Hepatitis C virus (HCV) is a leading cause of chronic viral hepatitis worldwide. The study of antibody-mediated virus neutralization has been hampered by the lack of an efficient and high-throughput cell culture system for the study of virus neutralization. The HCV structural proteins have been shown to assemble into noninfectious HCV-like particles (HCV-LPs). Similar to serum-derived virions, HCV-LPs bind and enter human hepatocytes and hepatoma cell lines. In this study, we developed an HCV-LP-based model system for a systematic functional analysis of antiviral antibodies from patients with acute or chronic hepatitis C. We demonstrate that cellular HCV-LP binding was specifically inhibited by antiviral antibodies from patients with acute or chronic hepatitis C in a dose-dependent manner. Using a library of homologous overlapping envelope peptides covering the entire HCV envelope, we identified an epitope in the N-terminal E2 region (SQKIQLVN...). This epitope was recognized by antibodies from patients with acute or chronic hepatitis C. The presence of antibodies with inhibition of binding activity was not associated with viral clearance.

In conclusion, antibody-mediated inhibition of cellular HCV-LP binding represents a convenient system for the functional characterization of human anti-HCV antibodies, allowing the mapping of envelope neutralization epitopes targeted by naturally occurring antiviral antibodies.

Hepatitis C virus (HCV), a member of the Flaviviridae, is a major cause of chronic viral hepatitis in the world (28, 31). Progression to chronic disease occurs in the majority of HCV-infected persons, and end-stage liver disease due to chronic HCV infection has become the main indication for liver transplantation (28, 31). Resolution of chronic infection is extremely rare; more often, chronic infection results in chronic hepatitis, liver cirrhosis, or hepatocellular carcinoma (23). Worldwide, an estimated 170 million people are infected with HCV. Treatment options for chronic HCV infection are limited, and a vaccine to prevent HCV infection is not available (15, 23).

Although the humoral and cellular immune responses induced by HCV have been analyzed in great detail, the mechanisms of viral clearance and persistence are still poorly understood (46). HCV can establish persistent infection despite a humoral and cellular immune response that is generally targeted against all viral proteins (30, 46). A strong cellular immune response appears to be important for virus clearance (28, 46). Chronic HCV infection results in the induction of a strong humoral immune response (30), and anti-HCV antibodies can be detected easily by using synthetic peptides or recombinant proteins in serologic assays (41). Using the chimpanzee model, antibodies with neutralizing properties have been described (18). These antibodies were directed against epitopes in the hypervariable region of the envelope glycoprotein 2 (E2) and appeared to be isolate specific. Antibody-mediated neutralization is also suggested by a study of patients undergoing liver transplantation for HCV- and hepatitis B virus-related liver cirrhosis. Infusion of anti-HBs hyperimmune globulin containing anti-HCV appeared to reduce HCV infection in the transplanted liver (19). Due to the pending development of convenient and efficient model systems for the analysis of large serum panels for virus neutralization, a systematic analysis of antibodies with neutralizing properties obtained from patients with acute or chronic HCV infection is lacking.

Several groups have recently described the synthesis of HCV-like particles (HCV-LPs) in insect cells using a recombinant baculovirus containing the cDNA of the HCV structural proteins core, E1, and E2 (6, 7, 13, 44, 45, 50). Recently, the formation of HCV-LPs in mammalian cells has also been described (10, 17). HCV-LPs exhibit morphological, biophysical, and antigenic properties similar to those of putative virions isolated from HCV-infected patients. In contrast to recombinant C-terminally truncated HCV E2 protein, the envelope proteins of HCV-LPs are presumably presented in a native, virion-like conformation. They may, therefore, interact with anti-HCV antibodies directed against nonlinear or conforma-
tional epitopes of HCV envelope proteins that may represent neutralizing epitopes (8, 13, 45). Indeed, a previous study demonstrated that antiviral antibodies in acute and chronic HCV infections interact with HCV-LPs when used as the capture antigen in an enzyme-linked immunosorbent assay (ELISA). Furthermore, HCV-LPs induce potent humoral and cellular immune responses in vivo and therefore offer a promising approach for vaccine development (7, 29, 33, 37).

