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

Saadia Bichr,1 Rosanna Rende-Fournier,1 Giovanna Vona,1 Ana-Maria Yamamoto,2 Erik Depla,3 Geert Maertens3 and Christian Bréchot1

1 Inserm U370, Faculté de Médecine Necker, 156 rue de Vaugirard, 75730 Paris Cédex 15, France
2 Inserm U25; Hôpital Necker; 75015 Paris, France
3 Innogenetics, Gent, Belgium

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; Bréchot, 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; Klenerman 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 administrating anti-HBV immunoglobulins, this observation being consistent with the presence in such immunoglobulin preparations of neutralizing antibodies to HCV (Feray 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 though 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
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.

<table>
<thead>
<tr>
<th>Patient</th>
<th>HCV genotype</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Histology</th>
<th>Sampling time</th>
<th>Neutralization E1</th>
<th>Neutralization E2</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3a</td>
<td>M</td>
<td>32</td>
<td>CAH</td>
<td>72</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>3a</td>
<td>M</td>
<td>36</td>
<td>CAH</td>
<td>84</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>3a</td>
<td>F</td>
<td>26</td>
<td>CAH</td>
<td>60</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>2a</td>
<td>M</td>
<td>38</td>
<td>CAH</td>
<td>6</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>1b</td>
<td>M</td>
<td>41</td>
<td>CAH</td>
<td>14</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>1b</td>
<td>F</td>
<td>61</td>
<td>CAH*</td>
<td>14</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>2a</td>
<td>F</td>
<td>63</td>
<td>CAH</td>
<td>12</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>2a</td>
<td>F</td>
<td>63</td>
<td>CAH</td>
<td>8</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>1a</td>
<td>F</td>
<td>29</td>
<td>CAH</td>
<td>6</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>1a</td>
<td>M</td>
<td>36</td>
<td>CAH</td>
<td>31</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

* Associated with cirrhosis.

In 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 (10^5 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 patients, and showed that this neutralizing antibody was also present in sera from patients with cirrhosis.

Detection of neutralizing antibodies to HCV
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); 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 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 
detected 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 
naturally 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 
envvelope 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.

We express our thanks to Dr Dina Kremsdorf and Dr Valérie Thiers 
for helpful discussions, and Sofie Priem, Marie-José Devau and Laurence 
Vendramme for excellent technical assistance. This work was supported 
by Inserm, French Ministry for Research (PRFMID and HCV network), 
ARC (Association pour La Recherche contre le Cancer), LNC (Ligue 
Nationale contre le Cancer), and EC (European Community).

References


Azzari, C., Resti, M., Moriondo, M., Ferrari, R., Lionetti, P. & Vierucci, 
A. (2000). Vertical transmission of HCV is related to maternal peripheral 
blood mononuclear cell infection. Blood 96, 2045–2048.

structure is disrupted and forms a new virus-like structure in 

Baumert, T. F., Wellnitz, S., Aono, S., Sato, J., Herion, D., Tilman 
Gerlach, J., Pape, G. R., Lau, J. Y., Hoofnagle, J. H., Blum, H. E. & 
Liang, T. J. (2000). Antibodies against hepatitis C virus-like particles 
and viral clearance in acute and chronic hepatitis C. Hepatology 32, 
610–617.

Blight, K. J., Kolykhalov, A. A., Reed, K. E., Agapov, E. V. & Rice, C. M. 
(1998). Molecular virology of hepatitis C virus: an update with respect to 
potential antiviral targets. Antiviral Therapy 3, 71–81.


Received 17 October 2001; Accepted 28 February 2002