Predictors of Long-Term Response to High-Dose Interferon Therapy in Type II Cryoglobulinemia Associated With Hepatitis C Virus Infection

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We have prospectively studied patients with type II cryoglobulinemia since 1985 to assess the efficacy of treatment with interferon-α at cumulative doses ranging from 234 to 849 MU. In the present study we retrospectively evaluated in this cohort parameters associated with complete response to therapy in 31 consecutive patients with type II cryoglobulinemia associated with hepatitis C virus (HCV) infection. Prevalence of complete response of cryoglobulinemia (disappearance of symptoms and signs of vasculitis and decrease of cryocrit below 10% of the initial value) was 62%, with a median response duration of 33 months and a range of 3 to 100 months. Three patients were putatively cured, as they remained in complete remission for more than 5 years off therapy. Eighteen patients (58%) had liver disease evidenced by histopathology and/or raised transaminase levels. Prevalence of normalization of transaminase levels was 100%, with a median response duration of 36 months. Relapse of hypertransaminasemia occurred in 100% and 8% of patients receiving less than or greater than 621 MU, respectively. By logistic regression analysis, the only pretherapy parameter that associated significantly ($P = .0393$) with complete response of cryoglobulinemia was the solitary anti-C22 (HCV core) antibody pattern, which was observed in 29% of patients. Association with older age and low cryocrit approached statistical significance ($P = .06$), while no significant correlations were found with serum IgM levels, duration of disease, HCV genotype, NS5a gene mutations, liver histology, HLA-DR phenotype, or WA cross-idiotype. Complete responses were also associated, on univariate statistical analysis, with low pretherapy HCV viremia. Responses were accompanied by decrease of viremia, of anti-HCV antibody levels and cryocrit. The usefulness of a high dose regimen is underscored by the higher rates of sustained responses of cryoglobulinemia and transaminase levels compared with previous studies.

MIXED CRYOGLOBULINEMIA IS characterized by serum cold-precipitable complexes and is associated with a systemic vasculitis.[1,2] Clinically, the syndrome is characterized by purpura, weakness, and arthralgias and progression to involvement of the kidneys, nervous system, and other vital organs. Clinical liver disease is present in 50% to 80% of patients.[3] Mixed cryoglobulins consist of polyclonal IgG and monoclonal (type II) or polyclonal IgM (type III) with rheumatoid factor (RF) activity. The etiology of mixed cryoglobulinemia is unknown, but in the past few years, a strong association with hepatitis C virus (HCV) infection has been widely and firmly established. Serologic markers of HCV occur in 90% of patients with mixed cryoglobulinemia[3] and, in addition, HCV has been shown to be specifically concentrated in the cryoprecipitates.[4]

Interferon-α, based on its antiproliferative activity, was used to treat patients with mixed cryoglobulinemia before the association with HCV infection was known, and these early trials produced a complete response rate of about 50%.[5-7] These results were remarkable in view of the poor responses to other therapies.[8,9] These initial studies yielded rates of long-term remissions considerably higher than those achieved in patients with chronic hepatitis C treated with interferon-α.[10] In this study, we retrospectively analyzed the response of patients with cryoglobulinemia to interferon in our long-term series to determine what factors may be responsible for the higher rate of long-term remission and thereby possibly gain insights into the pathogenesis of the disease that may lead to improved therapeutic protocols.

Because higher doses of interferon-α were administered to patients with cryoglobulinemia compared with the standard regimens used for treating patients with chronic hepatitis C, we evaluated the impact of this parameter on the type and duration of response. In addition, we evaluated the initial levels of viremia, the genotype of infecting virus, and the presence of mutations in the nonstructural protein 5a gene (NS5a) in patients with 1b genotype infection, three parameters that have been reported to be predictors of response to interferon-α therapy.[10,11] We also evaluated host factors, such as anti-HCV antibody responses, HLA-DR phenotype, and typed the cross-idiotype in the monoclonal IgM component of the cryoglobulins.

MATERIALS AND METHODS

Patients. Thirty-one patients (13 men and 18 women), 38 to 73 years old (median, 56 years), who had type II mixed cryoglobulinemia were enrolled in the present study, which began in 1985 before serologic assays for HCV became available[5]; subsequently, all patients were found to have markers of HCV infection. Entry criteria included the demonstration of type II mixed cryoglobulins and the presence of the clinical symptoms of systemic vasculitis with or without evidence of liver disease. Patients with decompensated cirrhosis, evidence of other autoimmune diseases, or with neoplasia were excluded from the study. None of the patients had a history

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of acute hepatitis or intravenous drug abuse or had received blood products. At the beginning of therapy, all patients had cutaneous involvement, varying from perimalleolar purpura to distal necrosis. Seventeen patients had clinical evidence of kidney disease, with chronic renal failure in 11. Eighteen patients had persistently abnormal transaminase levels; a pretreatment liver biopsy was available in nine patients, and the histological activity index was evaluated by the Knodell score.[12] Other major clinical findings were anemia (15 patients), sicca syndrome (nine patients), and paresthesias (22 patients). Most of the patients had previously been treated with steroids (26 patients), cyclophosphamide (16 patients) or plasmapheresis (nine patients) without sustained benefit. Four patients (patients 12, 13, 27, and 30) had not been treated previously.

Criteria of evaluation. Response of cryoglobulinemic vasculitis to therapy was assessed on the basis of clinical and hematologic parameters. A complete response was defined as the reduction of cryocrit to less than 10% of the initial value and the disappearance of all clinical symptoms of vasculitis, normalization of hemoglobin values, and normalization or improvement of kidney function tests, occurring all together at any time after the initiation of treatment. Chronic renal failure and the clinical or electromyographic signs of peripheral neuropathy were excluded from the evaluation of response. A minor response was defined as the persistence of cryocrit above 10% of the initial level and/or the persistence of at least one of the pretherapy manifestations of active cryoglobulinemic vasculitis other than chronic renal failure and peripheral neuropathy. Liver disease activity was monitored by monthly testing of transaminase levels, and it was considered in remission when they remained normal for at least 6 consecutive months.