Recent studies have demonstrated that HCV-LPs interact with defined human cell lines and hepatocytes similar to viral particles isolated from human serum. The interaction of HCV-LPs with target cell lines therefore represents a novel model system for the study of viral binding and entry (4, 44, 48, 50). In this study, we demonstrate that the HCV-LP-based system can be conveniently used for the functional characterization of anti-HCV antibodies in acute and chronic hepatitis C. We show that cellular binding of HCV-LPs is inhibited by human anti-HCV antibodies, and we define the envelope epitopes targeted by these antibodies. Furthermore, we use this system as a surrogate model to study the roles of neutralizing antibodies in viral clearance and the clinical outcome of HCV infection.

**MATERIALS AND METHODS**

**Patients.** Serial anti-HCV-positive serum samples (n = 93; sampled at time points from 0 to 18 months following the diagnosis of HCV infection) were obtained from 21 patients with acute symptomatic hepatitis C (8 patients with acute self-limited hepatitis and viral clearance and 13 patients with acute hepatitis C) to allow HCV-LP binding. After the cells were washed, the amount of HCV-LP bound to the cells was assessed as described above. All sera were screened for inhibition of HCV-LP binding using both mouse monoclonal anti-E2 and human polyclonal anti-HCV antibodies. Only sera demonstrating a decrease in cellular binding of >50% in both assays were considered inhibitory. Since the supply of human polyclonal anti-HCV antibody was limited, mouse monoclonal anti-E2 antibody was used for further characterization of inhibition of HCV-LP binding following the assessment of samples in screening assays. MFI values from HCV-LPs incubated with control serum (positive control), with anti-HCV-positive serum (experimental values), and control preparations (GUS) with control serum (negative control) were measured, and the specific neutralization was determined as described previously (38), according to the following equation: specific neutralization = [(positive control MFI − experimental MFI)/(positive control MFI − negative control MFI)] × 100. The inhibition of the binding titer was defined as the serum dilution that showed <50% inhibition of cellular HCV-LP binding.

IgG purification from human serum. In order to demonstrate that inhibition of binding was mediated by anti-HCV antibodies, IgG was purified from HCV-positive and HCV-negative control sera using a MabTrap kit (Amer sham, Buckinghamshire, United Kingdom) according to the manufacturer’s protocol. Prior to analysis by confocal laser scanning microscopy, the cover slides were mounted in antifade reagent (Fluoroguard; Bio-Rad Laboratories, Hercules, Calif.) to minimize photobleaching. The stained cells were analyzed in cross section using an LSM 410 laser scanning confocal microscope (Carl Zeiss Corp., Jena, Germany) with argon (488-nm wavelength) and helium-neon (543- and 633-nm wavelength) lasers. Digital images were acquired with LSM Image Examiner software (Zeiss, Jena, Germany).

**Antibody-mediated inhibition of cellular HCV-LP binding.** To study whether HCV-LP binding can be inhibited by serum-derived antibodies, HCV-LPs (0.1 μg of HCV-LP E2/ml) were mixed with human serum in various dilutions and incubated for 1 h at 37°C. Huh-7 cells (1.5 × 10⁶) were added to the mixture and incubated for 1 h at 4°C to allow HCV-LP binding. After the cells were washed, the amount of HCV-LP bound to the cells was assessed as described above. All sera were screened for inhibition of HCV-LP binding using both mouse monoclonal anti-E2 and human polyclonal anti-HCV antibodies. Only sera demonstrating a decrease in cellular binding of >50% in both assays were considered inhibitory. Since the supply of human polyclonal anti-HCV antibody was limited, mouse monoclonal anti-E2 antibody was used for further characterization of inhibition of HCV-LP binding following the assessment of samples in screening assays. MFI values from HCV-LPs incubated with control serum (positive control), with anti-HCV-positive serum (experimental values), and control preparations (GUS) with control serum (negative control) were measured, and the specific neutralization was determined as described previously (38), according to the following equation: specific neutralization = [(positive control MFI − experimental MFI)/(positive control MFI − negative control MFI)] × 100. The inhibition of the binding titer was defined as the serum dilution that showed <50% inhibition of cellular HCV-LP binding.

