA assay (Innogenetics). For each serum sample (10 ml), one strip (containing different peptides) was placed in a test tube, to which 1 ml of a sample diluent (phosphate buffer containing NaCl, Triton, protein stabilizers and 0.1% sodium azide) was added. Samples were incubated on the peptide-coated strips overnight on an orbital mixer at room temperature. After washing three times for 5 min each with wash solution (phosphate buffer containing NaCl, Triton and 0.5% sodium azide), 1 ml of prepared conjugate solution was added (goat anti-human IgG labelled with alkaline phosphatase in Tris buffer containing protein stabilizers and 0.1% sodium azide) and incubated for 30 min at room temperature. Again, the strip was washed, twice with wash solution and once with substrate buffer (Tris buffer containing NaCl and 0.1% sodium azide), and revealed with substrate solution (30 min at room temperature). The reaction was stopped with 0.1 mol/l sulphuric acid for 10 min.

Patient	HCV genotype	Sex	Age (years)	Histology	Sampling time	Neutralization	Reactivity	
							E1	E2
1	3a	M	32	CAH	72	+	+	-
2	3a	M	36	CAH	84	+	-	-
3	3a	F	26	CAH	60	-	+	-
4	2a	M	38	CAH	6	+	-	+
5	1b	M	41	CAH	14	-	+	+
6	1b	F	61	CAH*	14	+	+	+
7	2a	F	63	CAH	12	-	+	-
8	2a	F	42	CAH	8	-	-	+
9	1a	F	29	CAH	6	-	-	+
10	1a	M	36	CAH	31	-	-	+

* Associated with cirrhosis.

the present report, we demonstrate the ability of this experimental system to detect neutralizing antibodies.

As described previously (Loriot *et al.*, 1999), biliary cells could be maintained in culture with a well-differentiated status for at least 2 months (Fig. 1A, left panel). The expression of cytokeratin 7 was detected, indicating the purity and differentiation of these cells (Fig. 1A, right panel).

We then tested for HCV infection 5 days after cell inoculation with HCV⁺ 1b reference serum (10⁵ copies/ml) or heat-inactivated serum, which acted as a further negative control. Positive-stranded HCV RNA was not detected in extracts of cells incubated with heat-inactivated serum. In contrast, HCV RNA was specifically detected in extracts of cells incubated with HCV⁺ 1b reference serum (Fig. 1B, left panel). The specificity of RT-PCR was confirmed using Southern blotting (Fig. 1B, right panel). To further support the above findings, we used immunohistochemical analysis to monitor the expression of the HCV core antigen in HCV-infected biliary cells. A monoclonal antibody directed against the HCV core (aa 1–120) was used as the primary antibody (Euromedex). After washing, the cells were incubated in a peroxidase-labelled polymer system (EnVision anti-mouse horseradish peroxidase-conjugated antibody) (Dako) and the reaction was revealed with liquid DAB (Dako). Positive staining was observed in infected biliary cells (Fig. 1C, panel 1) but not in the negative controls, including both uninfected cells (Fig. 1C, panel 2) and infected cells treated without the primary

antibody (Fig. 1C, panel 3). The percentage of infected cells was estimated at about 10–20%.

Having validated HCV infection in our experimental model, we went on to test the ability of selected serum samples to neutralize HCV infection. The neutralization assay was performed using sera from patients who, after therapy, had recovered from infection (Table 1). We investigated ten patients with HCV-related chronic active hepatitis (CAH). All ten patients were long-term responders after interferon (IFN) or IFN + ribavirin therapy (normal liver tests and negative serum HCV RNA assay at least 1 year after the end of therapy). The HCV genotypes involved were: 1b (two patients), 1a (two patients), 2a (three patients) and 3a (three patients). Serum samples were obtained 6 to 84 months after the end of therapy. Fig. 2(A, C) shows representative results obtained with serum samples from patients 2 and 6; these patients had recovered from HCV infection with HCV genotypes 3a (patient 2) and 1b (patient 6). HCV RNA was undetectable when infectious reference serum 1b was incubated with undiluted sera or with 1:25 dilutions of sera from both patients. In contrast, HCV RNA was detected in the positive control of cells infected with HCV reference serum 1b only. HCV RNA was also observed when 1b reference serum was mixed with a 1:100 dilution of sera from patients 2 and 6. These results demonstrated neutralization activity in the sera from both patients.