Interferon therapy. Our standard treatment schedule was 3 MU of recombinant interferon-α 2a (Roferon-A; Hoffmann-La Roche, Basel, Switzerland) daily for the first 3 months and every other day for at least 9 additional months, reaching a cumulative dose of at least 621 MU. Three patients received from the beginning a reduced dosage of 3 MU every other day (total dose 234 MU), patients 26 and 28 because of leukopenia and patient 27 because of low compliance; in another patient (no. 13) therapy was tapered (total dose 468 MU) because of hematologic toxicity. Four patients (nos. 2, 19, 20, and 22) relapsed because of acquired resistance to recombinant interferon-α 2a, due to neutralizing antibodies in three of them and were successfully treated with natural interferon-α (Alfaferone; Ismunit, Pomezia, Italy). These patients have been described in detail in a separate report.[13] Seven patients, although nonrelapsers, received additional interferon-α therapy beyond the standard dose of 621 MU given in 1 year; they were either complete responders who were treated because of the persistence of paresthesias and/or electromyographic abnormalities or minor responders treated in an attempt to improve the quality of response. Additional therapy resulted in appreciable benefits on the residual clinical and laboratory abnormalities in none of these seven patients.

Characterization of cryoglobulins, HLA-DR typing, and WA cross-idiotype determination. Detection and characterization of cryoglobulins and HLA-DR typing were performed as previously described.[5,14] WA cross-idiotype determination was performed by both immunoblotting and enzyme-linked immunosorbent assay (ELISA) methods.[15]

Measurement of HCV antibodies, HCV RNA, and HCV genotyping. Serum samples were tested for anti-HCV antibodies by second generation recombinant immunoblot assay (RIBA; Chiron RIBA-2, Ortho Diagnostic Systems, Raritan, NJ) and by third generation ELISA assay (Ortho Diagnostic Systems). The assays were performed according to the manufacturer's instructions with sera diluted 1:20. Antibody concentrations were semiquantitatively evaluated by ELISA. Serial dilutions of pretherapy and posttherapy serum samples were tested and, for each patient, the serum dilution at which the pretherapy sample gave an optical density (OD) between 0.5 and 1.5 was selected; comparisons of antibody concentrations in pretherapy and posttherapy sera were done, for each patient, at the selected dilution. Results were expressed as (experimental OD at the selected dilution) − (background OD).

Qualitative and quantitative determinations of HCV RNA in serum were performed by reverse transcription-polymerase chain reaction (RT-PCR) as previously described.[4] The lower limit of detection was 3,000 genomic equivalents of HCV per milliliter (gE/mL). The intra-assay variation was 5.7%, and the interassay variation was 20%. Qualitative HCV PCR determinations were also obtained in Rome.[16] Replicate samples analyzed for HCV RNA in Rome and Burlington showed complete correlation. HCV RNA determinations were obtained when serum stored at −70°C and not previously thawed was available. Quantitative HCV RNA determination was performed when sufficient amount of serum stored at −70°C was available. Genotyping of HCV was performed as described by Widell et al[17] and confirmed in some patients by DNA sequencing.

Nucleotide sequencing of the NS5a gene. The sequence of the NS5a gene was determined using a strategy similar to that recently described.[11] RNA was extracted from the sera, and RT-PCR was performed using the outer primers to amplify the NS5a region (nucleotides 6703 to 7320). A second PCR was performed with nested 5′ and 3′ primers. An M13 forward and M13 reverse primer sequence was attached to the 5′ ends of the 5′ and 3′ nested primers, respectively, to facilitate direct DNA sequencing. The sequences of the primers were as follows: 5′ outer, 5′-TGGATGGAGTGCGGTTGCACAGGTA-3′, 3′ outer, 5′-TCTTTCTCCGTGGAGGTGGTATTGG-3′, 5′ inner, 5′-GTAAAACGACGGCCAGTCAGGTACGCTCCGGCGTGCA-3′, and 3′ inner, 5′- GGAAACAGCTATGACCATGGGGCCTTGGTAGGTGGCAA-3′ (M13 sequences are underlined). Both strands of the PCR products were sequenced using the Amplicycle sequencing kit (Roche Molecular Systems Inc, Branchburg, NJ) modified according to application note 2.0 for longer sequence reads. The sequencing primers were biotinylated M13 forward and M13 reverse primers for the sense and the antisense strands, respectively. The products of the sequencing reactions were resolved on either 6% sequencing gels or 5% Long Ranger gels (FMC Bio Products, Rockland, ME) and then transferred by electroblot to Immobilon-S membranes. The DNA was cross-linked by ultraviolet (UV) and detected by chemiluminescence using the Uniplex kit (Millipore, Bedford, MA) and BioMax film (Kodak, Rochester, NY). The films were scanned, the images were analyzed using DNAscan software (Scanalytics, Billerica, MA), and the resulting sequences were translated to amino acid sequences and compared with the NS5a sequence identified for HCV-J.

Statistical analyses. The Fisher's exact test, two-tailed Student's *t*-test, or Mann-Whitney's U test was used when appropriate for univariate statistical analyses. Stepwise logistic regression was used for the multivariate analysis of explanatory variables for complete response to interferon therapy. Variables were entered in a forward selection model, with a *P* value for entrance limit of .05. Statistical calculations were performed using the SOLO software (BMDP Statistical Software Inc, Los Angeles, CA).

RESULTS

Pretherapy variables associated with complete response of cryoglobulinemic vasculitis to interferon-α therapy. Complete remission of cryoglobulinemic vasculitis was observed in 17 of 31 (62%) HCV-positive patients treated with interferon-α. The pretherapy demographic, clinical, and laboratory data in these 31 patients are summarized in Table 1; their major clinical manifestations before therapy are reported in detail in Table 2. It should be noted that throughout the manuscript the definition of response to therapy refers

Table 1. Characterization of Patients With HCV-Associated Cryoglobulinemia Who Had Complete or Minor Responses to Interferon-α Therapy

Variable	Complete Responders n = 17	Minor Responders n = 14	*P* Value
Age (yr)*	57.7 ± 7.5	51.7 ± 10.2	.064
Sex (F/M)	9/8	10/5	NS
Duration of disease (yr)*	8.4 ± 4.5	8.4 ± 4.2	NS
Previous therapies (no. of patients):			
Steroids	15	13	NS
Cyclophosphamide	9	9	NS
Total follow-up (mo)*	52.5 ± 35.7	76.6 ± 24.7	NS
Liver disease (no. of patients)	10	8	NS
Initial cryocrit (%)*	14.9 ± 12.4	24.9 ± 18	.069
IgM level (mg/dL)*	338 ± 158	428 ± 247	.22
Solitary anti-C22 (no. of patients)	8	1	.02
Viremia*	7.9 ± 5.4†	47 ± 26†	.04
Genotype (no./23 patients tested)			
1a	4	0	.10
1b	5	6	NS
2b	4	4	NS

Abbreviation: NS, not significant.

