Reemergence of Hepatitis C Virus after 8.5 Years in a Patient with Hypogammaglobulinemia: Evidence for an Occult Viral Reservoir

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The question of whether viruses persist after apparent clearance of infection remains unanswered. Here, we describe a patient with hypogammaglobulinemia whose acute hepatitis C virus (HCV) infection appeared to resolve after receipt of interferon therapy, relapse immediately, and then clear spontaneously—only to relapse after receipt of corticosteroid therapy, and clear again, 8.5 years later. Sequencing indicated that the viruses detected during each relapse were virtually identical, with the hypervariable region 1 of E2 appearing to be monoclonal, which is typical of patients with hypogammaglobulinemia. Nonstructural 5A sequences exhibited quasispecies diversity initially but, after 8.5 years, had become monoclonal. The prolonged period of negativity for HCV RNA followed by relapse suggests that HCV may persist in apparent sustained viral responders.

Patients with hypogammaglobulinemia who become infected with hepatitis C virus (HCV) tend to have severe disease that may progress to cirrhosis and liver failure after only a few years [1, 2]. Responses to interferon (IFN) therapy have varied in these patients.

In patients with chronic HCV infection, the hypervariable region 1 (HVR1) of the viral E2 envelope glycoprotein exhibits a range of quasispecies variation that is considered to be the result of host humoral immune pressure that leads to viral adaptation and antibody-escape variants. By contrast, the sequence diversity of the nonstructural (NS) 5A protein–coding region has been shown to be associated with antiviral pressure from IFN defenses of the infected cell, such that quasispecies variation in the protein kinase R–binding domain (PKR-BD) and the included IFN sensitivity determining region (ISDR) of NS5A may influence intracellular defenses. These processes likely play a role in viral persistence by facilitating immune evasion and regulation. Studies of patients with hypogammaglobulinemia have demonstrated little quasispecies variation in the HVR1 over periods of up to 8 years of infection [3]. This lack of rapid sequence variation is attributed to a lack of humoral immune selection pressure, but an effect on the NS5A sequence of HCV in such patients has not been addressed.

Here, we describe the course of HCV infection in a patient with subclass 3 IgG deficiency who, in 1994, developed acute HCV infection and was treated with IFN-α2b for 5 months. Immediately after treatment, the patient experienced a brief, self-limited relapse. She then experienced a sustained virologic remission that lasted for 8.5 years, only to experience another brief relapse before the infection remitted again in 2003. We explore the virologic features of this unique infection pattern and report on the level of sequence heterogeneity within the HVR1 of the E2 protein and the PKR-BD of the NS5A protein during the 2 separate relapses.

**Case patient, materials, and methods.** A 23-year-old white woman with recurrent sinusitis and bronchitis/asthma received a diagnosis of subclass 3 IgG deficiency in late 1991 and began receiving treatment with intravenous immunoglobulin (IVIG) at 2-week intervals (20–25 g each dose). The results of her liver-function tests were entirely normal. In early March 1994, she began to develop nonspecific symptoms of fatigue and anorexia around the time her physician was notified of the contamination of units of Gammagard (Baxter Healthcare) by HCV, and screening for infection was suggested. Her amino-transferase levels were 217/347 (aspartate aminotransferase [AST]/alanine aminotransferase [ALT]) IU/L, increasing to peak values of 1850/2020 IU/L 1 month later (figure 1A). A polymerase chain reaction (PCR) assay for HCV RNA was initially positive, but then a quantitative PCR assay was negative (Roche Amplicor RT PCR Assay [limit of detection, <1000 copies/mL]). After 5 weeks, her aminotransferase levels had decreased to 54/281 IU/L. Her total bilirubin level remained normal.