In order to demonstrate that this neutralization was due to HCV antibodies, we purified IgG from the sera of cured

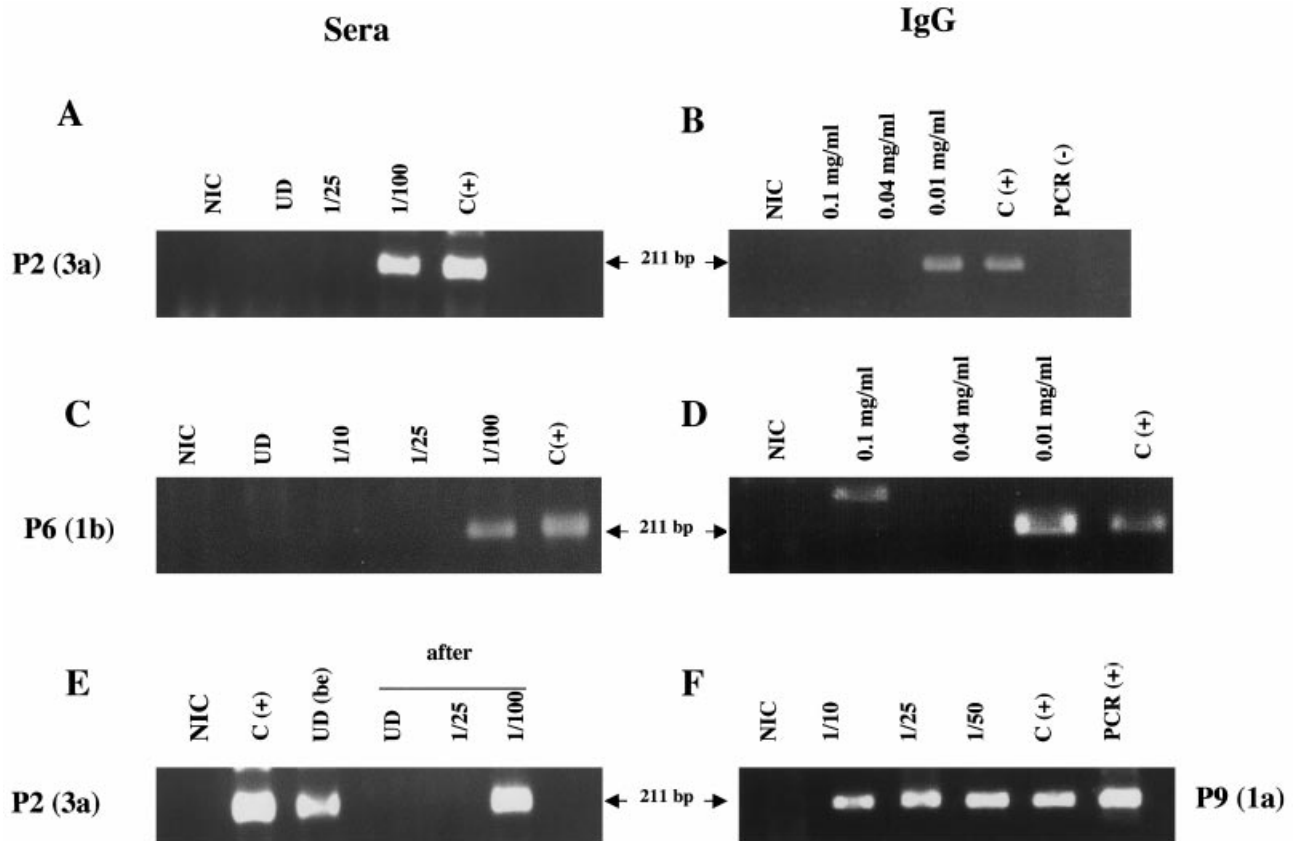


Fig. 2. Detection of neutralizing anti-HCV antibodies. HCV⁺ 1b reference serum was incubated with different dilutions of tested sera or purified IgG from recovered patients and the mixtures were used to infect biliary cells. Neutralization tests were repeated five times for patients 2 and 9 and twice for patient 6. (A, B) Neutralization assay with serum sample (A) and IgG (B) from patient 2. (C, D) Neutralization assay with serum (C) and IgG (D) from patient 6. (E) Neutralization assay with serum sample from patient 2 before (be) and after therapy. (F) Neutralization assay with IgG from patient 9. NIC, non-infected cells (negative control); UD, undiluted serum; 1:10 (0.1 mg/ml, 5 µg), 1:25 (0.04 mg/ml, 2 µg) and 1:100 (0.01 mg/ml, 500 ng), different dilutions of serum or IgG used; C+, positive control of cells incubated with infectious reference 1b serum; PCR-, negative control of PCR without cell extract; UD (be), undiluted serum sample from patient 2 before anti-viral therapy.

patients. Serum samples were diluted in 20 mM sodium phosphate buffer (pH 7) and applied to the High Trap Protein A column (Amersham). Purified fractions were eluted with 0.1 M citric acid, pH 4 (elution buffer). After elution, Tris-HCl (pH 9) was immediately added to the fractions collected so as to neutralize the acid pH of the elution buffer. Collected fractions containing immunoglobulins were dialysed using Slide-A-Lyser 10 K Dialysis Cassettes (Pierce) for 4 h in PBS at room temperature. Dialysed fractions were sterilized by filtration and the immunoglobulin concentrations determined using a micro-BCA protein assay (Pierce). Fig. 2(B, D) shows representative results obtained with IgG from patients 2 and 6. HCV RNA was undetectable when infectious serum was incubated with IgG at dilutions ranging from 1:10 (0.1 mg/ml) to 1:25 (0.04 mg/ml). In contrast, HCV RNA was detected when infectious serum was mixed with IgG at dilutions of 1:100 (0.001 mg/ml) in patients 2 and 6. Fig. 2(F) shows representative results obtained from patient 9 by mixing

