Effectiveness of leukocyte interferon in patients affected by HCV-positive mixed cryoglobulinemia resistant to recombinant alpha-interferon

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Abstract
Objective
Interferon is the first-choice therapy for HCV-positive mixed cryoglobulinemia, but only a small fraction of the patients show long-term recovery from the disease. In non-responders or relapsers, the second-line therapy (high dose interferon) generally is not effective. The aim of this study was to evaluate the effectiveness of leukocyte interferon as a second-line therapy in patients who are non-responders or relapsers to a first course of recombinant interferon.

Methods
Twenty-eight patients with HCV-positive mixed cryoglobulinemia were enrolled. In each case the HCV-RNA and HCV genotype, as well as the usual laboratory parameters, were determined before, at the end of therapy and 1 year after the end of therapy. All patients were treated following the same schedule: leukocyte interferon 3,000,000 three times a week for one year.

Results
Only 5 patients obtained complete recovery from viral infection as well as from all signs and symptoms of the disease. Most patients (80%) experienced relief from clinical symptoms without recovery from HCV replication. Responders to the second interferon course were “relapsers” to the first treatment. No patient considered as a “non-responder” showed complete remission from the disease after the second treatment.

Conclusions
A second leukocyte interferon course could be useful for patients affected by mixed cryoglobulinemia who relapsed after a first course of recombinant interferon therapy.

Key words
Mixed cryoglobulinemia, leukocyte interferon, hepatitis C virus, non-Hodgkin’s lymphomas.

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Introduction
Mixed cryoglobulinemia (MC) is a lymphoproliferative disorder charac-
terized by the presence of purpura, 
arthralgias and weakness, associated 
with the presence of circulating precip-
itating immunoglobulins (1). Since the 
majority of these patients show hepati-
tis C virus (HCV) infection, even with-
out clinical or biochemical evidence of 
chronic liver disease (CLD), this sug-

stantifies that HCV might be the main eti-
ologic factor in MC (2-5). This disease is 
frequently associated with various 
types of organ involvement such as skin 
or peripheral nervous system, while a 
fraction of patients experience severe 
or even life-threatening complications 
such as membrano-proliferative glom-
erulonephritis (6, 7) or non-Hodgkin’s 
lymphomas (NHL) (8, 9).

A standard treatment for MC does not 
exist and several drugs (steroids, cyclo-
sporine, colchicine and others) (10-13) 
and other therapies (diet, plasmaphere-
sis) (14, 15) have been used. Recently, 
several authors reported a good effica-
cy of alpha-interferon (IFN), a sub-
stance endowed with antiviral and anti-
 proliferative activity, able to inhibit 
HCV replication and to reduce cryo-
globulin production (16-18). Unfortu-
nately, only a minority of patients (10-
20%) obtain a complete response (19) 
while the largest fraction do not re-
spond or relapse at the end of the treat-
ment. In the past (before the intro-
duction of ribavirin), several approach-
es were tried for the treatment of non-
responders or relapsers such as higher 
IFN doses (20), a daily IFN dose (21), 
consensus IFN (22), or leukocyte IFN 
(23-25). Since leukocyte IFN seems to 
have milder side effects than recombi-
nant IFN (26), we carried out the pre-

Twentynine patients (20 women and 8 
men, mean age 51 ± 9 years) affected 
by mixed cryoglobulinemia (MC) were 
included in the study. The diagnosis 
was based on the presence of typical 
clinical, haematological and immuno-
logical findings associated with the 
presence of cold-precipitating proteins. 
The median duration of the disease 
before the first course of IFN therapy 
was 3 years (range 1 to 6). All patients 
had been previously treated with low-
dose (3 MU three times a week) recom-
binant alpha2b-IFN for 12 months, but 
a complete response had not been ob-
tained (13 could be considered as “re-
laspers” and 15 as “non-responders”). 
The interval between the end of the 
previous IFN treatment and inclusion 
in present study was variable, ranging 
from 6 months to 4 years. All patients 
were Italian, heterosexuals, and had no 
history of intravenous drug or alcohol 
abuse. All patients gave their informed 
consent before entry into the study, 
which had been previously approved by 
the Ethical Committee of Friuli 
Venezia Giulia.

