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Neutralizing Antibodies against Hepatitis C Virus and the Emergence of Neutralization Escape Mutant Viruses

YOHKO K. SHIMIZU,[1]* MINAKO HIJIKATA,[2] AIKICHI IWAMOTO,[2] HARVEY J. ALTER,[3] ROBERT H. PURCELL,[4] AND HIROSHI YOSHIKURA[2]

Department of Infectious Diseases and Vaccine Control, National Institute of Health, Tokyo 208,[1] and Department of Bacteriology, Faculty of Medicine, University of Tokyo, Tokyo 113,[2] Japan, and Department of Transfusion Medicine[3] and Laboratory of Infectious Diseases,[4] National Institutes of Health, Bethesda, Maryland 20892

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We developed an in vitro assay for antibodies to hepatitis C virus (HCV) that bind to virions and prevent initiation of the replication cycle in susceptible cells in vitro. These antibodies therefore appear to be capable of neutralizing the virus. Using this assay and a standard inoculum of HCV of known infectivity, we have measured the antibody in serial serum samples obtained from the same chronically infected patient over 14 years following onset of his hepatitis. Such antibody was found in sera collected within 5 years of onset of hepatitis but not in later sera. In double immunoprecipitation experiments with anti-human immunoglobulin, the same sera that contained neutralizing antibody were found to contain antibody that bound to HCV to form antigen-antibody complexes immunoprecipitable with anti-human globulin. Similarly, plasma collected from this patient in 1990, 13 years after onset of hepatitis, and which contained HCV that had diverged genetically from the 1977 strain, did not contain antibody capable of neutralizing either the 1977 or the 1990 strain of HCV. However, plasma collected a year later (1991, 14 years after onset of hepatitis) contained neutralizing antibody to the 1990, but not the 1977, strain of HCV. These results suggest that HCV does induce antivirion antibody, as measured by blocking of initiation of the replication cycle of virus in cells and by the formation of immunoprecipitable antigen-antibody complexes but that these antibodies are isolate specific and change over time. Thus, these antivirion antibodies function as neutralizing antibodies and are probably in vitro correlates of the attempt of the host to contain the emergence of neutralization-resistant variants of HCV over time.

Following viral infection, the host's immune system produces antibodies specific to the infecting pathogen. Antibodies that bind to and inactivate the virions, generally referred to as neutralizing antibodies, play an important role in prevention and possibly recovery from viral infections; viral antigens that stimulate neutralizing antibodies have been used successfully as vaccines for years.

In many viral infections, the appearance of neutralizing antibody coincides with the onset of recovery from the infection, and neutralizing antibody may play a role in removing virus from the circulation in infections that are characterized by viremia. However, hepatitis C virus (HCV) infection is persistent in over 50% of cases (1) and may result in various forms of chronic hepatitis and other liver disease, including cirrhosis and hepatocellular carcinoma (7). Several studies have documented the appearance of antibodies to the structural proteins of HCV (the nucleocapsid or core protein and the putative envelope proteins, E1 and E2/NS1) relatively early after onset of hepatitis; these responses are followed by antibody to one or more of the immunogenic nonstructural proteins (NS3, NS4, and NS5), often late after the onset of hepatitis (15). The persistence of HCV in most infected individuals, despite the development of antibodies to the envelope proteins, and the observation that chimpanzees can be reinfected with HCV upon challenge with heterologous or even homologous strains (3) have raised questions concerning the development of neutralizing antibodies in HCV infection and their clinical significance. It is vital to identify the critical epitopes that elicit neutralizing antibody responses in order to understand the pathogenesis of persistent infection and to develop effective vaccines.

To address these questions, there is a need to develop an assay for detecting neutralizing antibodies. To date, cell culture systems for the replication of HCV have not been useful for this purpose, and chimpanzees, the only animal host suitable for studies of HCV, are too expensive and difficult to obtain and maintain for more than a few carefully selected studies. For these reasons, an in vitro assay utilizing cultured cells is needed. We have recently shown that a clone (clone 10-2) derived from a human T-cell line, HPB-Ma, supported partial replication of HCV and that the relative ability of HCV to adsorb to presumed viral receptors on the surface of the cells correlated well with the relative infectivity of these strains of HCV for chimpanzees (14).

The mechanism of neutralization of most viruses by antibodies is poorly understood. Several mechanisms have been proposed. These include immune aggregation, resulting in an apparent diminished titer; physical blocking of attachment of the virion to the viral receptor on the cell surface, thereby preventing the first step in replication; interference with penetration of the virus through the cell membrane by perturbing essential virus functions such as proteolytic cleavage or fusion; and interference with uncoating by blocking intracellular dissociation of viral proteins. Furthermore, neutralization of viruses in vivo and in vitro may not be identical, but neutralizing antibodies are generally measured in vitro because it is much simpler. The end point of such neutralization is usually the prevention of virus-induced cytopathic effect in the cell

* Corresponding author. Mailing address: Department of Bacteriology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan. Phone: 3-3812-2111, ext. 3409. Fax: 3-5684-9374.