Because of a concern that her infection would persist and
Figure 1. Time course of alanine aminotransferase (ALT) levels and hepatitis C virus (HCV) RNA titers during acute infection, therapy, and the initial relapse (A) and during the second relapse (B). Acute infection developed after exposure to contaminated intravenous immunoglobulin in March 1994. Interferon therapy was initiated in April 1994 and was continued for 5 months until September 1994, and the initial relapse occurred promptly (within 5 days) after cessation of therapy. Viral clearance followed within 3 weeks, and the patient was well. She then had repeatedly documented normal aminotransferase levels and negative HCV RNA assays until March 2003, when hepatitis symptoms and elevations in aminotransferase levels and HCV RNA titers were once more observed. The infection cleared again without further treatment, and the patient has remained negative for HCV RNA and has had normal aminotransferase levels for an additional 18 months.
be aggressive in this setting, treatment with IFN-α2b (Intron A; 3 million units 3 times/week; Schering-Plough) was initiated on 25 April 1994. Her aminotransferase levels remained normal or near normal throughout treatment, HCV RNA was undetectable on 1 occasion during therapy, and the medication was discontinued at her request on 13 September 1994. Two days later, she developed fatigue, myalgias, anorexia, nausea, and vomiting, and she was hospitalized 5 days after discontinuation with aminotransferase levels of 8078/8531 IU/L (figure 1A). She was repeatedly positive for HCV RNA by PCR assays during this period, with the titers decreasing in parallel with her aminotransferase levels. Other causes of her high aminotransferase levels, such as acetaminophen ingestion, were excluded. Her total bilirubin level peaked at 4.2 mg/dL 3 days later, and her symptoms resolved rapidly, with her aminotransferase levels returning to normal within 3 weeks without further treatment. She continued IVIG treatment over the next decade with no evidence of ongoing HCV infection, having consistently normal aminotransferase levels and negative results of PCR assays documented in 1994, 1995, 1996, and 2000. However, in early March 2003, she complained of symptoms that were “like my previous hepatitis.” During the previous 6 months, she had been receiving intravenous methylprednisolone (125 mg) with each dose of IVIG as well as intermittent prednisone therapy for asthmatic episodes. On physical examination, there were no abnormalities. Her aminotransferase levels were elevated (1200/1085 IU/L), and she was again positive for HCV RNA (figure 1B); quantitative PCR indicated an HCV RNA titer of 267,000 IU/L. The genotype of the infecting virus was 1a. One week later, her HCV RNA titer had decreased to 15,200 IU/L. All corticosteroid therapy was discontinued, and, over the next 2 months, her HCV RNA titer again became undetectable without further treatment. Her aminotransferase levels returned to normal, and she has remained negative for HCV RNA during repeated testing (6 occasions) for 18 months. Liver biopsy was not performed.

Serum samples stored at −80°C were available from the 1994 treatment period and all later time points. HCV RNA was quantified in serum samples from 1994 by use of the Roche Amplicor RT PCR Assay (limit of detection, <1000 copies/mL). Negative assays were confirmed by use of more-sensitive assays (limit of detection, <200 copies/mL) after 1996 and, most recently, by a Roche Amplicor assay (limit of detection, <50 IU/L).

For sequence analysis, HCV RNA was amplified by reverse-transcription PCR (RT-PCR). Total RNA was isolated from 500 μL of the patient’s serum by use of Trizol (Gibco), in accordance with the manufacturer’s protocol. RNA products were precipitated in ethanol and were dissolved in 10 μL of 0.1% diethylpyrocarbonate-treated H2O containing 1.0 U of RNase inhibitor/μL of suspension (Gibco) in a final concentration of 1 mmol/L dithiothreitol. Resuspended RNA was stored at −80°C until use. Ten microliters of RNA was used for RT in the presence of 4.5 pmol of mixed hexamer primers and 200 U of Maloney murine leukemia virus reverse transcriptase, and reaction mixtures were incubated for 1 h at 37°C. cDNA products were stored frozen until use. Nested PCRs were conducted on 2 μL of the cDNA product with oligonucleotide primer pairs that targeted the HVR1 of E2 or the PKR-BD and flanking variable region of NS5A [4]. For the HVR1, primary PCR products were amplified as described elsewhere [5], but with 50 pmol of the primer pair 5′-gtgctacagggagtctg-3′ (sense primer encoding nt 1389–1409 of HCV genotype 1a) [6] and 5′-cattgg-agttcagggccgtcta-3′ (antisense primer encoding nt 1610–1633). The primer pair 5′-tgagctgctacaggtctag-3′ (sense primer encoding nt 6854–6873) and 5′-gctgcaaccgcatagat-3′ (antisense primer encoding nt 7360–7380) was used for primary amplification of NS5A sequences. Primary PCRs were conducted with 30 rounds of amplification at an annealing temperature of 54°C. Two microliters of primary PCR product was used to program the nested PCRs containing the HVR1 oligonucleotide primer pair 5′-tcagggctcagggcgtctagg-3′ (sense primer encoding nt 1428–1447) and 5′-tgccacatgctggtgctg-3′ (antisense primer encoding nt 1575–1604) or the NS5A oligonucleotide primer pair 5′-ccctccataacaggag-3′ (sense primer encoding nt 6875–6894) and 5′-gatggctgaggaccacctg-3′ (antisense primer encoding nt 7336–7354). Nested PCRs were conducted with 30 rounds of amplification at an annealing temperature of 52°C. All PCRs were conducted by use of the Advantage HF 2 proofreading-polymerase system (Clontech). The specificity of each PCR was controlled by conducting parallel reactions with HCV genotype 1a cDNA [6] and non–reverse-transcribed RNA products as positive and negative controls, respectively. PCR products were analyzed by agarose gel electrophoresis, were purified by gel extraction, and were cloned into the pCR2.1 vector by use of the TA cloning system (Invitrogen). Escherichia coli transformants that contained the cloned PCR products were selected on amplification medium. Plasmid DNA was isolated from 10 different clones of HVR1 or NS5A sequences, which represented 5 PCRs each. The nucleotide sequence of each clone was determined by use of plasmid-encoded primer sequences and an ABI automated sequencer. Nucleotide and deduced amino acid sequences were compared with the H77 HCV genotype 1a reference sequence [6] and analyzed by use of Vector NTI software (InforMax).

