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LONG-TERM EFFECTS OF α-INTERFERON THERAPY FOR TYPE II MIXED CRYOGLOBULINEMIA

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ABSTRACT

Background. **Several reports showed that mixed cryoglobulinemia (MC) is closely associated with hepatitis C virus (HCV) infection. Since several authors reported the efficacy of α-interferon in the treatment of MC, we investigated the long-term effects of this drug on clinical, hematological and virological parameters in a group of 18 patients (13 women and 5 men, mean age 56 ±11 years) affected by MC.**

Methods. **A bone marrow biopsy was performed in all patients, and a liver biopsy was obtained in those with biochemical signs of chronic liver disease. The presence of HCV-RNA in serum was assessed by detection of anti-HCV antibodies and by PCR amplification of the 5' untranslated region of HCV. All patients followed the same treatment schedule: three million units of recombinant interferon α-2b s.c., three times a week for 1 year.**

Results. **In 5 cases bone marrow histology showed the presence of a monoclonal lymphocytic infiltrate. Liver biopsies were performed in 13 (72%) of the patients and a chronic liver disease was found in all 13. Anti-HCV antibodies were present in 17 (95%) subjects. HCV-RNA was detected in all cases (100%) before therapy. Five (28%) patients achieved a complete response and 9 (50%) a partial response, while the others (4 cases, 22%) showed minor responses. Four patients cleared the virus and obtained a complete remission of the MC.**

Conclusions. **HCV may be a cause of MC. The disease is associated with a high incidence of monoclonal lymphocytic infiltrate of the bone marrow. α-interferon seems to be an effective agent for the treatment of MC.**

Key words: mixed cryoglobulinemia, α-interferon, hepatitis C virus, rheumatoid factor, immunoglobulins

Mixed cryoglobulinemia (MC) is considered a lymphoproliferative disorder characterized by the presence of variable levels of cryoglobulins and involvement of several organs and tissues.[1] Histopathological lesions are secondary to vasculitis caused by the deposition of immune complexes in small and medium-sized blood vessels. The disease is often associated with multiple organ involvement, such as chronic hepatitis,[2] glomerulonephritis[3] and peripheral neuropathy.[4]

In the last few years, several reports have shown that MC is closely associated with hepatitis C virus (HCV) infection.[5-7] Since this virus is able to infect both T and B lymphocytes,[8] and a recent report demonstrated the presence of the viral genome in the peripheral lymphocytes of patients affected by MC,[9] direct involvement of HCV in this disease is likely.

In the past, therapy was based on chronic glucocorticoid administration and periodical plasma-exchange.[10] Although useful in the acute phase of the disease, plasmapheresis has no current role in the long-term therapy of the dis-

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ease. In the presence of a viral infection corticosteroid (or other immunosuppressive) therapy can no longer be recommended, because of the risk of increasing viral replication and, consequently, the number of infected cells. Furthermore, corticosteroids cause significant toxicity, especially in elderly patients. Therefore there are compelling reasons to find alternative drugs for the treatment of this disorder.

Following reports by several authors on the efficacy of α-interferon (α-IFN) in the treatment of MC,[11-13] we investigated the long-term effects of this drug on clinical, hematological and virological parameters in a group of people suffering from this condition.

Patients and Methods

Patients

Eighteen patients (13 women and 5 men, mean age 56±11 years) affected by type II MC were included in the study. Diagnosis was based on clinical, hematological and immunological findings. Median duration of the disease before α-IFN therapy was 3 years (range 1 to 6). All participants showed cutaneous lesions (purpura) with varying degrees of severity. Polyneuropathy was present in 7 (39%) cases. Daily alcohol intake was under 30 gr for every patient, and all of them had been previously treated with corticosteroids and/or cytotoxic drugs.

Liver function tests and hematological parameters were determined by usual laboratory methods. HBV and HIV markers were detected by ELISA using commercially available kits. The presence of the following auto-antibodies was also determined in each patient: anti-smooth muscle (ASMA), anti-mitochondrial (AMA), organ non-specific antinuclear (ANA), anti-liver-kidney-microsomal (LKM) antibodies. Thyroid function was also assessed by standard methods. Rheumatoid factor (RF), C3 and C4 complement fractions were measured by rate nephelometry.

Cryoglobulin isolation

Twenty mL of blood were kept in a glass tube at 37°C for 2 hours. Sera were cleared by centrifugation at 4,000 rpm for 20 minutes at room temperature and stored at 4°C for 7 days. The cryoprecipitate was separated by centrifugation at 4000 rpm for 30 minutes at 4°C. Mixed cryoglobulins were classified as type II on the basis of the presence of monoclonal immunoglobulins with RF activity complexed with polyclonal IgG.