TABLE 1. Properties of HCV inocula[a]

Characteristic[b]	Source and inoculum					
	Patient H		Implicated donor (number 6)	Chimp, acute (number 34)	Patient F, chronic (F)	Patient N, chronic (number 4)
	Acute (H77)	Chronic (H90)				
Buoyant density (flotation at 1.063 g/ml)	Low	Low	Low	Low	High[c]	High[c]
Immunoprecipitation with anti-human immunoglobulin	No	No	No	No	Yes[c]	Yes[c]
Anti-HCV titer by immunofluorescence	<10	160	10	<10	160	160
In vivo infectivity in chimps (CID/ml)	$10^{6.5}$	+[d]	$10^{5.5}$	NT[e]	10^{0}	$<10^{2}$
In vitro infectivity per ml (TCID/ml)	10^{5}	+	$\geq 10^{2.5}$[f]	$10^{2.5}$	10^{1}	$<10^{1}$
Genome (titer/ml) by RT-PCR	10^{7}	10^{4}	10^{5}	10^{5}	10^{5}	10^{4}

[a] Data are from references 6, 9, and 14 or are unpublished.
[b] CID, chimpanzee infective dose; TCID, tissue culture infective dose.
[c] Antigen-antibody complexes were present.
[d] +, not titered.
[e] NT, not tested.
[f] A higher titer was obtained in some experiments.

substrate, but many other direct and indirect end points have been used. However, all are based on the assumption that neutralizing antibodies block replication of the virus regardless of the stage of infection at which neutralization occurs. Similarly, variant viruses that can no longer be neutralized by a given antiserum (neutralization escape mutants) are detected by their ability to replicate despite exposure to antibodies that neutralize the parent virus, regardless of the mechanism of neutralization.

In vivo neutralization of HCV has not been characterized, but the HPB-Ma cell line provides a possible host cell system for studying neutralization of this virus in vitro. We have shown previously (14) and in this study that HCV undergoes most, if not all, of its replication cycle in HPB-Ma, and we show herein that essential replicative steps, such as transcription, are prevented by neutralizing antibodies to HCV. Furthermore, we provide the first direct evidence for the emergence of neutralization escape mutants of HCV and the host's evolving immune response to such mutant viruses, mainly the development of specific neutralizing antibodies to sequentially emerging variant viruses.

MATERIALS AND METHODS

Cell line. The HPB-Ma cell line was obtained by infecting HPB-ALL cells with an amphotropic murine leukemia virus pseudotype of murine sarcoma virus (19). Cloning of the cells was performed by limiting dilution, and one of the clones, clone 10-2, which was found to be the most sensitive to HCV infection, was used in subsequent experiments.

Viruses. The inoculum used for the study of HCV infection in HPB-Ma clone 10-2 was a serum (serum 34) obtained from a chimpanzee infected with the fifth chimpanzee passage of HCV strain F. Serum 34 has been used as a principal inoculum in our previous studies (14) (Table 1). Plasmas H77, F, 6, and 4, which have been well characterized (9, 14; unpublished data), are summarized in Table 1 and were employed for further investigation of the relationship between replication in vitro and infectivity in vivo of the HCV strains they contain. Isolate H77, representing HCV in the acute-phase plasma, and H90, a genetically divergent isolate in a chronic-phase plasma obtained 13 years after infection of patient H (Table 1) (4, 12), were used as target viruses for the neutralization assay. The genome titer of H77 is 10^7/ml and that of H90 is 10^4/ml, as measured by PCR. Both have been shown to be infectious in chimpanzees (6; unpublished data).

Patient studied. Patient H developed biochemical evidence of hepatitis 7 weeks after transfusion in 1977 (4). The patient became positive for HCV RNA during week 2 and for antibodies to HCV capsid protein during week 8 (measured with recombinant C22 antigen) and to HCV nonstructural proteins NS3 and NS4 (measured with recombinant C33 and C100 antigens [Chiron, Emeryville, Calif.]) during week 11 and has remained positive for the past 15 years. The biochemical and virologic course of the infection in this patient are shown in Results. The serum samples used in this study were obtained on 12 July 1977 (H77), 23 June 1978 (H78), 16 April 1979 (H79-1), 27 November 1979 (H79-2), 11 June 1982 (H82), 9 February 1989 (H89), 1 August 1990 (H90), and 12 September 1991 (H91).

RT-PCR. For the detection of HCV RNA, extraction of nucleic acids, reverse transcription (RT), and a two-step PCR assay with nested primers were performed as described previously (14). For amplification of HCV RNA in the samples from patient H, the primers were synthesized, on the basis of the published sequences of H77 and H90 (12), to detect the putative NS5 region (from map positions 8395 to 8606 of the genome). In addition, we employed a primer set located in the 5′ noncoding region (from map positions 9 to 331) which was shown to be conserved among HCV strains (2). In the study of HCV replication in HPB-Ma clone 10-2, the positive and negative strands of HCV RNAs were detected by performing RT in the presence, respectively, of the antisense primer or the sense primer. An internal primer pair was used for the second round of PCR.

Restriction enzyme analysis of PCR product. Samples (50 μl) of PCR products were extracted with phenol-chloroform and precipitated with ethanol. The DNA fragments obtained were digested with a restriction enzyme, *Bfa*I, at 37°C for 60 min and analyzed by electrophoresis on 4% Nusieve agarose (FMC Bioproducts, Rockland, Maine).

Immunoprecipitation. Immunoprecipitation was performed as described both previously (9) and in Results.