* Mean ± 1 standard deviation.

† HCV copies/mL $\times$ 10^6. Results from four patients tested in each group.

to the remission of the symptoms and signs of cryoglobulinemic vasculitis and not, unless specifically stated, of those of chronic hepatitis C. Data concerning the response of liver disease in the subset of affected patients are described in a separate section of the Results.

HCV RNA sequences could be detected by qualitative PCR in plasma before therapy from 13 of 14 complete responders and from 11 of 13 minor responders (Table 2). The three patients who were PCR negative before therapy had antibodies against all the four recombinant HCV proteins on RIBA testing. Mean levels of viremia before therapy by quantitative PCR in samples from four complete and four minor responders were 7.9×10^6 and 4.6×10^7 gE/mL, respectively ($P = .04$).

HCV genotype was determined in 23 patients (Table 2), and no significant differences in distribution between complete and minor responders to interferon therapy were observed, except for a trend ($P = .10$) to a higher prevalence of infection with the 1a genotype in complete responders (Table 1). Because mutations in the NS5a gene of the 1b strain are associated with increased response to interferon-α therapy,[11] we sequenced NS5a in seven of the eight 1b isolates. Three sequences were wild type, three had a single mutation at position 2219, and one had mutations at positions 2218 and 2219, without any correlation with response to therapy. None of our complete responders had 4-11 mutations, as previously reported in patients with chronic hepatitis C,[11] and both complete and minor responders were found with one to two mutations similar to the intermediate group that had a prevalence of 13% complete response in the study from Japan.[11]

Anti-HCV antibodies were detectable by RIBA-2 in all patients (Table 2). Antibodies were directed to the HCV antigens C22 (100%), C33 (59%), C100-3 (34%), and C5-11 (28%). Purified cryoprecipitates and cryoglobulin-depleted sera had the same patterns of reactivity to HCV antigens. Eight of 17 complete responders and only one of 14 minor responders (Table 2) had isolated anti-C22 antibodies ($P = .02$). We investigated the pattern of anti-HCV antibody reactivity by RIBA-2 in a control group of 35 unselected patients with chronic hepatitis C without cryoglobulinemia and not treated with interferon, attending the Hepatology section of the Department of Clinical Medicine of the University of Rome (eight patients with chronic active hepatitis, nine with cirrhosis, and 18 with unknown histology). Isolated anti-C−22 reactivity was observed in one of these 35 patients only, compared with the nine of 31 patients with HCV-associated cryoglobulinemia ($P = .004$).

No differences between the complete responder and the minor responder groups were apparent in sex, duration of disease, previous immunosuppressive therapies, presence of liver disease, serum IgM levels (Table 1), and major clinical manifestations (Table 2). Also, there were no differences in the distribution of HLA-DR antigens and of the WA cross-idiotype of monoclonal RF (data not shown). The prevalence of the WA cross-idiotype among our patients was similar to that observed in the United States,[18] and the reported[19-22] absence of an association of histocompatibility antigen markers with type II cryoglobulinemia was confirmed.

By univariate analyses (Table 1), only the presence of solitary anti-C22 reactivity and low viremia associated significantly ($P < .05$) with complete response. The correlation of lower cryocrit ($P = .069$) and older age ($P = .064$) with complete response approached significance. These two parameters appeared to be interdependent, as linear regression analysis showed a near significant ($P = .057$) correlation of lower cryocrit with older age. Furthermore, there were strong correlations of cryocrit with IgM level ($P = .018$) and IgM level inversely with age ($P = .005$). Conversely, there was no correlation of cryocrit with viremia or duration of disease.

Table 2. Clinical and Laboratory Data in Patients With Type II Mixed Cryoglobulinemia Treated With Interferon