Purpura scoring system
A simple clinical scoring system was 
used to assess the severity of vasculitis. 
A score of 0 indicated the absence of 
skin lesions; a score of 1 the presence 
of less than 10 purpuric spots on the 
lower limbs; a score of 2 the presence 
of more than 10 spots on the lower 
limbs; a score of 3 the extension of the 
spots to the trunk and/or upper limbs; 
and a score of 4 the presence of skin 
ulcers and/or gangrene.

Patients and methods
Twenty-eight patients (20 women and 8 
men, mean age 51 ± 9 years) affected 
by mixed cryoglobulinemia (MC) were 
included in the study. The diagnosis 
was based on the presence of typical
**Histology**

In all patients a bone marrow biopsy was performed with a Jamshidi-like needle (Trapsystem, Kerna, Treviso, Italy). The sample was placed in B5 solution and 2 hours later in ethanol 70%. After decalcification, samples were stained following standard methods and immunohistochemistry was also performed. The bone marrow biopsy was performed in each case before the beginning of treatment and at the end of the follow-up, 12 months after the end of therapy. On the basis of histological and immunological findings, bone marrow was classified as “normal” in the absence of lymphocyte infiltration, as “reactive lymphocyte infiltration” in the presence of 2 or more paratrabeicular foci of small lymphocytes (with or without lymphoplasmacytoid features) that were polyclonal on FACS, as "monoclonal lymphocyte infiltration" in the presence of 2 or more foci of lymphocytes that were monoclonal on FACS, and as “non-Hodgkin’s lymphoma” in the presence of massive (more than 50%) infiltration by lymphoplasmacytoid lymphocytes that were monoclonal on FACS.

A liver biopsy was obtained only in the patients with biochemical and/or clinical signs of CLD. The biopsies were performed with a Menghini-like needle (Hepafix, Braun, Melsungen, Germany) with an internal diameter of 1.8 mm. Samples were placed in buffered formalin, stained with hematoxylin and eosin, and, for reticulum, with Gomori stain. The liver biopsy was performed before the first IFN therapy and never repeated. Liver biopsies were assessed using the METAVIR scoring system (27) for histological activity (score A0 to A3) and fibrosis (score F1 to F4).

**Phenotyping**

Mononuclear cells from marrow aspirate were separated on a Fycoll density gradient. Cells were stained with specific monoclonal antibodies and, after incubation and washing, immunofluorescence was measured by FACS flow cytometry (Becton Dickinson, Mountain View, 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 Diagnostic System (Raritan, NJ, USA); anti-CD19 (B4-RD1) from Coulter Immunology (Hialeah, FL, USA), anti-CD5, -16, -19, -57 (LEU1, LEU11c, LEU12, LEU7) from Becton Dickinson; and anti-IgM from Dako (Glostrup, Denmark). The monoclonality of the marrow lymphocytes was evaluated by FACS determination of the surface light-chain distribution.

**Virological studies**

The presence of anti-HCV antibodies was assayed by the second generation (four-antigen) immunoenzymatic screening test ORTHO-HCV (Ortho Diagnostic Systems, Raritan, NJ, USA). In all positive and negative tests an additional confirmatory test (RIBA, Chiron Corp., Emeryville, CA, USA) was carried out.

The presence of HCV-RNA in serum was assessed by PCR amplification of the conserved 5’ untranslated region (5’UTR) of HCV (28-30). Amplification was performed using the two-step “nested PCR” (31). The HCV genotype was determined by PCR amplification of the core region according to Okamoto et al. (32). This method was slightly modified by the addition of the primers able to detect type 3a (V) HCV (33). To detect the 2c genotype, after retro transcription and a first PCR with standard primers, a second PCR was performed with primers derived from the 2c prototype BEBE (34):

- Sense primer: 5’CTAAAGAYCG-GGCCNCTACT’3’
- Antisense primer: 5’CRKTRGGGCC-CCAYGAACGG’3’

Y = C or T; N = A, C, G or T; R = A or G; and K = G or T according the IUPAC ambiguity codes. On agarose gel electrophoresis, a specific 135 bp length product is obtained.