Histology

A bone marrow biopsy was performed with a Jamshidi needle in all patients, and the sample was placed in B5 solution and 2 hours later in ethanol 70%. After decalcification, samples were stained according to standard methods.

A liver biopsy was obtained in patients with biochemical and/or clinical signs of chronic liver disease. These biopsies were carried out with a Menghini-like needle having an internal diameter of 1.8 mm. Samples were placed in buffered formalin and stained with hematoxylin and eosin and (for reticulum) Gomori's stain.

Phenotyping

Mononuclear cells were separated from blood samples and from marrow aspirates on an Emagel density gradient. Cells were stained with specific monoclonal antibodies and, after incubation and washing, immunofluorescence was measured on a FACScan flow cytometer (Becton Dickinson, USA). Monoclonal antibodies against CD3, CD4, CD5, CD8, CD16, CD19, CD57 and IgM were used. Anti-CD3 -4 -8 (OKT3-4-8) were purchased from Ortho, anti-CD19 (B4-RD1) from Coulter, anti-CD5 -16, -19, -57 (LEU1, LEU11c, LEU12, LEU7) from Becton Dickinson, anti IgM from Dako.

The monoclonality of peripheral and/or marrow lymphocytes was evaluated by FACS determination of surface light-chain distribution. The summation curves of the histograms were analyzed with Kolmogorov-Smirnov statistics,[14] and a D value of more than 10.0 was considered significant for monoclonality.[15]

Virological studies

Anti-HCV antibodies. The presence of anti-HCV antibodies was assayed by the second gen-

eration (four-antigen) immunoenzymatic screening test ORTHO-HCV (Ortho Diagnostic Systems, Raritan, NY, USA). This assay detects specific reactivity to four HCV antigens, including three non-structural (C100-3, 5-1-1, C-33c) and one structural (C22). All positive and negative readings were checked with an additional confirmatory test (RIBA, Chiron Co, Emeryville, CA, USA). Sera showing two or more positive bands were considered *reactive,* whereas those with only one band (usually C22) were defined as *indeterminate,* and those without HCV antigen bands were *negative.*[16]

HCV-RNA detection

The presence of HCV-RNA in serum was assessed by polymerase chain reaction (PCR) amplification of the 5' untranslated region (5'UTR) of HCV, which is considered the most conserved region of the virus.[17-19] Amplification was performed in two steps, the so-called *nested PCR.*[20] To minimize the risk of contamination, a source of false positive results, a negative control was included in each batch of serum samples.

cDNA synthesis: 3.0 μL of serum were added to a mixture containing 5.0 μL of 5× buffer, 2.5 μL of dNTP (2.5 mM), 1.0 μL of Nonidet p-40 10%, 1.0 μL of HPRI, 50 pmoles of antisense external primer and H_2O/DEPC for a total volume of 22.0 μL. The mixture was incubated in ice cold water for 5', at 92.0°C for 30" and again in ice cold water for 5'. After centrifugation 4 U of reverse transcriptase were added and the sample was incubated for 60' at 42°C.

Nested PCR: First step: 25.0 μL of cDNA were placed in a mixture containing 10.0 μL of 10× buffer, 8.0 μL of dNTP (2.5 mM), external primers 50 pmoles, Taq polymerase 2 U/μL, and water for a total volume of 75 μL. Amplification was performed in a DNA thermal cycler for 35 cycles. Each reaction cycle included denaturation at 94°C for 1'; annealing at 45°C for 1' and extension at 72°C for 2'. In the first cycle the denaturation phase lasted 4'. Second step: three μL of the first amplification product were subjected to a second PCR (under the same conditions) for 25 cycles using internal primers.

The products of the second PCR were analyzed on agarose gel electrophoresis, stained with ethidium bromide and observed under u.v. light.

Primers: the following primers, synthesized with the Gene Assembler (Pharmacia, USA) were used:

external antisense:
5'GATGCACGGTCTACGAGACCTC 3' nt: -1 -21
external sense:
5'AACTACTGTCTTCACGCAGAA 3' nt:-289 -269
internal antisense:
5'GCGACCCAACACTACTCGGCT 3' nt:-70 -90
internal sense:
5'ATGGCGTTAGTATGAGTG 3' nt:-257 -240

Therapy

Schedule

All patients followed the same treatment schedule: three megaunits of recombinant interferon α-2b (Schering-Plough), i.m. or s.c., three times a week for 1 year. All patients also gave informed consent to treatment. The protocol was approved by the Ethical Committee of Pordenone General Hospital.