Patient No.	Major Clinical Manifestations Before Therapy	Total Dose of IFN (MU)	Duration of Response Off-Therapy (mo)	RIBA		HCV RNA		HCV Genotype	Outcome
				Pretherapy	Posttherapy	Pretherapy	Posttherapy*		
Complete responders									
1	P, LD, U, RF, A, PN, Hy	729	100	22	22	Pos	Neg (94)	1a	Alive off-therapy
2	P, PN, SS, Ar, U, A	849	6	22	22	NP	NP	NP	Relapse V; dead DIC
3	P, A, RF, Hy	753	91	22, 33, 5-11	22	Pos	Neg (75)	1b	Alive off-therapy
4	P, U, A, PN, RF	504	NE	22	22	Pos	NP	1b	Dead MI
5	P, Ar, U, PN, A	741	33	22	22	NP	Neg (39)	NP	Relapse V
6	P, PN, LD, RF, SS, A, Ar	717	53	22	22	Pos	Neg (72)	1b	Relapse V; dead Pne
7	P, Ar, PN, SS	621	76	22	22	Pos	Neg (44)	2b	Alive off-therapy
8	P, Ar, U, A, RF	80	NE	22	22	NP	NP	NP	LFU
9	P, LD, SS, A, Ar, PN	814	17	22, 33	22	Pos	NP	1b	LFU
10	P, LD, SS	621	2	22, 33, 100	22	Pos	Neg (49)	1a	Alive off-therapy
11	P, LD, RF, A	621	46	22, 33, 100, 5-11	22, 33, 100, 5-11	Pos	Pos (54)	2b	Alive off-therapy
12	P, LD, Ar	621	33	22, 33 100, 5-11	22, 33	Neg	Neg (12)	NP	Alive off-therapy
13	P, LD, PN, Ar, SS	468	3	22, 33, 100, 5-11	22, 33	Pos	Neg (12)	2b	Relapse LD
14	P, LD, Ar, A	621	18	22, 33	22	Pos	Neg (24)	1a	Alive off-therapy
15	P, LD, PN, RF, Hy	621	16	22, 33, 100, 5-11	22, 33	Pos	Neg (22)	1b	Alive off-therapy
16	P, LD, A, PN, Hy	315	NE	22	22	Pos	Neg (4)	2b	Relapse V; LFU
17	P, RF, PN, A, Hy	621	10	22, 33, 100	22	Pos	Neg (12)	1a	Alive off-therapy
Minor responders									
18	P, PN, Hy, Ar	621	0	22, 33	22, 33	NP	Pos (60)	NP	Episodes of P
19	P, RF, A, Hy, LD	847	60	22, 33	22, 33	Pos	Pos (76)	NP	Relapse V, RF; dead Sep
20	P, U, PN, Ar	814	0	22, 33	22, 33	Pos	Pos (78)	2b	Relapse V
21	P, PN, U	621	0	22, 33	22, 33	Pos	Pos (18)	2b	Episodes of P & U
22	P, LD, PN, Ar	814	72	22, 33	22, 33	Pos	Pos (84)	1b	Relapse V
23	P, LD, PN	621	77	22, 33	22, 33	Pos	Pos (72)	1b	No symptoms
24	P, LD, PN, Ar, SS	814	0	22, 33, 100	22, 33, 100	Pos	Pos (78)	1b	Relapse LD
25	P, PN, Ar	504	0	22, 33, 100, 5-11	22, 33, 100, 5-11	Neg	Neg (36)	NP	Persistent PN
26	P, LD, U, Ar, PN	234	0	22, 33, 100, 5-11	22, 33, 100, 5-11	Pos	Pos (72)	1b	Relapse LD; cirrhosis
27	P, LD, Ar, SS	234	1	22, 100, 5-11	22, 100, 5-11	Pos	Neg (6)	1b	Relapse V, LD
28	P, LD, PN, Ar	234	0	22, 100	22, 100	Pos	Pos (72)	2b	Relapse LD; cirrhosis
29	P, LD, PN	814	0	22	22	Pos	Pos (48)	2b	No symptoms
30	P, RF, Hy, A, PN	621	0	22, 33, 100, 5-11	22, 33, 100, 5-11	Neg	Neg (36)	NP	RF
31	P, RF, Hy, A, SS	621	0	22, 33	22, 33	Pos	Pos (29)	1b	Dead accident

Abbreviations: A, anemia; Ar, arthralgias; DIC, disseminated intravascular coagulopathy; Hy, hypertension; LD, liver disease; LFU, lost to follow-up; MI, myocardial infarction; P, purpura; PN, peripheral neuropathy; Pne, pneumonia; RF, renal failure; Sep, sepsis; SS, sicca syndrome; U, cutaneous ulcers; NE, not evaluable; NP, not performed.

* Month posttherapy HCV RNA tested.

Multivariate analysis of age, sex, duration of disease, presence of liver disease, solitary C22, and cryocrit and serum IgM levels before therapy were performed; other pretherapy variables were excluded because of inadequate sample size. Among the tested variables, only the solitary anti-C22 pattern associated significantly ($P = .0393$) with complete response, while older age ($P = .0629$) and lower cryocrit ($P = .0642$) approached statistical significance.

Outcome of clinical responses to interferon therapy. Duration of response after 12 months of therapy, evaluable in 14 of the 17 complete responders, ranged between 3 and 100 months, with a median of 33 months. Patients 1, 3, and 7, and potentially 14 and 15, were putative cures because they were asymptomatic with no detectable viremia or cryoglobulinemia after therapy had been stopped for more than 5 years in this first group and more than 16 months in the ''potential'' group. Fifteen patients had minor responses; in four patients (nos. 19, 20, 22, and 27), all features responded except cryoglobulinemia.

In all of the complete responders cryoglobulins became undetectable. Reduction of cryocrit levels correlated with remission of cutaneous symptoms and was generally rapid, occurring between 2 and 4 weeks after the initiation of therapy. Responses were generally accompanied by an increase of hemoglobin levels in patients with anemia and by reduction of serum creatinine in patients with renal failure. Paresthesias caused by sensorimotor axonal neuropathy, which was observed in 24 patients, improved significantly in the majority of patients, but responses occurred only after several months of therapy and were never accompanied by normalization of electroneurographic abnormalities.

Relapse of cryoglobulinemic vasculitis occurred in five

patients (patients 2, 16, 19, 20, and 22) during interferon therapy and in two patients (patients 5 and 6) after therapy had been stopped. Relapses were uniformly characterized by recurrence of purpura and increase of cryocrit values, which often reached the pretherapy levels, and by one or more of the following signs and symptoms: cutaneous ulcers, distal necrosis, acute decrease of renal function, macroscopic hematuria, anemia, increase of transaminase levels, or paresthesias. Of the patients whose disease relapsed during therapy, one (patient 16) had an early relapse and was lost to follow-up review shortly thereafter. In three of the remaining four patients (nos. 2, 19, and 20), relapses were accompanied by the production of anti-interferon-α antibodies, as has previously been reported.[13] Remission was reinduced in all four patients by treatment with natural interferon-α.

Response of cryoglobulinemia to interferon therapy associates with decrease of anti-HCV antibody titers and viremia. Comparison of the pretherapy and posttherapy anti-HCV antibody patterns by RIBA analysis (Table 2) showed that eight of nine complete responders with multiple antigen reactivity before therapy lost at least one reactivity after therapy, whereas in none of the minor responders did the pattern of antibodies change ($P = .009$). Antibodies to C22 antigen persisted in all patients irrespective of changes in clinical and virologic parameters. The only complete responder (patient 11) in whom multiple anti-HCV antibodies remained unchanged after therapy was also the only one in this group to remain viremic (Table 2) despite prolonged remission (57 months) of vasculitis and normalization of liver enzymes.

