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Interferon Treatment for Chronic Hepatitis C Infection in Hemophiliacs— Influence of Virus Load, Genotype, and Liver Pathology on Response

By John P. Hanley, Lisa M. Jarvis, Janet Andrews, Rosemary Dennis, Peter C. Hayes, Juan Piris, Robert Lee, Peter Simmonds, and Christopher A. Ludlam

In this study, we assessed the effectiveness of interferon treatment in 31 hemophiliacs with chronic hepatitis C virus (HCV) infection. Interferon alfa-2a (3 MU three times weekly) was administered for 6 months. Response was assessed by both serial alanine transaminase (ALT) and HCV RNA levels measured by a sensitive semiquantitative polymerase chain reaction (PCR) method. HCV genotype was determined by restriction fragment length polymorphism (RFLP), and evidence of changing genotypes during interferon therapy was sought. Severity of liver disease was assessed by both non-invasive and invasive methods, including laparoscopic liver inspection and biopsy. Sustained normalization of ALT levels occurred in eight patients (28%), and seven (24%) became nonviremic as assessed by PCR (<80 HCV/mL). Responders universally cleared HCV RNA within 2 months of starting interferon. Genotype 3a was associated with a favorable response to interferon. No evidence was found for a change in circulating genotype in patients who failed to respond to interferon or who relapsed. This study confirms that response rates to interferon are low in hemophiliacs as compared with other groups with chronic HCV infection. We have also demonstrated that virus load measurement over the first 8 to 12 weeks of treatment is an extremely useful method to identify responders at an early stage.
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ALMOST ALL HEMOPHILIACS who received clotting factor concentrates before the introduction of effective viral-inactivation techniques were infected with hepatitis C virus (HCV).[1,2] The majority of individuals exposed to the virus have become chronic carriers, characterized by both persistent fluctuating viremia and variable abnormalities in liver function tests. There is a wide spectrum of liver disease in these patients, ranging from only minor histologic evidence of chronic hepatitis to cirrhosis[3] and hepatocellular carcinoma.[4]

Interferon has been used to treat chronic HCV in both nonhemophiliacs and hemophiliacs. Response to treatment has been assessed by serial serum alanine transaminase (ALT) levels, clearance of viremia by polymerase chain reaction (PCR), and direct assessment of liver histology. Clearance of viremia is likely to be a prerequisite for a long-term response rather than normalization of ALT. To date, there have only been a few small studies on the efficacy of interferon alfa for the treatment of HCV infection in individuals with hemophilia.[5-9] Some of these studies have suggested that the response to interferon in hemophiliacs may be lower than in other groups infected with HCV. We report the results of interferon therapy in a group of 31 hemophiliacs with chronic HCV infection. We have monitored response to interferon using a sensitive semiquantitative PCR to detect HCV RNA. In addition, we have assessed the extent of liver disease in these patients using both invasive and noninvasive methods, and have critically evaluated whether these investigations are useful in predicting the response to interferon.

PATIENTS AND METHODS

Patient characteristics. A total of 31 patients (30 male and one female) were treated (Table 1). All patients were anti-HCV–positive by Abbott second-generation enzyme immunoassay (A-EIA; Abbott, Weisbaden-Dalkenheim, Germany) and were also positive on confirmatory testing by second-generation recombinant immunoblot assay (RIBA-2; Chiron Corp, Emeryville, CA) for antibody to nonstructural proteins 5-1-1 (NS4), c100-3 (NS4), c33c (NS3), and core-associated antigen c22-3.

All had previously received non–virus-inactivated factor concentrates, and 30 of 31 had persistently elevated serum ALT levels (ALT levels were measured on ≥3 occasions over the 6 months before treatment). Serum ALT level was normal in one individual. HCV RNA was detected in all patients by PCR as described below. Six patients were anti-HIV–positive (pretreatment CD4 counts, 4, 20, 140, 180, 250, and 360 cells/mm^3, respectively), and all were HBSAg-negative. In addition, all patients were negative for anti–smooth muscle and antimitochondrial antibody and had normal levels of α-fetoprotein, ceruloplasmin, and α1-antitrypsin.