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ing washing of the column with 7 ml of binding buffer, the purified IgG was
eluted using 5 ml of elution buffer and neutralized using 75 μl of neutralizing
buffer according to the manufacturer’s protocol. Fractions were analyzed for
the presence of anti-HCV antibodies using an HCV-LP-based ELISA (8).

Epitope mapping of antibodies inhibiting cellular binding of HCV-LPs. To
identify HCV-LP envelope epitopes targeted by human antibodies inhibiting
cellular HCV-LP binding, an anti-HCV-positive serum with strong inhibition
of binding activity was incubated with overlapping 15-mer peptides of the
HCV envelope glycoproteins (comprising amino acid positions 201 to 758)
derived from the HCV-J strain (26) or PBS (as a control) for 1 h at RT
(100 μg/ml; serum dilution, 1:50). The peptides were

provided through the European Research Network HCVacc (QLK2-1999-
00356) and were synthesized by Clonestar Corp. (Brno, Czech Republic).
HCV-LPs (derived from the homologous isolate as the peptides) were then
added to the peptide-antibody complexes and incubated for 1 h at 37°C.
Finally the HCV-LPs, antibodies, and peptides were added to HuH-7 cells
and incubated for 1 h at 4°C. Binding of HCV-LPs was detected by
flow cytometry using the monoclonal anti-E2 antibody AP33 as described above.

Statistical analysis. Comparison between subgroups was performed by the
chi-square test using the SAS Analyst statistical software package, version 6.12
(SAS Institute, Cary, N.C.).
RESULTS

HCV-LP-based model system for viral envelope binding and entry. To establish a convenient model system for the study of antibody-mediated inhibition of viral envelope binding to target cells, we first characterized the interaction of HCV-LPs with the hepatoma cell lines HuH-7 and HepG2. These liver-derived cell lines have been used as model systems to study the interaction of viral envelope glycoproteins with the host cell membrane (4, 20, 40, 44, 48). Furthermore, defined hepatoma cells have been shown to allow the entry of virus, virus-like particles, and retroviral HCV pseudoparticles (2, 5, 24, 44), and they may support low-level HCV infection (42). HCV-LPs of genotypes 1a and 1b were synthesized using recombinant baculoviruses containing the cDNA for the HCV structural proteins of strains HCV-J and HCV-H77. Using flow cytometry and anti-envelope antibodies for the detection of cellular HCV-LP binding, HCV-LPs demonstrated concentration-dependent and saturable binding to HuH-7 and HepG2 cells, respectively (Fig. 1A and B). This side-by-side analysis of HCV-LPs of genotypes 1a and 1b extends a previous study describing the binding of HCV-LPs of the infectious clone H77C to target cells (48).

The two hepatoma cell lines exhibited similar HCV-LP binding profiles, although saturation of HCV-LP binding to HuH-7 cells was reached at a slightly lower HCV-LP E2 concentration than for HepG2 cells. Saturation of HCV-LP binding was reached at an E2 concentration of ~0.5 to 1 μg/ml for HuH-7 cells (Fig. 1A) and 1 to 2 μg/ml for HepG2 cells (Fig. 1B). These data may indicate a higher number of HCV-LP E2 binding sites on HepG2 than on HuH-7 cells. HCV-LPs of genotypes 1a and 1b exhibited similar binding profiles (saturation of binding at 0.5 and 1 μg of E2/ml for HCV-LPs of...
genotype 1a or 1b and HuH-7 cells, respectively). Half-maxi-
mal saturation of HCV-LP binding was present at E2 concen-
trations between 0.2 and 0.4 μg/ml.

The next step after particle binding is uptake, or entry of
particles into the cell. To investigate whether HCV-LPs were
internalized into HuH-7 cells following binding to the cell
surface, we performed anti-E2-specific immunofluorescence
and confocal laser scanning microscopy of HuH-7 cells incu-
bated with HCV-LPs. As shown in Fig. 1C and D, incubation
of cells with HCV-LPs (genotype 1a) at 4°C resulted in the
exclusive detection of HCV-LP E2 at the cell surface, consis-
tent with HCV-LP binding to the cell membrane. By contrast,
incubation of HuH-7 cells with HCV-LPs at 37°C resulted in
the translocation of E2 immunoreactivity inside the cell, con-
sistent with temperature-dependent cellular HCV-LP entry.
These data indicate that HCV-LPs are taken up by HuH-7 in
a temperature-dependent manner following cell surface bind-
ing. These findings corroborate and extend previous experi-
ments using dye-labeled HCV-LPs (44).