Immunofluorescence. Immunofluorescence was performed as described previously (9, 14).

RESULTS

Correlation between infectivity in vivo and replication in vitro. Preliminary results suggested that the infectivity titer of HCV in vivo and the titer of virus as measured with HPB-Ma clone 10-2 cells paralleled each other. In order to further characterize the interaction of HCV virions and cells, positive-sense HCV RNA (the virion strand) and the negative strand

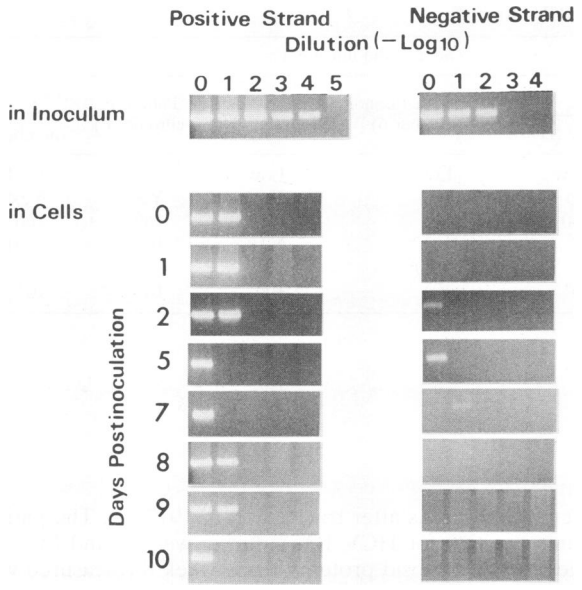

FIG. 1. Correlation between adsorption or penetration of HCV into cells and evidence for replication. Inoculum 34, containing HCV, was serially diluted in 10-fold increments. Aliquots were mixed with a suspension of cells and incubated as described in the text. After being washed, the cells were cultured, harvested at intervals, and tested by RT-PCR for the presence of HCV genomic RNA (positive strand and negative strand) in the cells. The results were compared with positive strand and negative strand detected in the inoculum (top).

(the replicative intermediate strand) were monitored at intervals in the cells after adsorption of the virus. Inoculum 34, containing HCV, was diluted serially in 10-fold increments. Two hundred microliters of each dilution was mixed with 2 ml of the cell suspension (5×10^5 cells per ml). After adsorption at 37°C for 2 h, the cells were washed twice and cultured. At intervals, the cells were harvested for analysis of intracellular HCV RNA by RT-PCR. As shown in Fig. 1, the negative strand was detected on days 2 and 5 in cells inoculated with a dilution of 10^0 and on day 7 in cells inoculated with a dilution of 10^{-1}. The positive strand persisted in cells inoculated with a 10^0 dilution for at least 10 days. Cells inoculated with the 10^{-1} dilution contained positive-strand RNA through day 2 and again on days 8 and 9. This experiment demonstrated that the infectivity titer as measured by detection of the positive or negative strand within cells was comparable to the titer of virus interacting with cells on day 0. We showed previously that appearance of intracellular positive- and negative-strand HCV RNA as measured by PCR correlated with synthesis of intracellular viral proteins as measured by immunofluorescence (9, 14). Thus, measurement of the early steps of viral replication (attachment and penetration) yielded the same results as measurement of later steps in the replication cycle (transcription and translation).

We next compared two HCV-containing samples (H77 and 6) with high-infectivity titers when tested in chimpanzees and two samples with low infectivity (F and 4) for their ability to replicate in HPB-Ma clone 10-2 cells (Table 1). The inocula were serially diluted in 10-fold increments, and 100 μl of each dilution was mixed with 1 ml of 5×10^5 cells. After incubation at 37°C for 2 h, the cells were washed twice with 20 ml of cell culture medium and were distributed into five bottles (5 ml each) and incubated at 37°C. One bottle was harvested daily and assayed by RT-PCR for the cell-associated positive-strand HCV RNA. As seen in Fig. 2, two striking differences between the inocula with high in vivo infectivity and those with low infectivity were observed: (i) the in vitro replication efficiency (the maximum dilution that yielded a positive HCV RNA signal in the cells on day 0 or 1 divided by the genome titer of the inoculum) was 1:10 for plasmas H77 and 6, whereas the ratio for samples F and 4 was 1:1,000; and (ii) the intracellular HCV RNA of the two highly infectious plasmas persisted for 5 to 15 days, whereas the intracellular HCV RNA following infection with the two low-titer plasmas was detected only transiently. We have shown previously that these two low-titer samples contained HCV virions complexed to antibodies, whereas the highly infectious plasmas contained HCV virions that were not complexed to antibody (9). Thus, antigen-antibody complexes appear to be unable to initiate sustained replication in the cells, whereas free virions are able to do so with an efficiency approaching that of their ability to initiate infection in vivo; therefore, the results of in vitro neutralization assays appear to parallel the results of neutralization in vivo.

Neutralization by sera taken at different stages of HCV infection. We attempted to determine whether neutralization of HCV virions in vitro could be demonstrated with sera obtained at intervals from the same patient over 15 years. The time of sampling is shown in Fig. 3. Before such testing, the samples were all tested at a 1:10 dilution for the presence of HCV that could infect the cells and thereby cause confusion in the interpretation of the experiment. Samples H78, H79-1, and H82 were all negative for HCV when tested by the in vitro infection assay, but H79-2, H89, H90, and H91 were all positive. However, after heating at 56°C for 30 min, all of the samples were negative for HCV in the infection assay (Fig. 4).