Evaluation criteria

Responses to treatment were classified according to the following clinical and laboratory criteria.[11]

Complete response: reduction of the cryocrit by more than 20% of the initial value, disappearance of all clinical manifestations of the disease (including purpura and neuropathy), normalization of hemoglobin.

Partial response: disappearance of all signs of the disease, normalization of hemoglobin, but a reduction of the cryocrit by less than 20%.

Minor response: reduction of the cryocrit by less than 20% associated with the disappearance of one or more signs of vasculitis (but not all).

Responses of the chronic liver disease were examined separately according to the evolution of serum ALT levels: patients with no or very little decrease in ALT serum activity were defined as *non-responders*. *Responders* were those whose ALT levels normalized during the

therapy period and remained normal for at least 12 months after discontinuation of therapy. *Relapsers* were those who showed an increase of serum ALT activity after cessation of therapy.

Follow-up

Biochemical and clinical parameters were determined each month during therapy and every two months after the discontinuation of α-IFN. Autoantibodies were measured every 3 months and thyroid function tests run every 6 months. Assessment of anti-HCV antibodies was performed before the beginning of therapy and at the end of treatment. All patients were followed for at least 12 months after the end of therapy.

Statistical analysis

Data are expressed as mean±standard deviation. Statistical analysis was carried out with the statistical package SPSS. One-way analysis of variance between two groups was calculated. For categorical variables a cross tabulation with Pearson's X^2 was used to test whether row and column variables were independent.

Table 1. Main clinical and laboratory findings in 18 patients affected by mixed cryoglobulinemia.

n.	age/sex	MC	RF (IU/mL) nv<80	C4 (mg/dL) nv>18	C3 (mg/dL) nv>100	CH50 (mg/dL) nv>200
1	58 F	IgM K	32	11	106	780
2	67 F	IgM K	35	11	93	650
3	68 M	IgM K	ND	8	ND	ND
4	50 M	IgM K	5120	10	76	200
5	47 M	IgM K	9060	2	51	200
6	68 M	IgM K	200	15	47	ND
7	56 F	IgM K	800	4	76	ND
8	60 F	IgM K	100	7	66	200
9	59 F	IgM K	128	3	81	200
10	71 F	IgM λ	40	13	78	500
11	56 F	IgM K	96	1	86	1500
12	43 M	IgM K	1800	22	97	500
13	65 M	IgM K	5050	4	87	150
14	62 F	IgM K	1200	8	82	430
15	31 F	IgM λ	277	3	44	490
16	45 F	IgM K	1250	11	90	634
17	57 F	IgM λ	22	16	100	666
18	60 F	IgM λ	20	3	71	100
Mean	57±10		1678±2564	8.4±5.5	78±17	480±345

MC: monoclonal component; RF: rheumatoid factor; C3 and C4: complement fractions C3 and C4; CH50: total complement; ND: not determined.

Results

Clinical and histological findings

The main clinical and laboratory findings are indicated in Table 1. The age of the patients refers to the time of diagnosis (mean age 57±11). All patients showed low levels of C4, whereas that of RF was variable, ranging from normal (5 cases) to 9,060 IU. The monoclonal component was IgM k in 14 (78%) subjects.

Bone marrow and liver histology is reported in Table 2. Normal bone marrow histology was found only in 7 patients (39%), while monoclonal (or monotypic) infiltration by plasmacytoid lymphocytes was present in the remaining 11 (61%). In these cases the monoclonality of marrow lymphocytes was evaluated by FACS determination of surface light-chain distribution. In 5 subjects (28%) the marrow infiltrate was monoclonal; in the other 6 a D value of less than 10 was found, indicating the presence of a non-neoplastic infiltrate (reactive). Four patients showed mild proteinuria and elevated serum creatinine (>150 mmol per liter). In these cases kidney biopsy revealed the presence of membranoproliferative glomerulonephritis.

Liver biopsy was performed in 13 (72%) of the patients and in all cases chronic liver disease was found, which ranged from mild persistent hepatitis to chronic active hepatitis associated with cirrhosis.

Virological findings

Anti-HCV antibodies were present in 17 (95%) subjects. HCV-RNA was detected in all cases (100%) before therapy. In one patient, negative for the presence of anti-HCV antibodies, amplification of the 5'NC region of HCV was positive; therefore the absence of anti-HCV

Table 2. Clinical, virological and histological findings of the patients.