Pretreatment assessment. Informed consent was obtained from all patients participating in the study. Before starting interferon treatment, a detailed assessment was undertaken of the extent of liver disease in each patient, with particular reference to the presence or absence of cirrhosis, using both noninvasive and invasive methods. The role of invasive investigations in the assessment of liver disease was discussed with all patients. Those with anti-FVIII or anti-FIX inhibitors were not offered invasive investigations, and individuals with von Willebrands disease were not offered liver biopsy. It was emphasized that the investigations were not essential for administration of interferon but would provide information to counsel the individual as to the extent of liver disease.

The following investigations were performed after appropriate factor concentrate replacement. (1) Upper gastrointestinal endoscopy was performed in 27 patients to identify the presence or absence of esophageal varices. (2) Laparoscopy was performed under sedation using a 5-mm pediatric laparoscope (Olympus, Tokyo, Japan) or microlaparoscope (Imagyn, Laguna Niguel, CA) in 21 patients. The degree of hepatic inflammation (none, mild, or marked), degree of surface fibrosis (none, moderate, or pronounced), and presence of cirrhosis and portal hypertension were assessed.[10,11] In 15 patients, a tru-cut biopsy was taken during the laparoscopy, and histology was assessed using both the Sheffield scoring system[5] (data not

Table 1. Patient Characteristics

Parameter	No. of Subjects	Median	Range
Hemophilia A			
Severe	11		
Moderate	8		
Mild	1		
Hemophilia B			
Severe	1		
Moderate	2		
Mild	5		
von Willebrand disease	3		
Total	31		
Age (yr)		35.0	13-67
Pretreatment ALT (U/L)		78	37-175
Pretreatment HCV RNA (copies/mL)		10^6	10^3->10^7

shown) and a visual classification. Using the latter method, overall histologic appearance was classified as mild, moderate, or severe. (3) Abdominal ultrasound scans to assess liver size, echogenicity, and presence of hepatocellular carcinoma, as well as spleen size, were performed on 30 patients. Baseline measurements are listed in Table 1. In addition, data concerning annual factor concentrate consumption and serum Igs (IgG, IgM, and IgA) and serum ferritin were collected.

Drug dosage and administration. Interferon alfa-2a (Roche, Welwyn Garden City, UK) 3 MU three times per week was given subcutaneously. The intention was to treat for 24 weeks.

Assessment of response to interferon treatment. Response was assessed on the basis of serial monthly ALT levels and HCV RNA quantitation. After 6 months' treatment, a complete ALT response (CR) was defined as normalization of ALT (<40 U/L) sustained for at least 2 months; a partial ALT response (PR) required a more than 50% reduction in pretreatment ALT. A HCV RNA CR required the virus to be undetectable in the serum, ie, PCR-negative (<80 HCV/ mL, see below); a PR required a 100-fold reduction in HCV RNA titer.

Typing and quantification of HCV RNA. All testing was performed using serum samples separated within 3 hours of collection and stored at −70°C. Virus RNA was extracted from 0.5 mL stored sera after pelleting of virus by centrifugation at 100,000g for 90 minutes at 4°C and incubation at 37°C for 2 hours with 1 mg/mL proteinase K in the presence of 40 μg/mL polyadenylic acid, 0.5% sodium dodecyl sulfate, 0.1 mol/L NaCl, 50 mmol/L Tris hydrochloride (pH 8.0), and 1 mmol/L EDTA. RNA was extracted with phenol, and after centrifugation the supernatant was reextracted successively with phenol/chloroform (1:1) and chloroform/isoamylalcohol (50:1). Nucleic acid was precipitated by the addition of .10 vol sodium acetate (pH 5.2) and 2 vol ethanol. The dried pellet was resuspended in 25 μL diethylpyrocarbonate-treated water.[12] RNA was reverse-transcribed by nested PCR using 5' noncoding region–specific primers 939, 209, 940, and 211.[13] For genotyping, product DNAs were cleaved with restriction enzymes HaeIII/RsaI and MvaI/HinfI as described previously,[14] and the fragments were separated by agarose gel electrophoresis using 4% Metaphor agarose (FMC BioProducts, Rockland, ME). Subtypes 1a and 1b and 2a and 2b were identified by the cleavage patterns resulting from digestion with BstUI and ScrFI, respectively.[15] Genotyping was performed on all patients before starting interferon and after 3 and 6 months' therapy.