For the neutralization experiment, we used a 10^{-3} dilution of acute-phase plasma containing $10^{3.5}$ chimpanzee infectious doses (10^2 tissue culture infectious doses) of HCV per ml (6, 9). This plasma has been shown to be negative for antibodies to HCV by immunofluorescence, enzyme immunoassay, immunoprecipitation, and determination of buoyant density (9). Sera or plasma obtained at intervals from patient H (H78, H79-1, H79-2, H82, H89, H90, and H91) were diluted in 10-fold increments, inactivated at 56°C for 30 min, further diluted in 2-fold increments, and examined for the presence of neutralizing antibodies as described below. To perform the neutralization assay, 60 μl of diluted H77 was mixed with 60 μl of a 1:10, 1:20, 1:40, or 1:80 dilution of the sample to be tested for antibody or a negative control serum and incubated at 4°C overnight. A 100-μl aliquot of each mixture was used for the neutralization assay, and the remaining 20 μl was used in an immunoprecipitation assay (9). The 100-μl sample was inoculated into 1 ml of a suspension of 3×10^5 cells. After incubation for 2 h at 37°C, the cells were washed twice in 20 ml of phosphate-buffered saline and extracted for detection of the cell-associated HCV genome by RT-PCR. The results are shown in Fig. 5 (left panels). Normal serum did not block the uptake of virus by the cells at any of the dilutions tested. In contrast, samples H78 and H79-2 inhibited attachment or penetration at dilutions of 1:10 and 1:20, and samples H79-1 and H82 similarly inhibited at dilutions as high as 1:40. In contrast, samples H89, H90, and H91 did not neutralize at any of the dilutions tested. We confirmed that the bands detected in the assay were, indeed, generated from HCV isolate H77 and not from HCV in sample H90, which was used as a source of putative antibody, by digesting the bands with *Bfa*I restriction enzyme (shown by an asterisk in the figure), an enzyme capable of cleaving the H77 but not the H90 sequence (the latter has lost the restriction site because of a C-to-T mutation

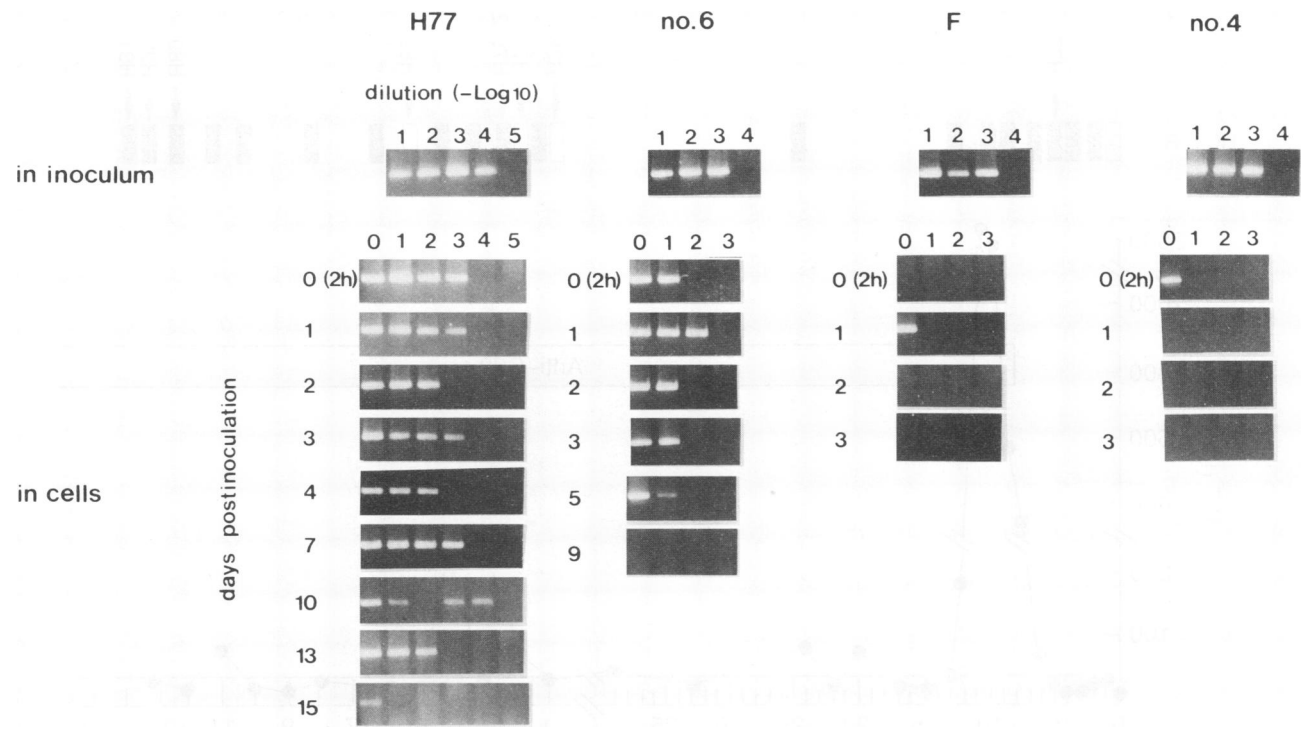

FIG. 2. Adsorption and penetration into cells and duration of detection of intracellular HCV (as measured by HCV RNA) compared with the titer of HCV RNA in the inocula. Two inocula with high in vivo infectivity, H77 and 6, and two with low infectivity, F and 4, were tested. For amplification of HCV RNA by RT-PCR, a primer set detecting the 5′ noncoding region was employed.

at nucleotide position 8485) (12). Thus, we detected neutralization of the H77 isolate of HCV by serum or plasma samples from the same chronically infected patient over a period bridging the first 5 years of chronic infection in this patient. By 1989, 12 years after infection with HCV, the patient no longer had antibodies that neutralized the H77 isolate of HCV.