Pts.	Liver histology	Bone marrow	Clinical findings	Anti-HCV antibodies	*PCR 5'UTR
1	NP	normal	—	–/–	+
2	CAH	NHL (A)	MPGN	±/+	+
3	CPH	NHL (A)	—	+/+	+
4	CPH	Lymphocytosis	—	+/+	+
5	CPH	NHL (B)	—	+/+	+
6	CPH	Normal	—	+/+	+
7	CAH	Normal	—	±/+	+
8	NP	Normal	MPGN	+/+	+
9	NP	Normal	MPGN	+/+	+
10	NP	NHL (A)	—	+/+	+
11	NP	Lymphocytosis	—	±/+	+
12	CAH+C	Lymphocytosis	—	+/+	+
13	CPH	Lymphocytosis	—	+/+	+
14	CAH+C	Normal	—	±/+	+
15	CAH	Lymphocytosis	—	+/+	+
16	CAH+C	Normal	—	±/±	+
17	CPH	NHL (B)	—	+/+	+
18	CAH+C	Lymphocytosis	—	+/+	+

*before therapy.
NP: liver biopsy not performed; CPH: chronic persistent hepatitis; CAH: chronic active hepatitis; CAH+C: presence of cirrhosis; MPGN: membranoproliferative glomerulonephritis; Anti-HCV antibodies: screening/confirmatory test.

antibodies does not exclude the presence of the virus (at least in MC).

Effects of the therapy

The effects of IFN treatment on the purpura score, on the cryoglobulin level, and on serum ALT and HCV-RNA are summarized in Table 3. In most of the patients a disappearance (or a marked improvement) of skin manifestations occurred within one to two weeks of starting treatment. A reduction of the cryocrit level was observed in the large majority of the patients, meaning the mean cryocrit level was significantly ($p < 0.01$) reduced. The hemoglobin level increased in all the patients showing anemia (3 cases, 17%). Based on the above reported parameters, 5 (28%) patients achieved a complete response and 9 (50%) a partial response, while the others (4 cases, 22%) showed a minor response. After the cessation of therapy cryoglobulins rose to pre-treatment levels (5.5±7.5 vs 5.3±8.1 p: NS) within few months, but purpura (0.5±0.7 vs 1.6±0.7 $p<0.05$), myalgias and other clinical signs of the disease did not return again. At the end of the follow-up period most of the patients needed no further therapy.

Of the 9 patients with abnormal ALT serum levels at the beginning of the study, 6 (66%) responded to α-IFN treatment with ALT normalization, but 4 of them relapsed immediately after suspension of the drug and were considered *relapsers*. At the end of the follow-up period, only 2 patients (28%) showed a sustained normalization of ALT and were considered *responders*. The level of anti-HCV antibodies did not change after therapy in either *responders* or *non-responders/relapsers*. Two patients who showed normal serum ALT levels at the time of diagnosis cleared the virus and became HCV-RNA negative at the end of therapy.

At the end of the follow-up HCV-RNA was detected in 14 (78%) patients. The 4 negative subjects showed complete remission of the MC and two of them also demonstrated complete remission of the liver disease.

Adverse effects

Minor side effects, including fever, fatigue and flu-like syndrome, were observed in most of the patients during the first week of treatment. These symptoms were usually improved by pre-treatment with paracetamol. Thrombocytopenia (platelets less than 100×10^9/dL) occurred in 3 cases, but there was no bleeding and therapy was not discontinued. One patient interrupted therapy after 10 months for deep depression and another after 9 months for the appearance of clinical and biological signs of hypothyroidism.

Discussion

The present study confirms previous observations on the close association between MC and HCV infection.[5-7] Anti-HCV antibodies were present in 95% of our 18 patients and HCV-RNA was found in 100%. It is noteworthy that the presence of the virus was associated with clinical and biochemical signs of chronic liver

Table 3. Effects of α-IFN therapy on clinical signs of mixed cryoglobulinemia.

Pts.	*Purpura (score)*			*Cryocrit level (%)*			*ALT (U/L)*			*5' NC*	
n.	B	A	F	B	A	F	B	A	F	A	F
1	2	0	1	7	0	2	16	16	18	+	+
2	2	0	0	7	6	10	35	16	12	+	+
3	2	0	0	3	0	0	18	8	10	–	–
4	2	0	0	2	0	4	28	20	24	+	+
5	2	0	0	1	0	0	32	20	37	–	–
6	3	0	2	1	1	2	52	16	72	–	+
7	2	0	0	11	7	12	84	20	56	–	+
8	1	0	0	1	1	1	24	27	25	+	+
9	2	1	2	3	3	4	12	12	29	+	+
10	1	0	0	30	19	31	16	28	39	+	+
11	3	2	2	20	10	21	20	20	41	+	+
12	1	0	0	1	1	1	68	32	75	–	+
13	1	0	1	3	2	4	80	20	109	–	+
14	2	0	0	3	2	3	104	80	121	+	+
15	1	0	0	1	1	0	92	32	30	–	–
16	1	1	0	1	1	0	188	96	104	+	+
17	0	0	0	2	0	0	52	24	16	–	–
18	1	1	1	2	1	2	88	92	58	+	+
	1.6±0.7	0.3±0.5	0.5±0.7	5.5±7.5	3.0±4.7	5.3±8.1	56±43	32±26	48±33	56%	78%