**Inhibition of cellular HCV-LP binding by serum-derived anti-HCV antibodies.** We next studied whether cellular HCV-
LP binding is inhibited by sera from HCV-infected individuals.
As shown in Fig. 2, anti-HCV-positive sera from patients with
hepatitis C inhibited cellular HCV-LP binding (Fig. 2). To
confirm that the observed inhibition of binding was due to
anti-HCV antibodies present in these sera, cellular HCV-LP
binding was studied in the presence of 10 anti-HCV-negative
control sera. None of these control sera inhibited cellular
HCV-LP binding (Fig. 2A and B). To exclude the possibility
that the observed inhibition of HCV-LP binding was due to
masking of HCV-LP epitopes by nonneutralizing anti-HCV
antibodies present in these sera, inhibition of binding was studied
using a human serum containing high-titer anti-E2 and anti-
HCV-LP antibodies (human polyclonal anti-HCV) (Fig. 2C).
Revealing the binding with antibodies from the same species as
the neutralizing serum (human-human) is critical because non-
neutralizing anti-envelope antibodies present in human serum
could cover E2 after it is bound to target cells and could,
therefore, interfere with the assessment of neutralization if the
binding was revealed with an anti-E2 antibody from a different
species (such as a mouse monoclonal antibody) (38). Only sera
demonstrating a reproducible decrease in cellular binding of
>50% were considered inhibitory.

Inhibition of HCV-LP binding was dependent on the serum
concentration (Fig. 2D), indicating a concentration-dependent
effect of inhibitory activity by anti-HCV antibodies present in
these sera. Titration of inhibition of binding activity by end-
point dilution revealed a maximal antibody titer of 1:200. In
order to determine whether inhibition of binding was mediated
by anti-HCV antibodies, IgG was purified from HCV-positive
and HCV-negative control sera using protein G antibody af-
ninity chromatography. Purified anti-HCV IgG inhibited cellu-
lar HCV-LP binding (Fig. 3B) similarly to anti-HCV serum
(Fig. 3A). Inhibition of HCV-LP binding by purified anti-HCV
IgG was concentration dependent, showing 50% inhibition of
binding at concentrations of ~100 μg/ml (Fig. 3C). Interest-
ingly, concentrations of purified IgG and serum IgG exhibited
similar levels of inhibition of binding profiles (Fig. 3C).

**Antibody-mediated inhibition of binding and viral clearance in acute and chronic hepatitis C.** Among the 21 patients with

### FIG. 3. Inhibition of HCV-LP binding by purified human anti-
HCV IgG.  
(A) Inhibition of cellular HCV-LP binding by anti-HCV-
positive or control serum (dilution, 1:25).  
(B) Inhibition of cellular HCV-LP binding by purified anti-HCV IgG (1 mg/ml) from
the same sera shown in panel A.  
(C) Concentration-dependent inhibition of HCV-LP binding by purified anti-HCV IgG (squares) and
serum containing anti-HCV antibodies (circles).  
Analysis of inhibition of HCV-LP binding was performed as described in the legend to Fig.
2.  
Serum IgG concentrations were determined as described in Materials and Methods.
acute symptomatic hepatitis C, 8 resolved acute infection; the other patients developed chronic infection. A total of 13 out of 21 patients with acute symptomatic hepatitis C demonstrated inhibition of cellular HCV-LP binding. Quantitative assessment of antibodies revealed inhibition of binding titers ranging between 1:50 and 1:200. To study whether antibody-mediated inhibition of HCV-LP was strain or isolate specific, we used HCV-LPs derived from genotypes 1a and 1b as ligands for cellular binding (Fig. 4). Two inhibitions of binding profiles were observed. The majority of patients (9 of 13) with acute symptomatic hepatitis C demonstrated strain-specific inhibition of HCV-LP binding (Fig. 4A and B). These patients were all infected with HCV genotype 1b and were characterized by antibody-mediated inhibition of HCV-LP binding restricted to HCV-LPs of genotype 1b. Four out of 13 patients with acute symptomatic hepatitis C (infected with HCV genotypes 1a, 1b, and 3) and inhibition of HCV-LP binding activity exhibited cross-strain inhibition of binding activity (Fig. 4C and D). Patients without inhibition of HCV-LP binding activity were infected with genotypes 1b (four of eight), 3 (three of eight), and 4 (one of eight).