In the patients who showed no change in the RIBA pattern, anti-HCV antibody concentrations were further evaluated semiquantitatively by ELISA. In eight of nine complete responders in this category, but only in one of the 14 minor responders, there was a decrease of antibody titers; the mean difference between the pretherapy OD value and the lowest OD value posttherapy was 0.739 in complete responders and 0.026 in minor responders ($P < .001$). The only minor responder with a solitary C-22 antibody (patient 29) showed an increase in titer. To rule out that the decrease of specific anti-HCV antibody titers was the result of a generalized reduction of immunoglobulin synthesis, the serum immunoglobulin levels before and after 1 year of treatment were measured (not shown). Only one patient (patient 31) showed a significant reduction of IgG levels from 2,400 mg/dL to 678 mg/dL, but anti-HCV antibody titer remained unchanged.

HCV RNA after therapy (Table 2) became undetectable in 10 of 11 complete responders and in only one of 10 minor responders ($P = .0002$). In the 10 complete responders, clearance of HCV RNA (that is, HCV RNA no longer detectable by PCR assay) occurred as early as 1 month (median, 10 months) after treatment. Early and late patterns of virus clearance were observed. Early clearance was documented within 1 year of therapy in seven patients (Table 2). Very rapid clearance, within 1 month, could be documented in three of these patients (nos. 14, 15, and 16). Late clearance was instead observed in three patients (nos. 3, 6, and 7) who remained positive for 44, 47, and 22 months, respectively, after completion of therapy (not shown); subsequently, the virus ''spontaneously'' cleared and they remained negative over the subsequent 47, 11, and 53 months, respectively (not shown). In contrast, 10 minor responders remained viremic for 18 to 84 months (median, 72 months) after the start of therapy.

Viremia was quantitated in serial samples from eight patients. Rapid clearance of HCV was found in three of four complete responders. In contrast, only a moderate reduction of HCV titers was observed in three of four minor responders. In one minor responder (patient 27), initial clearance of viremia with therapy was followed by reappearance of viremia with clinical relapse.

No relationship between viral clearance and HCV genotype was apparent. Of particular note, the 1b genotype infection in patient 16 cleared within 1 month, and the only minor responder to clear viremia was infected with 1b genotype. Conversely, the only complete responder (patient 11), who remained viremic during a sustained remission, was infected with the 2b genotype.

In 10 complete responders in whom data on all serologic parameters were obtained, there was a correlation of clearance of viremia, loss of populations of HCV antibody or decrease in antibody titer, and clearance of cryoglobulins with clinical response to interferon therapy (Table 2 and data not shown). The correlation of these parameters with clinical disease activity was particularly apparent in patient 16 who, after an initial remission of symptoms with clearance of viremia and reduction of antibody titers and cryocrit, had a clinical relapse concomitant with a flare-up of these laboratory parameters. There were, however, some exceptions. For example, in patients 6, 7, and 11, complete clinical remission and suppression of cryoglobulins occurred despite constant levels of anti-HCV antibodies and persisting viremia. In one of these patients (no. 11) viremia persisted throughout the follow-up period, whereas in patients 6 and 7, HCV RNA became undetectable and antibody levels fell after several months of follow-up (Table 2). Of the two patients who relapsed while off-therapy (nos. 5 and 6), one (no. 6) was notable, as relapse occurred 53 months after withdrawal of interferon, at which time HCV viremia had cleared for 8 months and was characterized by the recurrence of purpura and renal involvement, but not of abnormal transaminase levels and by persistently negative HCV viremia.

Response of liver disease to interferon therapy. Eighteen patients (58%) had elevation of transaminase levels (Table 3), and liver biopsy was performed before therapy in nine of them. Levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) returned to normal levels within the first month of therapy in all of the 18 patients, 10 of whom were complete responders. Normalization of transaminase levels lasted for 1 to 100 months (median, 33 months) in the overall group of patients. No relationship between histologic activity and duration of response was apparent. Although older age tended to correlate with the severity of histologic activity grading and staging scores ($P = .055$ and $P = .15$, respectively), the mean age of patients with liver disease, 51.7 ± 10.5 years, was significantly ($P = .024$) lower than that of patients without liver disease, 59.3 ± 6.1 years. Relapses of hepatic disease were observed

Table 3. Characterization and Response to Interferon Therapy of Liver Disease in Patients With HCV-Associated Cryoglobulinemia

Patient No.	Response of Cryoglobulinemia	Liver Histology*	HCV Genotype	Initial ALT†	Total Dose of Interferon	Relapse of Abnormal ALT	Months Off-Therapy With Normal ALT
1	Complete	ND	1a	37	729	No	100
6	Complete	6 + 1	1b	36	717	No	53
9	Complete	ND	1b	30	814	No	17
10	Complete	5 + 2	1a	38	621	No	62
11	Complete	ND	2b	105	621	No	46
12	Complete	4 + 2	ND	108	621	No	33
13	Complete	5 + 2	2b	79	468	Yes	3
14	Complete	4 + 1	1a	64	621	No	18
15	Complete	ND	1b	70	621	No	16
16	Complete	9 + 4	2b	66	315	No	Lost to follow-up
19	Minor	2 + 1	ND	48	847	No	60
22	Minor	ND	1b	85	814	No	96
23	Minor	ND	1b	97	621	No	77
24	Minor	4 + 2	1b	47	814	Yes	6
26	Minor	ND	1b	125	234	Yes	1
27	Minor	5 + 1	1b	147	234	Yes	1
28	Minor	ND	2b	35	234	Yes	3
30	Minor	ND	2b	61	621	No	60

Abbreviation: ND, not determined.

* Histological activity is expressed by the Knodell score[12]; the first number refers to grading (scores 1 + 2 + 3), and the second to staging (score 4).

† Highest ALT value observed within 3 months before the initiation of therapy; normal values <22 U/L.

in five of the 18 patients (Table 3). All relapses occurred 1 to 6 months after withdrawal of interferon therapy. Noteworthy, four of these five patients, but none of the 12 patients without relapse of liver disease, were treated with less than 621 MU of interferon ($P = .0021$; Table 3). In the group of 12 patients treated with greater than 621 MU, remission of liver disease lasted between 16 and 100 months (median, 60 months).

DISCUSSION

Although the effectiveness of interferon therapy for type II cryoglobulinemia associated with HCV infection is now firmly established,[5-7,13,23-27] an optimum regimen has not yet been determined or have the criteria for selecting patients likely to have long-term responses been accurately delineated.