infectious serum with IgG dilutions of between 1:10 (0.1 mg/ml) and 1:50 (0.02 mg/ml). In contrast with patients 2 and 6, HCV RNA was detectable at all serum dilutions.

Attempts were then made to determine whether neutralization activity might differ before and after the end of HCV multiplication in given patients. Fig. 2(E) shows representative results obtained with serum from patient 2, before anti-viral therapy and after a long-term response to treatment. Using serum obtained before therapy, HCV RNA was detected when infectious serum was incubated with undiluted serum, suggesting an absence of detectable neutralizing anti-HCV antibodies before therapy; in contrast, when using serum samples obtained after therapy, neutralizing anti-HCV antibodies were shown at a serum dilution of 1:25 but not 1:100. These results indicated that neutralizing antibodies were identified after a long-term response but not before therapy.

Globally, this report demonstrates that *in vitro* infection of primary biliary cells may be used efficiently for studies aimed

at detecting and characterizing neutralizing antibodies to HCV. Biliary cells can easily be obtained during gallbladder surgery and primary biliary cells retain a well-differentiated status for up to 2 months. *In vitro* infection of biliary cells can be achieved reproducibly and we were able to confirm our initial observations by combining the immunohistochemical detection of HCV antigens with HCV RNA detection. No robust experimental system currently exists for the culture of HCV. Although interesting data have been reported in primary human hepatocytes and the levels of HCV multiplication reported with that system are clearly higher than those obtained in our model, it nonetheless remains difficult to obtain this material on a sustained basis and, furthermore, the differentiation status of donor hepatocytes markedly influences the quality of results.