Virus levels were measured semiquantitatively by limiting-dilution analysis of cDNA reverse-transcribed from RNA.[16,17] Centrifugation of 0.5 mL sera provided a level of detection of approximately 800 HCV/mL. To increase sensitivity of the PCR method, samples

that were negative at this level of detection (<800 HCV/mL) were further analyzed by centrifugation of 5.0 mL sera, providing a cutoff point of approximately 80 HCV/mL.

Statistical analysis. The relationships between ALT response or HCV RNA response and baseline measurements were assessed using Fisher's exact test for nominal baseline variables, chi-square test for trend for ordered categorical baseline variables, Wilcoxon rank-sum test for non–normally distributed continuous baseline variables, and two-sample *t*-test for normally distributed continuous baseline variables. All significance tests were two-sided.

RESULTS

Of 31 patients, 29 completed 6 months' treatment with interferon. One stopped after 2 months due to leukopenia, and one stopped after 5 months due to a subjective hearing loss that subsequently recovered. The 29 patients who completed 6 months of treatment were evaluated. Normalization of ALT (CR) occurred in eight of 29 (28%); a more than 50% reduction in pretreatment ALT (PR) was achieved in four of 29 (14%), and 17 of 29 (59%) were nonresponders (NR). Seven of 29 (24%) became PCR-negative for HCV RNA (CR). In addition two of 29 (7%) achieved at least a 10^2-copies/mL reduction in HCV RNA (PR). In 20 of 29 (69%), serum HCV RNA levels were unchanged (NR).
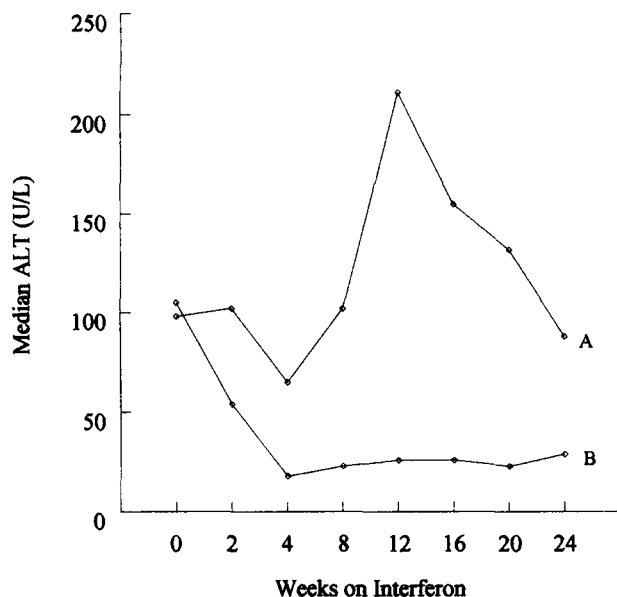
There was a statistically significant difference in age between responders (mean age, 46 years) and NR (mean age, 33 years; two-sample *t*-test, $P = .044$). However, multivariate analysis showed no independent relationship between age and response after adjusting for the effect of genotype on response (see below).

Response was not associated with HIV status, body weight, severity of hemophilia, duration of infection, annual factor concentrate consumption, serum ferritin, or serum IgS (data not shown).