The 62% frequency of complete response of cryoglobulinemia to interferon therapy in this study was similar to other reports,[24-27] but the extended period of observation permitted documentation of complete responses of as long as 100 months and a median response duration of 33 months. These sustained responses are considerably longer than any other results reported for patients with cryoglobulinemia,[24-27] and our results suggest that extended interferon therapy with a total dose of 621 MU or greater can produce putative cures in 15% to 20% of patients. Because this was not a randomized, controlled study to compare high and low interferon-α dosages, definitive conclusions cannot be made regarding the efficacy of higher dose schedules. However, our results with high dosages are consistent with recent studies showing greater efficacy of high dose interferon-α in the treatment of chronic hepatitis C.[28,29] The conclusion that high dose interferon-α may yield longer remission of cryoglobulinemia is in agreement with our initial studies[6] and with the more recent studies of Misiani et al.[26]

Complete response of cryoglobulinemic vasculitis was usually associated with clearance of viremia and decrease of anti-HCV antibodies. This is consistent with the hypothesis that HCV antigens, either free or complexed to specific antibodies, stimulate the production of type II cryoglobulins[23] and, therefore, that disease activity directly correlates with viral load. However, a correlation of response with the reduction of viremia and of antibody titers was not always apparent, because in some complete responders, both parameters remained unaltered and in others, viral clearance occurred long after the clinical remission. These observations suggest the contribution of immunomodulatory rather than antiviral effects of interferon and of late-acting host effector mechanisms.[30]

Our study highlights the greater efficacy of high-dose interferon therapy not only on cryoglobulinemic vasculitis, but also by separate analysis, on the response of liver disease. The 100% normalization of transaminase levels and the 75% rate of normalization lasting for more than 6 months off-therapy with a mean duration of 35.9 months, were considerably better than the results reported for the broad population of patients with chronic hepatitis C treated with interferon.[10] The finding that relapses of liver disease occurred in 100% of patients receiving less than 621 MU cumulative dose, compared with an 8% relapse rate in patients receiving more than 621 MU, strongly supports the greater efficacy of higher cumulative doses of interferon on liver disease. These findings are consistent with recent studies on chronic hepatitis C that showed higher response rates and longer sustained responses with higher doses of interferon-α[28,29]; indeed, the highest cumulative doses used in those studies were comparable to the doses used in our study.

Concerning our analysis of pretherapy variables predictive of complete response of cryoglobulinemic vasculitis to inter-

feron therapy, the most striking association was with the presence of solitary anti-C22 antibody. The 29% prevalence of solitary anti-C22 in our patients is similar to the 34% observed in another study on mixed cryoglobulinemia,[31] while this pattern is uncommon (3% to 4%, this study and Chemello[32]) among patients with chronic hepatitis C. The solitary anti-C22 antibody pattern did not appear to result in our patients from immunosuppressive therapies, and therefore its high prevalence in HCV-infected patients with type II cryoglobulinemia could be due either to their genetic background of immune reactivity or, perhaps more likely, to the fact that in these patients, HCV antigenic load tends to be low and thus capable of eliciting only responses directed to immunodominant epitopes. This possibility is supported by the known immunodominance of C-22 antigen[33,34] and by the frequent finding of isolated antibody to C22 in chronic hepatitis patients with minimal disease[35,36] or treated with interferon-α.[37] A tendency to low viral load in type II cryoglobulinemic patients with HCV infection is also consistent with the relatively benign course of chronic hepatitis in these subjects.[38] It is tempting to speculate that the lower severity of liver disease in HCV-infected patients with type II cryoglobulinemia could be due to the cryoglobulins themselves. In fact, the selective concentration of HCV and very low density lipoproteins (VLDL) in the cryoprecipitates[4,39] suggests that monoclonal rheumatoid factors could bind HCV complexed to specific antibodies and to VLDL, therefore preventing endocytosis via Fc or LDL receptors.[40]

In our study, the mean age of complete responders tended to be higher than that of minor responders, although only approaching significance by multivariate analysis. This is noteworthy because it is the reverse of the finding in chronic hepatitis C. It should be noted, however, that our clinical and laboratory criteria of response relied on the manifestations of cryoglobulinemic vasculitis and not on the indexes of liver disease activity as in studies done in patients with chronic hepatitis C. Thus, the most likely explanation for our observation is that because age correlated inversely with serum IgM level and with cryocrit, older patients responded better because they produced less monoclonal rheumatoid factor.

Another substantial difference with previous studies in chronic hepatitis C is that we had rapid viral clearance and long-lasting response of both cryoglobulinemic vasculitis and abnormal transaminase levels in patients infected by strain 1b, which is widely reported[41] to be resistant to interferon therapy. Our high rate of response in strain 1b-infected patients was not due to mutations in the NS5a gene[11] and, consistent with recent reports,[28,29,42] could be more likely attributed to the high dosage of interferon used in our study.

The distribution of genotypes among the patients with type II cryoglobulinemia in this study reflected the distribution in the normal population,[41] consistent with all previous studies,[43-47] except a recent report on an association of the 2a genotype with mixed cyroglobulinemia.[48] In the latter study, however, a 2a primer was used that did not differentiate among 2a, 2b, and 2c sequences and dual infections were detected using a 1b primer, which has been shown to produce false-positive dual infections.[49] Moreover, considering the wide temporal and geographic variations in genotypes, there may be differences in the prevalence of genotypes between northern and southern Italy.

The present study includes, to our knowledge, the longest durations of complete responses reported among patients with HCV-associated type II cryoglobulinemia treated with interferon and has both clinical and theoretical implications. Clinically, an important observation is that a total dose of interferon of $\geq$621 MU can produce apparent cures, albeit only in a small percentage of patients. Our data indicate that, as in chronic hepatitis C, low viremia is associated with the likelihood of complete response of HCV-associated cryoglobulinemia. Interestingly, the solitary anti-C−22 antibody pattern observed in about one third of our patients was also significantly associated with complete response, hopefully providing a much simpler and less expensive predictive tool than the quantitation of viremia in patients with cryoglobulinemia. Finally, our data suggest that levels of viremia and of anti-HCV antibodies may be used to monitor disease activity and response to therapy. Future prospective studies exploiting these laboratory parameters and employing higher cumulative doses of interferon will hopefully permit us to more accurately delineate predictors of response and to increase the prevalence of long-lasting complete remission of cryoglobulinemic vasculitis.