To obtain information on the relationship between the inhibition of binding titers and the outcome of acute or chronic HCV infection, we retrospectively studied serial serum samples from the patients. The presence of antibodies with inhibition of HCV-LP binding activity did not correlate with viral clearance and the outcome of infection (Fig. 5A). Two representative individuals with self-limited HCV infection are shown in Fig. 5. One patient (infected with genotype 3) cleared the viral infection in the absence of measurable antibodies with
inhibition of HCV-LP binding activity (Fig. 5C). The other patient (infected with genotype 1b) cleared the infection in parallel with inhibition of binding activity (Fig. 5D). Interestingly, this patient had generated antibodies with cross-strain inhibition of HCV-LP binding activity in two patients with acute self-limited hepatitis C and virus clearance. To assess whether the appearance of antibodies with HCV-LP inhibition of binding activity was associated with a defined phase of infection, we compared the presence of inhibitory antibodies in samples in the acute phase of infection (≤6 months following the diagnosis of acute infection) with the presence of antibodies in the chronic phase of infection (≥6 months following the diagnosis of infection). As shown in Fig. 5B, the frequency of antibodies with HCV-LP inhibition of binding was higher in the acute (48%) than in the chronic (39%) phase of infection, although this difference was not statistically significant (P = 0.6).

**Epitope mapping of antibodies inhibiting cellular binding of HCV-LPs.** To identify HCV-LP envelope epitopes targeted by human antibodies inhibiting cellular HCV-LP binding, an anti-HCV-positive serum with marked inhibition of binding activity was preincubated with overlapping 15-mer peptides of the HCV envelope glycoproteins (comprising amino acid positions 201 to 758) derived from the HCV-J strain (26) prior to the addition of HCV-LPs. As shown in Fig. 6, three E2 peptides were able to reverse inhibition of binding activity (peptides 408 to 422, 568 to 582, and 616 to 630). Whereas preincubation with E2 peptide 568 to 582 and 616 to 630 resulted in a partial reversion of antibody-mediated inhibition of cellular HCV-LP binding, E2 peptide 408 to 422 (amino acid sequence, SQKIQLVNTNGSWHI) completely reversed inhibition of the binding activity of human anti-HCV antibodies. Reversion of antibody-mediated inhibition of binding was concentration dependent, as shown in Fig. 6B. These data indicate that the viral envelope E2 epitope SQKIQLVNTNGSWHI is targeted by human anti-envelope antibodies, resulting in blocking of HCV-LP binding to the target cell surface.

**DISCUSSION**

In this study, we demonstrate for the first time that cellular binding of HCV-LPs to target cells represents a novel, convenient model system for the functional characterization of antiviral antibodies present in sera from patients with acute or chronic HCV infection. The system is characterized by a fast, simple, and highly standardizable flow cytometry-based method for antibody characterization. Our model system allowed us, by studying a large panel of serial serum samples from
HCV-infected patients, to define the roles of antibodies inhibiting cellular binding of the viral envelope to target cells in virus clearance in HCV infection.

A major obstacle in assessing the relevance of the antibody responses in HCV infection is the lack of a convenient in vitro or in vivo neutralization assay for HCV. Various cell lines and primary hepatocytes or biliary epithelial cells have been shown to be susceptible to HCV infection (3, 9, 32, 39, 43, 52). However, variability in infection efficiency and the limited availability of primary cells hamper the use of these assays for the analysis of large serial serum panels. Other models for the study of the HCV-host interaction include binding of unpurified or sucrose gradient-purified virions to human cell lines (1, 49). These systems use partially purified virions but require either the use of ultrasensitive detection methods, such as reverse transcriptase PCR and in situ hybridization, or flow cytometry-based systems using less standardized reagents, such as polyclonal anti-HCV antibodies derived from human serum, for the detection of virus binding.