Patterns of ALT and HCV RNA responses. Normalization of ALT levels correlated with clearance of viremia as measured by PCR (Spearman rank correlation, $r = .53$, $P = .003$; Table 2). Of eight patients who had a normalized ALT, five also became HCV RNA–negative, whereas in three there was no corresponding reduction in virus load. Of four patients who achieved a partial reduction in ALT, only one became nonviremic. Fifteen of 17 in whom ALT level was unchanged also had no change in virus load. Only one individual who had no change in ALT became nonviremic. Median ALT values in patients who became nonviremic versus those who failed to clear HCV RNA are shown in Fig 1. In one patient only, there was a transient HCV RNA clearance at 2 months, but at 3 months the HCV RNA titer returned to the pretreatment level of 10^7 HCV/mL. This was associ-

Table 2. ALT Response and HCV RNA Response

ALT Response	HCV RNA Response			
	CR	PR	NR	Total
CR	5	—	3	8
PR	1	1	2	4
NR	1	1	15	17
Total	7	2	20	29

Fig 1. Association of virus clearance with normalization of ALT. Patients with a partial or no reduction in virus load (line A, n = 22) showed no corresponding reduction in ALT, whereas patients who became nonviremic (line B, n = 7) achieved a reduction in ALT during treatment with interferon.

cirrhosis. Inflammation was identified laparoscopically in all patients, being mild in 14 (70%) and marked in six (30%). Evidence of portal hypertension was visible in five (25%).

Liver histology was evaluated in 14 patients and showed mild, moderate, and severe (including cirrhosis) histologic changes in four (29%), seven (50%), and three (21%), respectively.
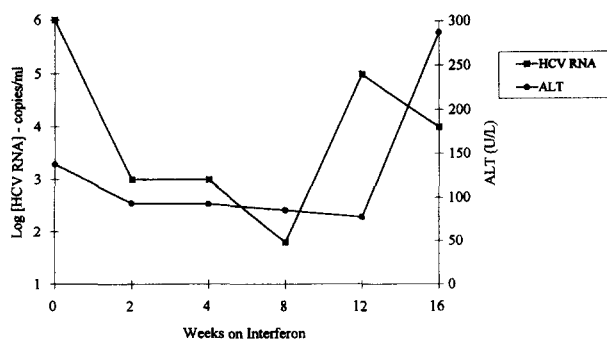
Analysis of response to interferon against laparoscopic liver appearance, liver histology, presence/absence of hepatomegaly, splenomegaly, and esophageal varices showed no evidence that any of these variables were associated with response to interferon.

Follow-up information. Within 3 months of discontinuing interferon, five of seven patients who became nonviremic have relapsed, with reappearance of serum HCV RNA and elevated ALT levels. In two patients who achieved a partial reduction in virus load, HCV RNA levels returned to pretreatment values within 1 month of stopping interferon. Only two patients remain HCV RNA–negative with normal ALT. The three patients who showed normalized ALT levels without a corresponding reduction in HCV RNA developed elevated ALT levels within 2 months of stopping interferon.

DISCUSSION

We have shown that the response to interferon therapy in hemophiliacs with chronic HCV infection is poor and appears inferior to that of other groups of infected patients. In this study, only 28% had a normalized serum ALT after 6 months' treatment with interferon, and 24% became PCR-negative for HCV RNA. Furthermore, sustained responses were uncommon. This compares with initial ALT CR of 50% and sustained responses of 20% to 25% reported in nonhemophiliacs.[18] Early investigators used ALT to assess long-term response.[19,20] More recent studies have confirmed that clearance of HCV RNA is a prerequisite for long-term response.[21]

The importance of HCV quantitation has been increasingly recognized. We describe herein a sensitive semiquantitative PCR method for HCV RNA detection with a lower

ated with a parallel ALT reduction and subsequent relapse (Fig 2). There was no relationship between pretreatment ALT level and response.

Rate of HCV RNA clearance in responders. The rate of HCV RNA clearance in seven CR was assessed (Fig 3). In six of seven, a prompt reduction in HCV RNA occurred to less than the limit of detection within 8 weeks of commencing interferon. In one individual (patient no. 2, Fig 3), initial clearance of HCV RNA at week 4 was followed by a transient increase in virus titer to 10^4 copies/mL at weeks 8 and 12 before sustained clearance between weeks 16 and 24.

Predictive factors for response to interferon. There was a striking relationship between HCV genotype and a favorable response to interferon. A total of six of eight hemophiliacs infected with genotype 3a became nonviremic. In the other 21 patients, only one individual (genotype 2b) became HCV RNA–negative (Fisher's exact test, $P = .0002$). No changes in HCV genotype, as detected by RFLP analysis, were identified in any patients during interferon therapy (Table 3).