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REFERENCES

1. Meltzer M, Franklin EC, Elias K, McCluskey RT, Cooper N: Cryoglobulinemia−A clinical and laboratory study. II. Cryoglobulins with rheumatoid factor activity. Am J Med 40:837, 1966

2. Brouet JC, Clauvel JP, Danon F, Klein M, Seligmann M: Biologic and clinical significance of cryoglobulins: A report of 86 cases. Am J Med 57:775, 1974

3. Agnello V, Romain PL: Mixed cryoglobulinemia secondary to hepatitis C virus infection. Rheum Dis Clin North Am 22:1, 1996

4. Agnello V, Chung RT, Kaplan LM: A role for hepatitis C virus infection in type II cryoglobulinemia. N Engl J Med 327:1490, 1992

5. Bonomo L, Casato M, Afeltra A, Caccavo D: Treatment of idiopathic mixed cryoglobulinemia with alpha interferon. Am J Med 83:726, 1987

6. Casato M, Lagana B, Antonelli G, Dianzani F, Bonomo L: Long-term results of therapy with interferon-alpha for type II essential mixed cryoglobulinemia. Blood 78:3142, 1991

7. Knox TA, Hillyer CD, Kaplan MM, Berkman EM: Mixed cryoglobulinemia responsive to interferon-alpha (letter). Am J Med 91:554, 1991

8. Geltner D: Therapeutic approaches in mixed cryoglobulinemia. Springer Semin Immunopathol 10:103, 1988

9. St Clair WE: Vasculitis, in Weisman MH, Weinblatt ME (eds): Treatment of the Rheumatic Diseases: Companion to the Textbook of Rheumatology. Philadelphia, PA, Saunders, 1995, p 137

10. Davis GL, Lau JY, Lim HL: Therapy for chronic hepatitis C. Gastroenterol Clin North Am 23:603, 1994

11. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C: Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus lb infection. N Engl J Med 334:77, 1996

12. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ: Classification of chronic hepatitis: Diagnosis, grading and staging. Hepatology 19:1513, 1994

13. Casato M, Antonelli G, Maggi F, Pucillo LP, Di Lullo L, Leoni M, Currenti M, Dianzani F, Bonomo L: Resistance to recombinant alpha interferon therapy in idiopathic mixed cryoglobulinemia: Reinduction of remission by natural alpha interferon both in antibody-positive and -negative patients. J Biol Regul Homeost Agents 8:56, 1994

14. Olerup O, Zetterquist H: HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: An alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens 39:225, 1992

15. Agnello V, Barnes JL: Human rheumatoid factor cross-idiotypes. I. WA and BLA are heat-labile conformational antigens requiring both heavy and light chains. J Exp Med 164:1809, 1986

16. Cha TA, Beall E, Irvine B, Kolberg J, Chien D, Kuo G, Urdea MS: At least five related, but distinct, hepatitis C viral genotypes exist. Proc Natl Acad Sci USA 89:7144, 1992

17. Widell A, Shev S, Mansson S, Zhang YY, Foberg U, Norkrans G, Fryden A, Weiland O, Kurkus J, Nordenfelt E: Genotyping of hepatitis C virus isolates by a modified polymerase chain reaction assay using type specific primers: Epidemiological applications. J Med Virol 44:272, 1994

18. Agnello V, Zhang QX, Abel G, Knight GB: The association of hepatitis C virus infection with monoclonal rheumatoid factors bearing the WA cross-idiotype: Implications for the etiopathogenesis and therapy of mixed cryoglobulinemia. Clin Exp Rheumatol 13:S101, 1995

19. Czaja AJ, Carpenter HA, Santrach PJ, Moore SB: Immunologic features and HLA associations in chronic viral hepatitis. Gastroenterology 108:157, 1995

20. Nightingale SD, Pelley RP, Delaney NL, Bias WB, Hamburger MI, Fries LF, Steinberg AG: Inheritance of mixed cryoglobulinemia. Am J Hum Genet 33:735, 1981

21. Migliorini P, Bombardieri S, Castellani A, Ferrara GB: HLA antigens in essential mixed cryoglobulinemia. Arthritis Rheum 24:932, 1981

22. Berliner S, Weinberger A, Zamir R, Hazaz B, Pinkhas J: Familial cryoglobulinemia and C4 deficiency. Scand J Rheumatol 13:151, 1984

23. Agnello V: The aetiology of mixed cryoglobulinaemia associated with hepatitis C virus infection. Scand J Immunol 42:179, 1995

24. Durand JM, Kaplanski G, Lefevre P, Richard MA, Andrac L, Trepo C, Soubeyrand J: Effect of interferon-alpha 2b on cryoglobulinemia related to hepatitis C virus infection (letter). J Infect Dis 165:778, 1992

25. Ferri C, Marzo E, Longobardo G, Lombardini F, La Civita L, Vanacore R, Liberati AM, Gerli R, Greco F, Moretti A, Monti M, Gentilini P, Bombardieri S, Zignego AL: Interferon-alpha in mixed cryoglobulinemia patients: A randomized, crossover-controlled trial. Blood 81:1132, 1993

26. Misiani R, Bellavita P, Fenili D, Vicari O, Marchesi D, Sironi PL, Zilio P, Vernocchi A, Massazza M, Vendramin G, Tanzi E, Zanetti A: Interferon alfa-2a therapy in cryoglobulinemia associated with hepatitis C virus. N Engl J Med 330:751, 1994

27. Dammacco F, Sansonno D, Han JH, Shymala V, Cornacchiulo V, Iacobelli AR, Lauletta G, Rizzi R: Natural interferon-alpha versus its combination with 6-methyl-prednisolone in the therapy of type II mixed cryoglobulinemia: A long-term, randomized, controlled study. Blood 84:3336, 1994

28. Kasahara A, Hayashi N, Hiramatsu N, Oshita M, Hagiwara H, Katayama K, Kato M, Masuzawa M, Yoshihara H, Kishida Y, Shimizu Y, Inoue A, Fusamoto H, Kamada T: Ability of prolonged interferon treatment to suppress relapse after cessation of therapy in patients with chronic hepatitis C: A multicenter randomized controlled trial. Hepatology 21:291, 1995