**Therapy**

Since the number of patients was low, we did not consider a randomisation between two arms, one with higher dose of the same IFN and one with leukocyte IFN. Therefore, all patients followed the same treatment schedule: 3,000,000 I.U. of leukocyte interferon (Alfaferone, Alfa Wasserman, Italy) 3 times a week for 1 year.

**Evaluation criteria**

The response to treatment was determined at the end of therapy and one year later by a new set of clinical and laboratory criteria. In fact, in previous clinical trials we observed several discrepancies for different aspects of the disease such as the disappearance of the purpura or the cryoglobulins and the persistence of viral replication. Therefore, to establish homogeneous criteria, the response was split into four separate items: (i) viral response; (ii) biochemical response; (iii) immune response; and (iv) clinical response. These were defined as follows:

(i) Viral response, i.e. the effect of the treatment on HCV-RNA: complete response = loss of HCV-RNA at the end of treatment and the end of follow-up; relapse = loss of HCV-RNA at the end of the treatment, but positivity at the end of the follow-up; no response = persistent positivity during therapy and at the end of the follow-up.

(ii) Biochemical response; i.e. the effect of therapy on liver function tests. Complete response = normalisation of the serum ALT level during the treatment followed by normal ALT values lasting for 12 months after discontinuation of therapy. Partial response = reduction (but not normalisation) of ALT by more than 50%. No response = ALT stable or reduction of less that 50% during treatment. Relapse = normalisation of the serum ALT level during the treatment followed by return to pre-treatment values at the end of the follow-up.

(iii) Immune response, defined as the effect of therapy on the serum rheumatoid factor (RF) concentration and cryocrit level. Complete response = normalisation of serum RF concentration and disappearance of circulating cryoglobulins. Partial response = reduction (but not normalisation) of RF and cryoglobulins by more than 50%. No response = Stable levels or a reduction of less than 50% in the RF and cryocrit levels. Relapse = normalisation of serum RF and cryoglobulins during therapy followed by a return to pre-treatment values after the end of treatment.
Table I. Clinical, biochemical and histological characteristics of the patients.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age/gender</th>
<th>C4 (g/liter)</th>
<th>Monoclonal component</th>
<th>Liver histology</th>
<th>Bone marrow histology</th>
<th>HCV genotype</th>
<th>Outcomes of previous therapy</th>
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<td>1</td>
<td>58 F</td>
<td>0.40</td>
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<td>A1F2</td>
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<td>REL</td>
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</tr>
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<td>NR</td>
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<td>NP</td>
<td>REL</td>
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<tr>
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<td>2c</td>
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<td>ML</td>
<td>UC</td>
<td>REL</td>
</tr>
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<td>REL</td>
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<td>A1F1</td>
<td>ML</td>
<td>1b</td>
<td>NR</td>
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<td>2c</td>
<td>NR</td>
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<td>A1F1</td>
<td>RL</td>
<td>2a</td>
<td>REL</td>
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<td>ML</td>
<td>1b</td>
<td>REL</td>
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<td>Absent</td>
<td>A1F2</td>
<td>RL</td>
<td>1b</td>
<td>NR</td>
</tr>
<tr>
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<td>64 F</td>
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<td>IgMk</td>
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<td>NR</td>
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</tr>
<tr>
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<td>1b</td>
<td>NR</td>
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<td>IgMk</td>
<td>A2F2</td>
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<td>1b</td>
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</tr>
<tr>
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<td>A1F1</td>
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<td>RL</td>
<td>2a</td>
<td>NR</td>
</tr>
</tbody>
</table>

Means 51 ± 9 0.12 ± 0.007

NP: Not performed. For the marrow histology: N=normal, ML=monoclonal, NHL=non-Hodgkin’s lymphoma, RL=reactive lymphocytosis. Liver histology was according to META VIR (see text for details). UC: unclassifiable genotype. Outcome of previous therapy: REL=relapser, NR=non-responder.