There was no relationship between pretreatment virus load and either response to interferon (Fig 4) or HCV genotype (Fig 5).

Severity of liver disease was assessed by methods outlined earlier. Of 28 abdominal ultrasound scans evaluated, five (18%) demonstrated splenomegaly and seven (25%) showed an abnormal liver (five with hepatomegaly and two with an abnormal liver texture). In seven of 25 (28%), esophageal varices were demonstrated at endoscopy. Of 20 laparoscopic liver inspections, 10 (50%), four (20%), and six (30%) showed none, mild, or pronounced surface fibrosis, respectively, with five patients with pronounced fibrosis having

Fig 2. Breakthrough hepatitis during interferon therapy. This patient achieved an initial response to interferon with clearance of HCV RNA by week 8 and corresponding reduction in ALT level. As interferon was continued, viremia recurred by week 12 and there was an associated increase in ALT. Such episodes of breakthrough hepatitis during interferon treatment may be associated with the development of neutralizing antibodies to interferon.

Fig 3. Rate of virus clearance in HCV RNA responders (n = 7). In 6 of 7 patients who became nonviremic, there was a prompt reduction in HCV RNA to < the limit of detection within 8 weeks of commencing interferon. In one individual (patient no. 2), initial clearance of HCV RNA at week 4 was followed by a transient increase in virus titer to 10^4 copies/mL at weeks 8 and 12, before sustained clearance between weeks 16 and 24.
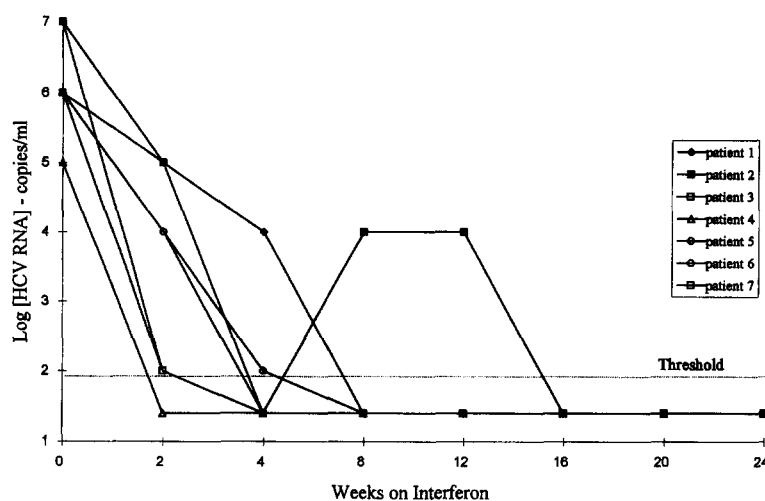
limit of detection of 80 HCV/mL. This compares with 3.5 × 10^5 equivalents/mL when the branched DNA (bDNA) assay is used.[22] Using bDNA, we would have failed to detect viremia in four (13%) of our patients and incorrectly classified six (20%) as responders who merely showed a partial reduction in HCV RNA levels. Clearly, sensitive quantitative assays are essential to monitor response to treatment, and some studies may have overestimated response rates by using relatively insensitive methods for RNA quantitation. We

found that HCV RNA was cleared within 8 weeks of starting interferon in seven patients who responded. Using this method, it would therefore be possible to differentiate responders from nonresponders at an early stage of treatment and discontinue interferon in nonresponders. Alternatively, these individuals could be offered a dose-escalation, although there are few data available in hemophiliacs concern-

Table 3. Predictive Factors for Response to Interferon

| Factor | HCV RNA | | | |
	CR	PR/NR	Total	P
Genotype				
1a	0	11	11	
1b	0	7	7	
2a	0	1	1	
2b	1	1	2	
3	6	2	8	.0002*
Anti-HIV				
Positive	0	5	5	
Negative	7	18	25	.30
Splenomegaly (ultrasound)				
Yes	0	5	5	
No	7	16	23	.29
Hepatic fibrosis (laparoscopy)				
None	3	7	10	
Mild	1	3	4	
Pronounced	3	3	6	.64
Hepatic inflammation (laparoscopy)				
Mild	5	9	14	
Marked	2	4	6	1.00
Esophageal varices (endoscopy)				
Yes	1	6	7	
No	6	12	18	.63
Liver histology				
Mild	1	3	4	
Moderate	3	4	7	
Severe	1	2	3	1.00