Binding of individually expressed recombinant glycoprotein E2 to human cell lines has been used as a surrogate model for the binding of HCV to host cells, allowing the study of antibody-mediated neutralization of binding (25, 38). However, it is still unclear whether individually expressed recombinant E2 protein reflects the natural interaction of the viral envelope with target cells. The use of C-terminally truncated E2 protein as a surrogate ligand for virus binding is limited by the fact that the proper conformation of the envelope protein requires the coexpression of both the E1 and E2 proteins (36). The signal sequences of the C termini of the E1 and E2 transmembrane domains are important for membrane anchoring, heterodimerization, and membrane retention (14). The relevance of this finding is reflected by the recently described antigenic differences between C-terminally truncated E2, full-length E1/E2 complexes, and HCV-LPs, pointing to the existence of structural differences with important functional implications (13, 34). In contrast to recombinant C-terminally truncated E2, HCV-LP E2 is present as an E1/E2 heterodimer expressed from a full-length E1/E2 cDNA. Several studies have demonstrated that HCV-LPs contain E2 in a native conformation, which may resemble properly folded E2 in the virion (13, 45, 48). HCV-LPs and virions share distinct features in their cellular binding profiles, suggesting that cellular binding of HCV-LPs represents an appropriate model system for the study of HCV-host cell membrane interaction (44, 48, 50). In contrast to previous model systems for the study of virus-host interaction (1, 49), our model system uses a clearly defined ligand and

FIG. 6. Epitope mapping of antibodies inhibiting cellular binding of HCV-LPs. (A) To identify HCV-LP envelope epitopes targeted by human antibodies inhibiting cellular HCV-LP binding, an anti-HCV-positive serum (HCV+/H11001) with marked inhibition of binding activity (Fig. 3) was incubated with overlapping 15-mer peptides of the HCV envelope glycoproteins (comprising amino acid positions 201 to 758) derived from the HCV-J strain (26) or PBS (as a control) for 1 h at RT (peptide concentration, 100 μg/ml; serum dilution, 1:50). HCV-LPs (derived from the homologous isolate as the peptides) were then added to the peptide-antibody complexes and incubated for 1 h at 37°C. The HCV-LPs, antibodies, and peptides were added to HuH-7 cells and incubated for 1 h at 4°C. Following the removal of nonbound HCV-LPs, antibodies, and peptides by washing the cells in PBS, binding of HCV-LPs was detected by flow cytometry as described above. Inhibition of HCV-LP binding (as indicated on the y axis) was calculated as described in the legend to Fig. 2. The error bars indicate standard deviations of results of a representative experiment performed in triplicate. (B) Concentration-dependent reversion of HCV-LP inhibition of binding activity by E2 peptide 408. Anti-HCV-positive serum (dilution, 1:50) was preincubated with E2 peptide 408 (SQKIQLVNTNGSWHI; corresponding to amino acid positions 408 to 422) at the peptide concentrations indicated on the x axis. Cellular HCV-LP binding (corresponding to ΔMFI, indicated on the y axis) in the presence of the antiviral antibody-peptide complex was determined as described in Materials and Methods.
highly standardized conditions to quantify cellular binding (monoclonal antibodies and flow cytometry). Binding could easily be detected and quantified by mouse monoclonal anti-E2 antibodies, whereas human polyclonal anti-HCV antibody served as a reagent to confirm the specificity of the inhibition of binding.

In this study, we demonstrate for the first time that cellular HCV-LP binding is inhibited by anti-HCV-positive sera from HCV-infected patients. Specific inhibition of HCV-LP binding by antiviral antibodies is demonstrated by the following experimental findings: (i) only sera from HCV-infected patients, but not from uninfected control subjects, inhibited cellular HCV-LP binding in a concentration-dependent manner (inhibitor-specific inhibition of binding [Fig. 2 and 3]); (ii) only purified IgG from HCV-infected patients, but not from uninfected control subjects, inhibited cellular HCV-LP binding in a concentration-dependent manner (antibody-specific inhibition of binding [Fig. 3]); (iv) antibody-mediated inhibition of binding is reverted by preincubation with a defined viral envelope peptide (Fig. 6).