We reported previously the genetic drift of HCV strain H over 13 years in patient H (12). To determine whether this genetic drift was reflected in a serologic drift over this interval of time as a possible explanation for the loss of neutralizing antibodies, we performed the neutralization assay in the same manner as described above but with a 1:10 dilution of the H90 isolate of HCV (unheated) instead of the H77 isolate as the target virus. As shown in the right panel of Fig. 5, the H82 sample from patient H failed to neutralize the H90 isolate even though it neutralized the H77 strain at a dilution of 1:40. In contrast, the H91 sample neutralized the H90 isolate of HCV up to a dilution of 1:40 but did not neutralize the H77 isolate at any dilution tested. Thus, it appeared that a serological variant of HCV arose during the course of chronic infection in patient H and that neutralizing antibody was reactive with the temporally most recent prior isolate of HCV in each case but not subsequent or temporally distant prior isolates.

Immunoprecipitation by sera containing neutralizing antibody. The 20 μl of the mixture remaining from the neutralization assay was mixed with an equal volume of undiluted rabbit anti-human immunoglobulin (Cappel, Durham, N.C.). The mixtures were incubated overnight at 4°C, centrifuged at 680 × *g* for 15 min, and separated into supernatants and pellets. Both were extracted and tested by RT-PCR for the presence of HCV RNA. As shown in Fig. 6, HCV isolate H77 plus normal serum was not immunoprecipitated at any dilution tested. In contrast, isolate H77 mixed with sample H78 or H82 was immunoprecipitated at dilutions that paralleled the neutralization titers of the samples, as shown in Fig. 6. These results indicate that the ability of certain samples to neutralize HCV correlates with their ability to form antigen-antibody complexes with HCV.

DISCUSSION

The replication of HCV in cell culture is clearly not optimum. Only a small proportion of cells are infected, even when selected clones of the cells are employed, and the level of replication varies over time in culture, as seen in Fig. 1 and 2 and as reported previously for HPB-Ma cells (14) and for another continuous T-cell line, molt-4 Ma (13). This may be related to the growth rate of the cells (13) or to other factors. However, at least partial replication of HCV in these cell lines can be convincingly demonstrated by detection of intracellular positive and negative strands of HCV RNA by PCR, detection of intracytoplasmic negative-strand HCV RNA by in situ hybridization, and detection of expressed viral core and NS4 proteins by immunofluorescence with monoclonal antibodies (13, 14).

The titers of anti-HCV neutralizing antibody detected in this study were not high but were similar to those for neutralizing antibodies against many other viruses as measured by conventional in vitro neutralization tests. The titer of antibody appeared to increase over time before disappearing. Although there were slight variations in titer over the 4 years during which neutralization could be detected, it is surprising that it was as consistent as it was. Similarly, subsequent samples from patient H were consistently negative for antibody to the H77 virus. The limited neutralization data obtained with the H90