* Fisher's exact test.
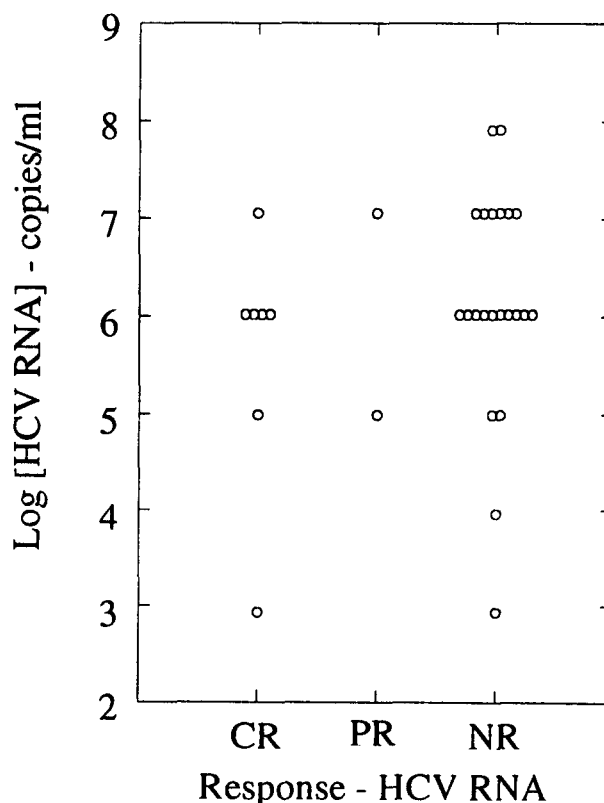
Fig 4. Pretreatment virus load and response to interferon. There was no significant relationship between pretreatment virus load and response to interferon (χ^2 test for trend, P = .073).
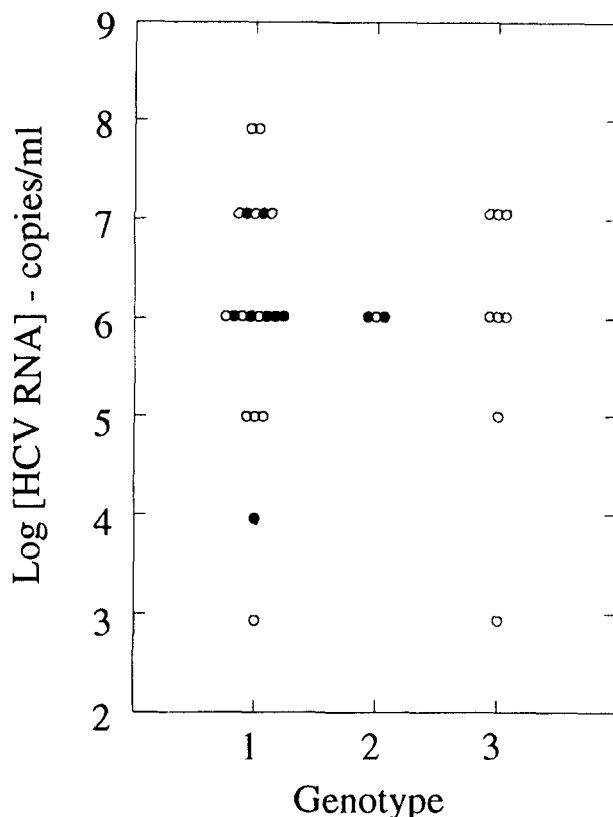
Fig 5. Pretreatment virus load and HCV genotype. There was no significant relationship between pretreatment virus load and HCV genotype (Kruskal-Wallis test, P = .77). (O) subtype a; (●) subtype b.

ing the value of this to increase response rates. In view of the generally poor overall responses to interferon in hemophiliacs, we believe this approach is unlikely to benefit a significant number of patients.