The C4 serum concentration was not considered in the immune response because it does not change over time, even in long-term responders.

(iv) Clinical response, i.e. the effect of therapy on the main clinical manifestations of the disease (including purpura, arthralgias and weakness). Complete response = disappearance of all clinical signs of the disease. Partial response: improvement in the clinical symptoms (reduction in the global score by more than 50%). No response = stable, or a reduction in the global score of less than 50%. Relapse = normalisation of clinical symptoms during therapy followed by a return to the pre-treatment score after the end of treatment.

Follow-up: Biochemical and clinical parameters were determined each month during therapy and every 2 months after discontinuation of the interferon. Autoantibodies were determined every 3 months and thyroid function tests every 6 months. Determinations of HCV-RNA were performed before beginning therapy, at the end of the treatment, and at the end of the follow-up. All patients were followed for at least 12 months after the end of therapy. We did not perform a second liver biopsy in our patients because most of them (19 subjects) refused it.

Statistical analysis: Data are expressed as the mean ± standard deviation. Statistical analysis was carried out using the statistical package SPSS for Windows (35). The analysis of variance between two groups was calculated (one-way), where p represents the probability of Snedecor’s F that the means of numerical continuous outcomes. For categorical variables, cross tabulation with a Pearson’s χ² was used to test whether the row and the column variables were independent. The partial association was studied using the hierarchical log-linear analysis in a multiway cross-tabulation.

Results

Biochemical and histological findings

The main clinical, laboratory, and histological findings of patients are indicated in Table I. Age refers to the patient’s age at the beginning of therapy (mean 51 ± 9 years). All patients had low C4 levels, whereas the level of RF was rather variable, ranging from normal (2 cases) to 6,090 kIU/liter. The monoclonal component was IgM in 25 subjects, while in 3 cases no monoclonal component was found. Accordingly, in these 3 patients the mixed cryoglobulinemia was defined as type III.

Liver biopsy was performed in 27 patients (96%). In all cases a chronic liver disease was found, ranging from chronic hepatitis (24 cases, 89%) to cirrhosis (A3F4) (3 cases, 11%). A normal bone marrow histology was found in only 7 patients (25%), while varying degrees of lymphocyte monomorphous infiltration were present in the remaining 21 patients (75%). In these cases, FACS determination of the surface light-chain distribution showed the presence of a monoclonal infiltrate in 15 cases (54%) and a non-monoclonal (reactive) infiltrate in the other 6 (21%). Among the 15 subjects showing monoclonal infiltrate, 6 (40%) had histological findings suggestive of the diagnosis of non-Hodgkin’s lymphoma: lymphoplasmacytic or lymphoplasmacytoid immunocytoma (small lymphocytic lymphomas, group A of the WF). This diagnosis was supported by the presence of a > 50% infiltrate with lymphoplasmacytoid lymphocytes (CD19 and CD20 positive). The 3 subjects affected by type III mixed cryoglobulinemia showed bone marrow with moderate lymphocyte infiltration, but monoclonality was not found.
Table II. Effects of interferon therapy on main biochemical parameters.