B: before therapy; A: after therapy; F: end of the follow-up period (1 year after the cessation of therapy); 5'NC positivity before therapy is reported in Table 2 (100%); *p<0.05; **p<0.01.

disease in only a fraction of the patients (13 out 18, 72%). The severity of liver disease was not related to the degree of clinical manifestations of MC or to the cryocrit level.

In the presence of a chronic viral infection, the use of corticosteroids or other immunosuppressive agents should no longer be recommended. These drugs are able to determine transitory responses in MC, but they can increase viral replication and the number of infected cells. This study shows that α-IFN is an effective agent in MC. α-IFN was able to determine a response rate as high as 78% in patients resistant to corticosteroids. This favorable response rate was obtained without severe side effects in one year of therapy. It is worth noting that immunosuppressive therapy had shown much greater toxicity in these patients, due to the need to use high dosages for very long periods. A recent report[21] confirms the efficacy of a-IFN in MC, but clinical and laboratory data on the patients unable to eliminate the virus are surprisingly lacking in that paper. Therefore a comparison with our work is not possible. The mechanism of action of α-IFN in MC is at present not clear, but several possibilities have to be considered. It is known that α-IFN has a good antiproliferative effect, as indicated by the use of this drug in several myelo- and lymphoproliferative disorders like chronic myelogenous leukemia,[22] primary thrombocythemia,[23] chronic lymphocytic leukemia,[24-25] and low-grade non-Hodgkin lymphomas.[26] Since MC is caused by proliferation of monoclonal[27] (or monotypic) lymphocytes, α-IFN could suppress the lymphoid cell clones producing the cryoglobulins. The observation of extensive monoclonal lymphocytic bone marrow infiltration in our group of patients supports this hypothesis.[28-29] Another possible mechanism of

action could be inhibition of immunoglobulin synthesis[30] and/or an effect on B-cell differentiation.[31] Finally, α-IFN may be able to decrease (even in non-responders) the amount of viral antigens complexed to specific antibodies, thus reducing the number of target molecules for the monoclonal rheumatoid factor.

In addition to its effects on the immune system, α-IFN has a direct antiviral action. The effects of α-IFN on HCV replication are well known, and several studies indicated that a fraction (about 30%) of patients carrying HCV-positive chronic liver disease can recover and permanently eliminate the virus.[32-33] Since HCV infection is also present in all cases of MC, viral replication could be the target of α-IFN therapy. The mechanism by which HCV infection determines MC remains obscure: the virus is able to infect B- and T-lymphocytes, and negative-stranded (replicative) forms have been found in the peripheral blood mononuclear cells of chronically infected patients.[8] Persistence of the virus in the immune system could greatly expand clones of Ig-producing cells by direct or indirect mechanisms.[34] In minks infected by Aleutian disease virus (a DNA parvovirus), infection of the immune system at first induces polyclonal and then monoclonal production of immunoglobulins, often IgM.[35]

Though the mechanism is obscure, this virus seems to be the etiological factor of the disease. In fact, our 4 patients who permanently eliminated the virus (two of whom had liver disease) also recovered from MC. In the patients who relapsed at the end of treatment, biochemical signs of the disease returned within a few months. Conversely, the clinical signs of MC (purpura, arthralgias, weakness) did not reappear immediately after the cessation of therapy. This may indicate that the pathologic lesions (and related symptoms) are due to slow deposition of immune complexes and complement in the small vessels of various organs and tissues. Unfortunately, only a minority of our patients were able to clear the virus; in addition, treatment is costly and has many side effects (two of our patients permanently interrupted the therapy). From the small number of patients in this series, it is impossible to identify subjects likely to respond well to treatment. Since the majority of patients showed relief from clinical symptoms during therapy, low-dose long-term treatment with α-interferons may be suggested.

In conclusion, α-IFN seems to be an effective agent for the treatment of MC, though further studies are needed to determine whether it is able to modify the natural history of the disease.

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