Using antibody-mediated inhibition of HCV-LP binding as a marker for virus neutralization at the level of envelope binding to the host cell membrane, our study provides for the first time a detailed functional characterization of anti-HCV antibodies in acute and chronic HCV infections. The key findings of our study are the following: (i) antibodies inhibiting HCV-LP binding are present in acute or chronic HCV infection; (ii) antibodies that inhibit HCV-LP binding are of low titer (<1:200); (iii) in the majority of studied individuals, antibody-mediated inhibition of cellular HCV-LP binding is subtype or isolate specific; (iv) the presence of antibodies with inhibition of HCV-LP binding activity may not predict virus clearance.

Using antibody-mediated inhibition of HCV-LP binding as a surrogate marker for virus neutralization, we conclude that virus-neutralizing antibodies are present in acute and chronic HCV infections. Since the presence of such antibodies did not correlate with the outcome of infection, we conclude that neutralizing antibodies presumably do not play a major role in virus clearance. Our data indicate that limited induction of high-titer antibodies with neutralization capacity, as well as isolate-specific restriction of neutralization, most likely contributes to the failure of antibody-mediated virus clearance in HCV infection.

The absence of a high-titer humoral immune response with neutralizing properties in patients with self-limited acute hepatitis C is in line with the concept that a robust and multispecific antiviral cellular immune response is central for sustained virus clearance in acutely infected individuals (22, 23, 30, 46). Several studies with humans have shown that virus clearance is associated with a strong HCV-specific CD4+ T helper and cytotoxic-T-cell response (for a review, see references 23, 30, and 46).

Recently, pseudotyped retroviral particles containing HCV glycoproteins have been suggested as a model system for HCV entry (5, 5a, 24). Whether the entry of retroviral HCV pseudoparticles containing heterologous retroviral proteins represents HCV entry leading to viral infection is not yet known. In a previously published pilot study, Bartosch et al. analyzed sera from four HCV-infected chimpanzees and one HCV-infected human for the presence of antibodies neutralizing pseudoparticle entry (5a). Interestingly, the authors could detect antibodies (titer, >1:320) neutralizing pseudoparticle entry in the single human individual and in two out of four chimpanzees that progressed to chronic HCV infection. Cross-neutralization of pseudoparticle entry was observed in one of the two animals and the single human (5a). In contrast, no antibodies with neutralizing properties were observed in two chimpanzees with self-limited infections (5a). Since only one human individual and four experimentally infected chimpanzees were assessed in this study (5a), it is difficult to compare the results obtained in the HCV pseudoparticle and HCV-LP models at the present time. Interestingly, both systems indicate the presence of cross-neutralizing antibodies in chronic HCV infection. A prospective study assessing serum samples from a large number of HCV-infected humans side by side is needed to compare findings obtained using the two systems. Studies are under way to address this important question.

We cannot exclude the possibility that anti-envelope antibodies with binding-inhibitory properties are present in antigen-antibody immune complexes. In this case, these antibodies are not detected by this assay or by ELISAs based on HCV-LPs (8) or individual envelope proteins (12, 21) or by other neutralization-of-binding assays (25, 38). Virus neutralization can also occur at stages of the viral life cycle following virus binding to the cell membrane (16). These stages include fusion of the viral envelope with the cell membrane, blocking of viral uncoating, and replication and would not be detected by our assay.

Interestingly, antibody-mediated inhibition of HCV-LP binding did not correlate with the immunoreactivity of anti-HCV antibodies against HCV-LPs or recombinant E1/E2 proteins as capture antigens in an ELISA (8). Several sera from patients with chronic HCV infection containing high-titer antibodies against HCV-LPs (>1:128,000) (8) or recombinant E1/E2 proteins (>1:64,000) did not result in inhibition of HCV-LP binding (data not shown). In contrast, several sera from patients with acute self-limited hepatitis C and low-titer anti-HCV-LP or anti-E1/E2 antibodies exhibited marked inhibition of HCV-LP binding (data not shown). The difference between the presence (as measured by HCV-LP [8] or E1/E2 [48, 29] ELISAs) and function (as measured by inhibition of HCV-LP binding [this study]) of anti-envelope antibodies is also reflected by the interaction of HCV-LPs with anti-HCV antibodies from patients infected with different HCV genotypes. Using HCV-LPs as capture antigens in an ELISA format, HCV-LPs of genotype 1b cross-interacted with antibodies of patients infected with HCV genotypes 1a, 2, 3, 4, 5, and 6 (8). In contrast, cross-reactivity between the same HCV subtypes (1a or 1b) was a less frequent event (4 of 13 patients) in antibody-mediated inhibition of HCV-LP binding. Although we cannot exclude the possibility that cross-reactivity observed in studies using the HCV-LP ELISA may be partly due to the interaction of antibodies with partly deenveloped nucleocapsids present in the HCV-LP preparation, our data indicate that HCV elicits abundant antibodies directed against the HCV structural proteins (as demonstrated in HCV-LP ELISA) (8) that have little or no ability to block envelope docking to the target cell (this study).