In one individual, initial clearance was followed by a transient increase in virus titer before further clearance. The reasons for this are not entirely clear. Temporary noncompliance with treatment is a possible explanation, or there may be a small number of individuals who achieve a slower reduction in HCV RNA rather than the rapid and sustained reduction observed in the majority of responders.

There is usually a good correlation between normalization of ALT and HCV RNA clearance in responders to interferon. However, discrepancies between ALT and HCV RNA responses have been described previously.[23] We have identified some individuals who achieved a biochemical response without a corresponding reduction in virus load. Clearly, these patients are not true responders, and biochemical relapse is inevitable once interferon is discontinued. Since ALT is not an entirely accurate measure of response, studies using ALT alone to assess response may also overestimate true responders. Interestingly, there was one individual who cleared HCV RNA without an associated ALT response. This raised the possibility of other non-HCV coexisting liver pathology resistant to interferon treatment, but none has been identified. Another individual relapsed during interferon

therapy. Such episodes of "breakthrough hepatitis" are well recognized and may be associated with the development of neutralizing antibodies to interferon.[24] A change in HCV genotype may also be an explanation for breakthrough hepatitis, and a change in the dominant genotype in hemophiliacs treated with interferon has been reported.[25] In this study, we did not detect any changes in the circulating genotype in any patients during interferon therapy. However, it is recognized that the RFLP method will not detect co-infecting genotypes circulating at low frequencies. In addition, interferon NR may be associated with a change in variants of the same genotype (termed quasi-species). Analysis of variants within an individual before and after interferon treatment is in progress.

Several studies assessing response to interferon have been performed on cohorts of hemophiliacs infected with HCV.[5-9] Some of these studies have suggested that the overall initial response rates are somewhat lower in hemophiliacs than in other groups with HCV. Interestingly, response rates in the earlier studies are superior to those performed more recently. This may be a reflection of the relatively small number of patients studied, or may have been caused by progression of liver disease in cohorts of hemophiliacs, leading to diminished responses to interferon. In addition, although the data available provide conflicting results, long-term response appears unusual. In the largest study,[9] only one of 20 (5%) achieved a sustained response to interferon. In the only trial that assessed response by liver biopsy,[5] four of seven responders had a long-term response. Our study, which contains the largest treated group of hemophiliacs with HCV reported to date, supports the view that long-term responses to interferon are uncommon in hemophiliacs. Sustained response rates may be improved by longer courses of interferon.

Attempts have been made to identify factors that may predict response to interferon.[26] Absence of cirrhosis, younger age, low serum HCV RNA level, and genotypes 2 and 3 are all factors associated with a favorable response to interferon. As yet, the presence of adverse factors has not been considered sufficient to absolutely exclude some individuals with HCV from interferon therapy. There has been particular concern, in view of the generally poor response to interferon in hemophiliacs, that treatment with interferon is inappropriate in the majority of individuals. Not only are patients exposed to a potentially toxic drug with unpleasant side effects, but the cost of a course of interferon is considerable.

We have tried to identify parameters that may predict response to interferon in hemophiliacs with HCV. Assessment of liver disease has included both invasive and noninvasive methods. We have shown that genotype 3a is associated with a favorable response, but we failed to identify any other statistically significant independent variables, including pretreatment virus load, associated with a poor response to interferon. In view of this, despite the apparent poor response to interferon in hemophiliacs, it is not possible to predict accurately which individuals are likely to respond.

In conclusion, the results of interferon treatment for HCV in hemophiliacs are disappointing. Interferon alfa-2a 3 MU

three times per week for 6 months is unlikely to result in a long-term sustained response. Monitoring response with a sensitive semiquantitative PCR to quantify HCV RNA is extremely useful to identify responders at an early stage of treatment. Those who fail to clear HCV RNA should discontinue interferon. Dose-escalation is unlikely to benefit many patients, but may be attempted in selected individuals.

ACKNOWLEDGMENT

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