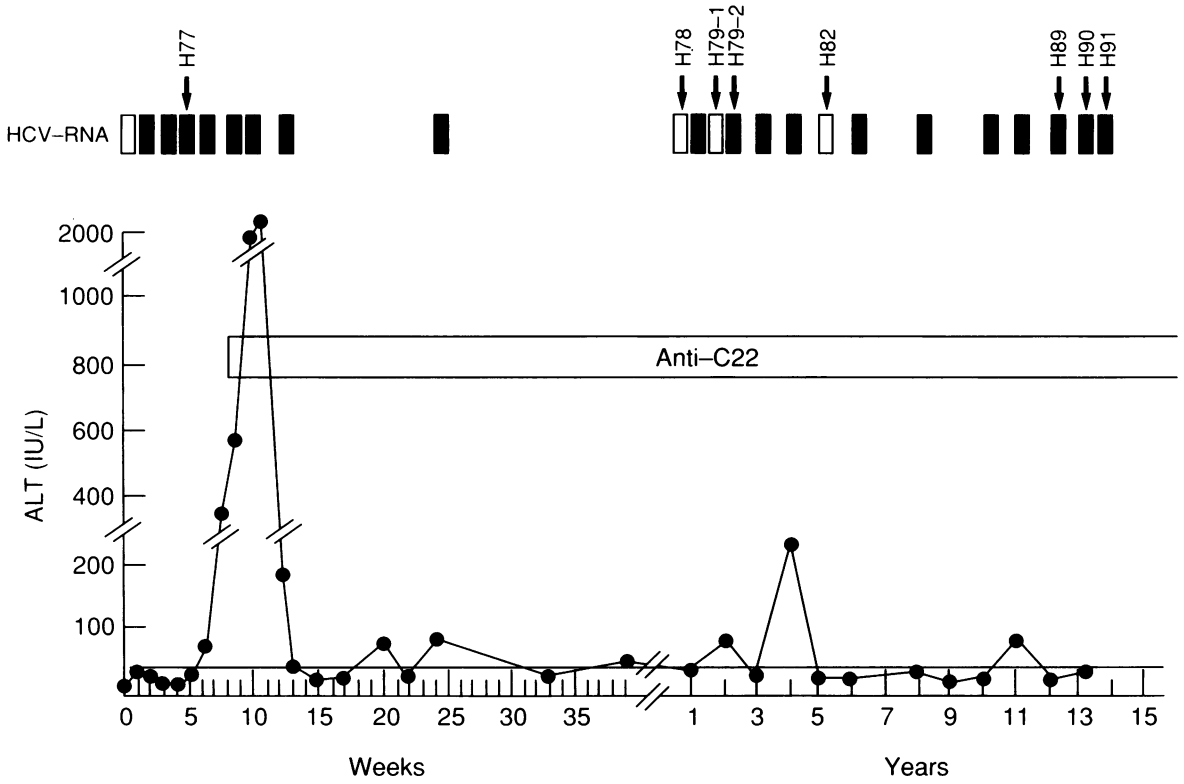

FIG. 3. Biochemical and virological course of HCV infection in patient H. Open and solid bars indicate, respectively, negative and positive assays for HCV RNA. Arrows identify the samples that were tested for neutralization and immunoprecipitation. See text for details. The horizontal bar indicates the interval during which serum was positive for antibody to HCV capsid protein (anti-C22).

virus strongly suggests that the host immune response responds to the sequential emergence of variants of HCV by the sequential development of variant-specific antibodies.

Although these neutralization studies have been carried out with only two variants of one strain of HCV, the evaluation of other strains for the presence of antigen-antibody complexes (9) and the demonstration of reduced infectivity of such complexes both in vivo and in vitro (14) strongly suggest that neutralization of HCV is a general phenomenon not limited to this patient. Additional studies are in progress, but these are difficult to perform because of the need for well-characterized viruses that have been serially collected over time and properly stored. It will, of course, be important to extend these studies to include both closely related and genetically diverse strains of HCV in order to determine the breadth of the immune response. These studies are currently in progress.

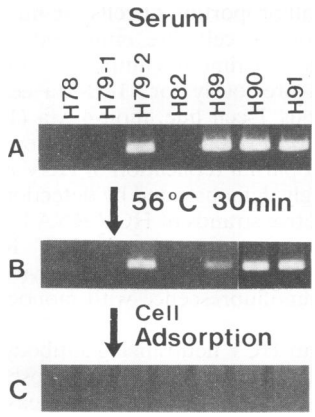

FIG. 4. Inability to detect the adsorption and penetration of HCV into cells after heat inactivation of the test sera from patient H. Fifty microliters of a 1:10 dilution of the sera was tested by RT-PCR for HCV RNA before (A) or after (B) treatment at 56°C for 30 min. Fifty microliters of the heat-treated sera was inoculated into the cell suspension and incubated at 37°C for 2 h. After being washed, the cells were pelleted and tested for cell-associated HCV RNA (C).

We have shown that the in vitro infectivity titer of selected strains of HCV parallels the infectivity titer of these strains in chimpanzees and that binding of virus to tissue culture cells is a necessary first step in replication. We have also shown that the titer of native virus bound to the cells in the first 2 h of incubation is an accurate predictor of the infectivity titer of virus as measured by assays for viral transcription and translation later in the replication cycle. In contrast, virus that has been altered by complexing with antibody or heating does not bind efficiently to cells and, hence, does not initiate the replication cycle. That these effects involve the surface of the virion is suggested by other studies (unpublished) in which HCV was exposed to UV light, an inactivation procedure that inactivates the viral RNA but not the structural proteins. Such irradiated HCV efficiently bound to the HPB-Ma cells but was incapable of initiating replication. Because the degree of binding of HCV to HPB-Ma cells was an accurate predictor of replication of virus, we were able to modify the neutralization test to encompass only the initial steps of attachment, penetration, and possibly early uncoating. However, as we have

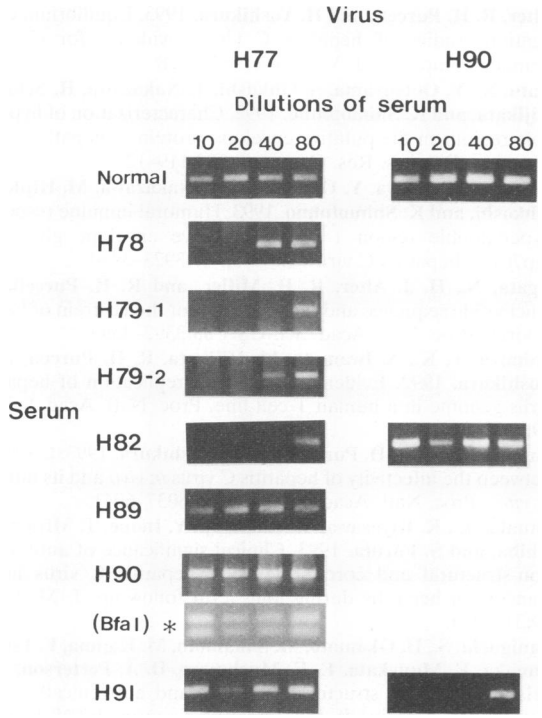

FIG. 5. Neutralization of HCV isolates H77 and H90 by sera obtained from chronically infected patient H over time. Asterisk (*) indicates fragments obtained after digestion with *Bfa*I of the PCR product obtained after incubation of H77 with H90 (left panel), confirming that the PCR product was derived from the H77 virus and not the H90 virus.