29. Poynard T, Bedossa P, Chevallier M, Mathurin P, Lemonnier C, Trepo C, Couzigou P, Payen JL, Sajus M, Costa JM, Vidaud M, Chaput JC, and the multicenter study group: A comparison of three interferon alfa-2b regimens for the long-term treatment of chronic non-A, non-B hepatitis. N Engl J Med 332:1457, 1995

30. Vilcek J, Sen GC: Interferons and other cytokines, in Fields BN, Knipe DM, Howley PM (eds): Fields Virology. Philadelphia, PA, Lippincott, 1996, p 375

31. Misiani R, Bellavita P, Fenili D, Borelli G, Marchesi D, Massazza M, Vendramin G, Comotti B, Tanzi E, Scudeller G, Zanetti A: Hepatitis C virus infection in patients with essential mixed cryoglobulinemia. Ann Intern Med 117:573, 1992

32. Chemello L, Cavalletto D, Pontisso P, Bortolotti F, Donda C, Donadon V, Frezza M, Casarin P, Alberti A: Patterns of antibodies to hepatitis C virus in patients with chronic non-A, non-B hepatitis and their relationship to viral replication and liver disease. Hepatology 17:179, 1993

33. Nasoff MS, Zebedee SL, Inchauspe G, Prince AM: Identification of an immunodominant epitope within the capsid protein of hepatitis C virus. Proc Natl Acad Sci USA 88:5462, 1991

34. Ferrari C, Valli A, Galati L, Penna A, Scaccaglia P, Giuberti T, Schianchi C, Missale G, Marin MG, Fiaccadori F: T-cell response to structural and nonstructural hepatitis C virus antigens in persistent and self-limited hepatitis C virus infections. Hepatology 19:286, 1994

35. Zanella A, Conte D, Prati D, Mozzi F, Capelli C, Zanuso F, Fraquelli M, Bosoni P, Vianello L, Pappalettera M, Velio P, Montagnolo G, Bianchi PA, Sirchia G: Hepatitis C virus RNA and liver histology in blood donors reactive to a single antigen by second-generation recombinant immunoblot assay. Hepatology 21:913, 1995

36. Botarelli P, Brunetto MR, Minutello MA, Calvo P, Unutmaz D, Weiner AJ, Choo QL, Shuster JR, Kuo G, Bonino F, Houghton M, Abrignani S: T-lymphocyte response to hepatitis C virus in different clinical courses of infection. Gastroenterology 104:580, 1993

37. Yuki N, Hayashi HN, Hagiwara H, Takehara T, Katayama K, Kasahara A, Fusamoto H, Kamada T: Quantitative analysis of antibodies to hepatitis C virus during interferon-alpha therapy. Hepatology 17:960, 1993

38. Monti G, Galli M, Invernizzi F, Pioltelli P, Saccardo F, Monteverde A, Pietrogrande M, Renoldi P, Bombardieri S, Bordin G, Candela M, Ferri C, Gabrielli A, Mazzaro C, Migliaresi S, Mussini C, Ossi E, Quintiliani L, Tirri G, Vacca A, and the GISC: Cryoglobulinaemias: A multi-centre study of the early clinical and laboratory manifestations of primary and secondary disease. Q J Med 88:115, 1995

39. Agnello V, Zhang QX, Elfahal M, Abel G, Knight G: Association of hepatitis C virus (HCV) with VLDL in type II cryoglobulinemia. Ninth International Congress of Immunology, San Francisco, CA, July 23–29, 1995, p 631

40. Agnello V, Abel G, Zhang QX, Elfahal M, Knight GB: Low density lipoprotein receptor mediated endocytosis of hepatitis C virus. IX Triennial International Symposium on Viral Hepatitis and Liver Disease, Rome, Italy, April 21–25, 1996, p 158

41. Bukh J, Miller RH, Purcell RH: Genetic heterogeneity of hepatitis C virus: Quasispecies and genotypes. Semin Liver Dis 15:41, 1995

42. Zeuzem S, Schmidt JM, Lee JH, Rster B, Roth WK: Effect of interferon alfa on the dynamics of hepatitis C virus turnover in vivo. Hepatology 23:366, 1996

43. Willems M, Sheng L, Roskams T, Ramdani B, Doutrelepont

JM, Neven F, Durez P, Treille S, Adler M, Desmet V, Fevery J, Yap SH: Hepatitis C virus and its genotypes in patients suffering from chronic hepatitis C with or without a cryoglobulinemia-related syndrome. J Med Virol 44:266, 1994

44. Pozzato G, Mazzaro C, Crovatto M, Modolo ML Ceselli S, Mazzi G, Sulfaro S, Franzin F, Tulissi P, Moretti M, Santini GF: Low-grade malignant lymphoma, hepatitis C virus infection, and mixed cryoglobulinemia. Blood 84:3047, 1994

45. Pawlotsky JM, Roudot-Thoraval F, Simmonds P, Mellor J, Ben Yahia MB, Andre C, Voisin MC, Intrator L, Zafrani ES, Duval J, Dhumeaux D: Extrahepatic immunologic manifestations in chronic hepatitis C and hepatitis C virus serotypes. Ann Intern Med 122:169, 1995

46. Mazzaro C, Tulissi P, Moretti M, Mazzoran L, Pussini E, Crovatto M, Santini GF, Pozzato G: Clinical and virological findings in mixed cryoglobulinaemia. J Intern Med 238:153, 1995

47. Sinico RA, Ribero ML, Fornasieri A, Renoldi P, Zhou J, Fasola M, Portera G, Arrigo G, Gibelli A, D'Amico G, Tagger A: Hepatitis C virus genotype in patients with essential mixed cryoglobulinaemia. Q J Med 88:805, 1995

48. Zignego AL, Ferri C, Giannini C, Monti M, La Civita L, Careccia G, Longobardo G, Lombardini F, Bombardieri S, Gentilini P: Hepatitis C virus genotype analysis in patients with type II mixed cryoglobulinemia. Ann Intern Med 124:31, 1996

49. Andonov A, Chaudhary RK: Genotyping of Canadian hepatitis C virus isolates by PCR. J Clin Microbiol 32:2031, 1994