Results. Quasispecies diversity was assessed by analysis of 2 coding regions, the HVR1 of E2 and the PKR-BD of NS5A, which includes the ISDR [5, 7]. Ten independent clones, isolated by RT-PCR, were sequenced for each serum sample. At the time of the patient’s initial relapse in 1994, HVR1 sequences were identical among the clones and were separated into 2 quasispecies populations by virtue of a single nucleotide substitution encoding a K→M mutation at HCV polyprotein amino acid position 370, which is outside the HVR1 (figure 2). In
contrast, we identified 5 NS5A quasispecies variants, each harboring mutations within the PKR-BD and the ISDR, as well as 1 sequence with an additional mutation in a previously described variable region, termed "V4," that flanks the PKR-BD [7]. Sequence analysis was also performed for the virus associated with the patient’s relapse in 2003, and these sequences were compared with those associated with her previous infection. With the exception of a P→Q reversion at position 2235 in the NS5A coding region, the sequences of the HVR1 and the NS5A coding region of 10 independent clones at this time point were identical to the dominant sequences of the previous isolates (figure 2).

**Discussion.** Humoral immunity appears to play little role in HCV clearance, possibly because of immune escape by way of rapid mutation of the HVR1. Viral clearance is associated with a broad and vigorous T cell response and is likely influenced by intracellular antiviral defenses [8, 9]. Innate and IFN-induced antiviral pathways may determine viral clearance by interfering with viral replication and translation and by enhancing cell-mediated immunity [10]. Examination of the response to HCV infection in patients with hypogammaglobulinemia provides an opportunity to study these responses.

Following an episode of severe active hepatitis (with remarkably high aminotransferase levels), our patient had complete resolution of infection after discontinuation of IFN therapy. Resolution—as evidenced by long-term normalization of aminotransferase levels and clearance of the virus for 8.5 years—then was followed by relapse with a nearly identical viral species. The initial resolution of infection after a striking disease flare appears to have resulted from strong intracellular and/or cell-mediated immune processes that were independent of the humoral immune response.

As was expected, when we sequenced multiple clones from the time of initial relapse and from the time of the second relapse 8.5 years later, we found virtually no evidence of heterogeneity in the HVR1 of the viral envelope [3, 11]. This finding is consistent with previous evidence indicating that HVR1 diversity results from the selection of escape variants in response to humoral immune pressure but contrasts with the level of quasispecies variation within NS5A at the time of the initial relapse. Higher numbers of amino acid mutations in the ISDR, compared with the previously described HCV genotype 1a viral sequence, correlate with increased sensitivity of HCV to the intracellular antiviral response and to IFN in general [4,
The viral evolution to a single NS5A sequence at the time of the second relapse could have resulted from intracellular immune pressure and selection of a single persistent variant. The documentation of viral mutation in response to this host pressure in the absence of antibody argues that the immune selection of viral quasispecies that are resistant to intracellular defenses and/or T cell immunity may be responsible for viral persistence in this patient.

Acute hepatitis C responds to IFN, and successful viral clearance in patients with hypogammaglobulinemia has been reported [12]; spontaneous viral clearance without treatment is rare [13]. The role of IFN in our patient’s disease course is unclear. Our patient experienced an immediate relapse after 5 months of IFN therapy, which was followed by viral clearance for 8.5 years. The very-delayed relapse of infection after >8 years may have resulted from transient immunosuppression due to repeated corticosteroid use. However, other factors, including abnormal immunity and cytokine signaling associated with hypogammaglobulinemia, could also have effected this relapse pattern [14]. That the phenomenon did not represent reinfection was proved by the fact that only a single amino acid difference in the NS5A region was found over the 8.5-year period. We interpret the minimal shift in quasispecies diversity and the repeatedly negative serum HCV RNA PCR assays to represent a low level of viral replication during this long quiescent period. We interpret the minimal shift in quasispecies diversity and the repeatedly negative serum HCV RNA PCR assays to represent a low level of viral replication during this long quiescent period. Although considered to be a sustained viral responder, our patient continued to have a reservoir of low-replicating virus that was held in check but not eradicated by her immune system until the corticosteroid-induced immune suppression led to the relapse. Recent studies in immunocompetent patients support the presence of such a reservoir [15]. An HCV reservoir that requires continued innate or T cell immune surveillance to prevent disease activity even years after the infection appeared to have resolved may exist in at least some sustained viral responders.

References