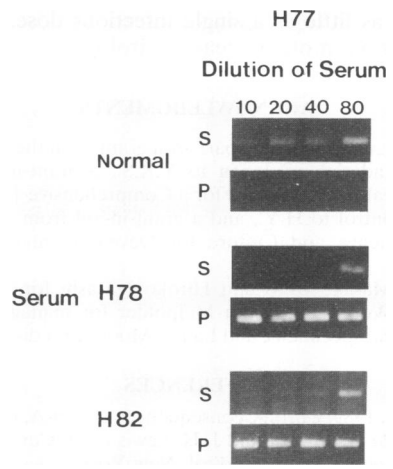

FIG. 6. Double immunoprecipitation with anti-human immunoglobulin. The assay was carried out as described previously (9) and in the text. After separation, each supernatant (s) and pellet (p) was examined for the presence of HCV RNA by RT-PCR.

demonstrated, such a test fulfills the definitions of in vitro neutralization.

The genetic sequence of the N-terminal region of the E2/NS1 gene of HCV is highly variable and has been called hypervariable region 1 (HVR1) (8, 17). This region has been shown to have a high rate of mutation in HCV strains recovered from the same chronically infected patient over time (10, 12, 18). In some patients, the rate of emergence of variants of HCV does not appear to be constant but corresponds with exacerbations of hepatitis. These emerging variants of HCV appear to be neutralization escape mutants of the virus. Evidence for this comes from serologic studies in which antibodies that were HVR1 specific were shown to appear sequentially in response to the emergence of variant viruses that were genetically altered in the HVR1 (11, 16). Our neutralization and immunoprecipitation assays confirm that, after infection, HCV-induced antibodies that bound to the virions and prevented initiation of viral replication emerged and that these antibodies persisted for several years but eventually declined and were replaced by neutralizing and immunoprecipitating antibodies that were directed against a subsequent variant of the virus. We have not established that the neutralizing antibodies are directed against epitopes of the putative envelope proteins, but the fact that they correlate with the detection of antibodies that bind to the surface of the virion, coupled with the knowledge that neutralizing antibodies to other enveloped viruses are almost always directed against the envelope proteins, make it likely that these are anti-envelope antibodies. This conclusion is also supported by reports of the emergence of variants of HCV that differ genetically and serologically in the HVR1 of the E2/NS1 gene product and that stimulate sequential immune responses to the sequentially emerging HVR1 variants, as noted above (11, 18). The existence of variant viruses with mutated envelope proteins could explain why chimpanzees convalescent from infection with HCV were not protected against subsequent challenges with similar or even the identical strain of HCV (3).

An important question is whether in vitro neutralization studies performed with continuous T-lymphocyte cell lines have relevance to neutralization in vivo. Preliminary studies of in vitro neutralization, in which the putative antigen-antibody complexes were tested for residual infectivity by inoculating chimpanzees, has revealed an exact correlation between in vitro neutralization by using cell culture and in vitro neutralization by using chimpanzees for detection of viral replication (5). Thus, the neutralization assay described herein may be a very useful tool for evaluation of passive and active immunoprophylaxis against HCV infection in chimpanzees and humans.

However, the demonstrated clonal specificity of the neutralizing antibodies may be an impediment to vaccine development. It will now be necessary to search in the HCV envelope proteins for neutralization-related epitopes that are widely conserved among HCV strains. It will also be important to determine the breadth of neutralizing capacity of naturally occurring and vaccine-induced neutralizing antibodies. Finally, it will be important to determine the biological constraints to the sequence variation found within the HVR1, if, in fact, this is an important site of neutralization. It may be that a limited number of sequences are permissible in this region and that a mixture of variant sequences spanning the range of variability of HVR1 could be incorporated into a polyvalent vaccine. Our observation that, following infection, HCV actually induced neutralizing antibodies in humans is a very encouraging first step in answering these questions.

The use of PCR to study virus-antibody interactions on the one hand and the adsorption of viruses to cells on the other provides new and sensitive alternatives to techniques that utilize radiolabelled virus particles, because the latter often are associated with a high background of nonspecific binding of unincorporated radioisotopes or damaged virions. In addition, the sensitivity of the new PCR-based assays, which permit the

detection of as little as a single infectious dose, should have wide application in other areas of virology.