Several mechanisms may explain this finding. First, the majority of anti-HCV antibodies are targeted against epitopes of
the HCV structural proteins that are not required for virus neutralization. Envelope epitopes presented on the surfaces of HCV-LPs have been mapped recently (13, 45). Our data are consistent with the hypothesis that many epitopes exposed on the HCV-LP surface and recognized by human antiviral antibodies are most likely not target epitopes for virus neutralization. Epitopes mediating viral envelope binding and targeted by virus-neutralizing antibodies are still poorly defined. So far, two epitopes in envelope glycoproteins E1 and E2 (amino acids 197 to 207 and 640 to 653) have been proposed to play a potential role in mediating cellular HCV-LP binding (44). In chimpanzees, the E2 HVR-1 has been inferred to represent a potential B-cell epitope associated with virus neutralization (18). In contrast, antibodies directed against several epitopes of the HCV core protein were not able to block HCV-LP binding to target cells, suggesting that the nucleocapsid is not involved in mediating HCV-LP cellular binding (M. Triyatni and T. J. Liang, unpublished observations). Using a panel of overlapping envelope peptides covering the entire HCV envelope, we identified an epitope of the N-terminal E2 region (SQKOLVNTNGSWHI; amino acid positions 408 to 422) as the target of human antiviral antibodies inhibiting cellular particle binding. This epitope overlaps with the E2 hypervariable region and corroborates the importance of this region for the binding of the viral envelope to the cell surface. Interestingly, the same region has been shown to be involved in E2-CD81 interaction (34, 35) and in mediating cellular binding and entry of HCV pseudoparticles (5, 24).

Second, the presence of HCV as a highly variable pool of rapidly mutating viral quasispecies in an individual patient may contribute to virus escape from antibody-mediated virus neutralization. A previous study elegantly demonstrated that a monoclonal antibody raised against the E2 hypervariable region from HCV recovered from a single patient induced protection against homologous HCV infection in chimpanzees but not against the emergence of neutralization escape mutants that were found to be already present in the complex viral quasispecies of the inoculum (18). This study, performed with serum samples from a single patient, suggested that neutralizing antibodies can be induced but isolated-specific virus neutralization and the rapid emergence of viral escape mutants may represent a major limitation for antibody-mediated virus neutralization in HCV infection. Our data identifying antibodies with strain-specific neutralization of viral envelope binding in a large serum panel of 21 well-characterized patients indicates that the proposed mechanism may indeed contribute to the failure of antibody-mediated virus neutralization in patients with HCV infection.

Third, the virus has developed highly specific escape strategies to evade antibody-mediated neutralization. These strategies may include conformational masking of receptor binding sites following envelope-antibody interaction (27) or alteration in envelope N-glycosylation motifs (11, 47), as described recently for human immunodeficiency virus. The HCV-LP-based model system described in this study may be helpful in defining similar mechanisms for HCV.

In summary, this model system may be useful in providing a detailed map of epitopes targeted by human antiviral antibodies on the level of envelope-cell surface receptor interaction. Furthermore, the HCV-LP-based model system may allow the elucidation of viral escape mechanisms for the evasion of antibody-mediated neutralization. Understanding these mechanisms may ultimately provide important clues for the development of new antivirals inhibiting virus-cell surface interaction, as well as define strategies for the efficient induction of virus cross-neutralizing antibodies for vaccine development.

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