Using this material, we were able to detect antibodies capable of neutralizing *in vitro* infection in the serum of patients who had recovered from HCV infection after IFN or IFN + ribavirin therapy. The fact that neutralizing antibodies were indeed present in these serum samples was shown by inhibition of neutralization upon serum dilution and by the neutralization achieved with purified IgG. Several interesting features are suggested by this study: firstly, amongst the few subjects tested, neutralizing anti-HCV antibodies were detected after the end of HCV multiplication but not before therapy, thus suggesting an association between their detection and the efficacy of therapy. Some previous reports had suggested that in chronically infected chimpanzees neutralizing anti-HCV could be detected at some but not all time-points, several years after contamination (Farci *et al.*, 1994). On the other hand, anti-HCV antibodies detected using baculovirus-engineered virus particles (HCV-LPs) (Baumert *et al.*, 2000) have been transiently detected in patients with self-limited acute hepatitis. Studies based on a binding neutralization assay have also revealed an association between the detection of anti-NOB (neutralization of binding) antibodies and the resolution of HCV infection (Ishii *et al.*, 1998). Thus, although our experimental system now needs to be tested in a large series of patients, our findings were consistent with an association between the detection of anti-HCV antibodies with neutralizing activity and the inhibition of HCV multiplication. Nevertheless, an intriguing point remains: the long-term persistence of neutralizing anti-HCV antibodies that we identified during this study (antibodies identified up to 84 months after the end of therapy). Our result contrasts with those of other studies, during which it was not possible to detect such neutralizing activity or where there was a marked decline in the anti-HCV-LP antibody titres in sera obtained long after the end of HCV multiplication (Baumert *et al.*, 2000). In long-term responders to IFN or IFN + ribavirin, there is evidence for eradication of the virus, as shown by negative PCR tests for HCV RNA in serum, PBMCs and liver (Romeo *et al.*, 1993; Zeuzem *et al.*, 1998). Further studies will be needed to determine the kinetics of the anti-HCV antibodies detected in our assay and to correlate these findings with virus

elimination patterns. Secondly, our findings demonstrate that infection with our reference HCV 1b inoculum could be neutralized by sera obtained from patients infected with different HCV genotypes. Antibodies to HCV-LPs detected with HCV 1b proteins expressed in baculovirus have been detected in patients infected by different HCV genotypes. Consistent with these observations, conserved domains have been identified in the hypervariable region 1 sequence of the envelope protein E2. Thus, taken together, our data support the possibility of inducing neutralizing anti-HCV antibodies with cross-reactivity between different genotypes, which may have major implications in the prevention of an HCV infection. Thirdly, in most, if not all, sera with neutralizing activity, our results demonstrated serological reactivity against different peptide-derived E1 and E2 envelope proteins (Table 1) (Lechner *et al.*, 1998; Rosa *et al.*, 1996; Weiner *et al.*, 1992; Zhou *et al.*, 1999; Zibert *et al.*, 1995). Several reports have, however, shown that antibodies to E2, as determined using the tests currently available, can be detected throughout the course of HCV infection, whether it has resolved or is progressing to chronicity, and their detection is not correlated with HCV clearance (Cerny & Chisari, 1999; Hassoba *et al.*, 1997; Tanaka *et al.*, 1999). During the present study, one patient did not exhibit any detectable anti-E2 and anti-E1 reactivity, although this patient's serum showed neutralizing activity. Our data suggest the importance of conformational antibodies and, perhaps, that of other virus epitopes in eliciting neutralizing antibodies.

Taken together, the present study provides a valuable *in vitro* infection model to test the potential neutralizing activity of various anti-HCV antibodies.

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