ACKNOWLEDGMENTS

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REFERENCES

1. **Alter, H. J.** 1989. Chronic consequences of non-A, non-B hepatitis, p. 83–97. *In* L. B. Seeff and J. H. Lewis (ed.), Current perspective in hepatology. Plenum Medical, New York.
2. **Bukh, J., R. H. Purcell, and R. H. Miller.** 1992. Sequence analysis of the 5′ non-coding region of hepatitis C virus. Proc. Natl. Acad. Sci. USA **89:**4942–4946.
3. **Farci, P., H. J. Alter, S. Govindarajan, D. C. Wong, R. Engle, R. R. Lesniewski, I. K. Mushahwar, S. M. Desai, R. H. Miller, N. Ogata, and R. H. Purcell.** 1992. Lack of protective immunity against reinfection with hepatitis C virus. Science **258:**135–140.
4. **Farci, P., H. J. Alter, D. Wong, R. H. Miller, J. W. Shih, B. Jett, and R. H. Purcell.** 1991. Long-term study of hepatitis C virus (HCV) replication in the course of non-A, non-B hepatitis: correlation with antibody response and clinical outcome. N. Engl. J. Med. **325:**98–104.
5. **Farci, P., H. J. Alter, D. Wong, M. Shapiro, and R. H. Purcell.** 1993. Prevention of HCV infection in chimpanzees following antibody-mediated *in vitro* neutralization. Hepatology **18:**111A. (Abstract.)
6. **Feinstone, S. M., H. J. Alter, H. Dienes, Y. K. Shimizu, H. Popper, D. Blackmore, D. Sly, W. T. London, and R. H. Purcell.** 1981. Non-A, non-B hepatitis in chimpanzees and marmosets. J. Infect. Dis. **144:**588–598.
7. **Gilliam, J. H., K. R. Geissinger, and J. E. Richter.** 1984. Primary hepatocellular carcinoma after chronic non-A, non-B post-transfusion hepatitis. Ann. Intern. Med. **101:**794–795.
8. **Hijikata, M., N. Kato, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, and K. Shimotohno.** 1991. Hypervariable regions in the putative glycoprotein of hepatitis C virus. Biochem. Biophys. Res. Commun. **175:**220–228.
9. **Hijikata, M., Y. K. Shimizu, H. Kato, A. Iwamoto, J. W. Shih, H. J. Alter, R. H. Purcell, and H. Yoshikura.** 1993. Equilibrium centrifugation studies of hepatitis C virus: evidence for circulating immune complexes. J. Virol. **67:**1953–1958.
10. **Kato, N., Y. Ootsuyama, S. Ohkoshi, T. Nakazawa, H. Sekiya, M. Hijikata, and K. Shimotohno.** 1992. Characterization of hypervariable regions in the putative envelope protein of hepatitis C virus. Biochem. Biophys. Res. Commun. **189:**119–127.
11. **Kato, N., H. Sekiya, Y. Ootsuyama, T. Nakazawa, M. Hijikata, S. Ohkoshi, and K. Shimotohno.** 1993. Humoral immune response to hypervariable region 1 of the putative envelope glycoprotein (gp70) of hepatitis C virus. J. Virol. **67:**3923–3930.
12. **Ogata, N., H. J. Alter, R. H. Miller, and R. H. Purcell.** 1991. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. Proc. Natl. Acad. Sci. USA **88:**3392–3396.
13. **Shimizu, Y. K., A. Iwamoto, M. Hijikata, R. H. Purcell, and H. Yoshikura.** 1992. Evidence for *in vitro* replication of hepatitis C virus genome in a human T cell line. Proc. Natl. Acad. Sci. USA **89:**5477–5481.
14. **Shimizu, Y. K., R. H. Purcell, and H. Yoshikura.** 1993. Correlation between the infectivity of hepatitis C virus *in vivo* and its infectivity *in vitro*. Proc. Natl. Acad. Sci. USA **90:**6037–6041.
15. **Tanaka, E., K. Kiyosawa, Y. Nakatsuji, Y. Inoue, T. Miyamura, J. Chiba, and S. Furuta.** 1993. Clinical significance of antibodies to non-structural and core proteins of hepatitis C virus in post-transfusion hepatitis during long term follow-up. J. Med. Virol. **39:**318–324.
16. **Taniguchi, S., H. Okamoto, M. Sakamoto, M. Kojima, F. Tsuda, T. Tanaka, E. Munekata, E. E. Muchmore, D. A. Perterson, and S. Mishiro.** 1993. A structurally flexible and antigenically variable N-terminal domain of the hepatitis C virus E2/NS1 protein: implication for an escape from antibody. Virology **195:**297–301.
17. **Weiner, A. J., M. J. Brauer, J. Rosenblatt, K. H. Richman, J. Tung, K. Crawford, F. Bonino, G. Saracco, Q. L. Choo, M. Houghton, and J. H. Han.** 1991. Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. Virology **180:**842–848.
18. **Weiner, A. J., M. Geysen, C. Christopherson, J. E. Hall, T. J. Mason, G. Saracco, F. Bonino, K. Crawford, C. D. Marion, K. A. Crawford, M. Brunetto, P. J. Barr, T. Miyamura, J. McHutchinson, and M. Houghton.** 1992. Theoretical and empirical evidence for immune selection of HCV E2/NS1 glycoprotein variants: putative role in chronic hepatitis C virus infections. Proc. Natl. Acad. Sci. USA **89:**3468–3472.
19. **Yoshikura, H.** 1989. Thermostability of human immunodeficiency virus (HIV-1) in a liquid matrix is far higher than that of an ecotropic murine leukemia virus. Jpn. J. Cancer Res. **80:**1–5.