<table>
<thead>
<tr>
<th>Cryocrit level (%)</th>
<th>ALT (NV: 0 - 0.67 µkat/liter)</th>
<th>Rheumatoid Factor (NV &lt; 50 kIU/liter)</th>
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<tr>
<td>Pts B A F</td>
<td>B A F</td>
<td>B A F</td>
</tr>
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<td>3.20 0.65 0.45</td>
<td>0.84 0.60 0.33</td>
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<td>0.66 0.43 0.40</td>
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<td>1.01 0.62 0.56</td>
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<td>1.06 0.66 0.58</td>
</tr>
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<td>2.35 2.00 1.55</td>
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<td>2.30 1.70 1.46</td>
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<td>2.75 0.66 0.58</td>
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<td>1.90 0.96 1.02</td>
</tr>
<tr>
<td>23</td>
<td>1.53 ± 15.3 ± 7.4 ± 8.1</td>
<td>1.15 ± 0.69 ± 0.84* ± 0.84#</td>
</tr>
<tr>
<td>24</td>
<td>1.06 ± 15.3 ± 7.4 ± 8.1</td>
<td>1.15 ± 0.69 ± 0.84* ± 0.84#</td>
</tr>
<tr>
<td>25</td>
<td>0.84 ± 15.3 ± 7.4 ± 8.1</td>
<td>1.15 ± 0.69 ± 0.84* ± 0.84#</td>
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<tr>
<td>26</td>
<td>0.66 ± 15.3 ± 7.4 ± 8.1</td>
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<td>27</td>
<td>0.43 ± 15.3 ± 7.4 ± 8.1</td>
<td>1.15 ± 0.69 ± 0.84* ± 0.84#</td>
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<tr>
<td>28</td>
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<td>1.15 ± 0.69 ± 0.84* ± 0.84#</td>
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<tr>
<td>29</td>
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<tr>
<td>30</td>
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<td>1.15 ± 0.69 ± 0.84* ± 0.84#</td>
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<tr>
<td>31</td>
<td>0.33 ± 15.3 ± 7.4 ± 8.1</td>
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</tbody>
</table>

Six patients had normal ALT serum levels at the beginning of the study, and therefore were not taken into consideration in our analysis of the biochemical response. During the treatment 13 patients responded with a normalisation of their ALT levels, but one relapsed immediately after suspension of the drug. At the end of the follow-up period, 12 patients (43%) had a sustained normalisation of ALT and were considered as biochemical “complete responders”.

In most patients (23 cases, 82%) a complete remission or a marked improvement of the main clinical manifestations of the disease (skin manifestations, weakness, arthralgias) occurred within 2 or 3 weeks of treatment. Unfortunately, most patients (16 cases, 70%) relapsed a few weeks after the end of the treatment. At the end of the follow-up, only 5 patients (18%) obtained a complete response, and one additional patient maintained the partial response. The outcome of treatment is indicated in Table III.

Fever, fatigue and flu-like syndrome were observed in most patients during the first 2 to 3 weeks of treatment. These symptoms usually improved with acetaminophen pre-treatment. Two patients interrupted the treatment due to side-effects: severe depression in one case at the 5th month and diffuse erythema in another at the 6th month. Thrombocytopenia (platelets less than 100 x 10^9/L) occurred in 5 cases but therapy was not discontinued.

Discussion
This study confirms that leukocyte interferon is an effective agent in the treatment of MC patients who relapsed after a previous course of recombinant interferon therapy. These results are of interest because they suggest the possibility of a complete recovery from HCV infection in patients treated once with interferon. Interestingly, no patient who failed to respond to the first IFN treatment showed a complete response to the second IFN course; in fact, all patients from this group who initially responded to re-treatment (# 1, 17, 20, 24, 26 see Tables I and II) relapsed. Similar results are usually obtained in
patients affected by HCV-positive chronic hepatitis without MC who are treated with a second interferon course or with combination therapy (interferon + ribavirin) (36-37).

In this study the response to the treatment was split into four items, which allowed us to determine separately the effects of IFN on different aspects of the disease. From a virological point of view, the response was rather unsatisfactory: in only 5 patients (18%) was the virus eliminated, while the majority (82%) could be considered as “non-responders”. Interestingly, no patient was a “relapser”, which might indicate that the first course of IFN selected highly resistant HCV strains. In fact, the fraction of patients who showed a virological response under therapy was larger (46%) during the first IFN treatment than during the second one (18%).

A fraction of the patients could be not evaluated for liver function since they showed normal serum AST/ALT/GGT/ALP levels before treatment. In the others, a normalisation was obtained in 13 patients during therapy and in 12 at the end of the follow-up. Interestingly, the normalisation of liver function tests did not occur only in the cases whose virus was eliminated, but even in a fraction of patients (7 cases, 25%) with persistent HCV-RNA positivity. All of these cases showed variable levels of cryoglobulins and rheumatoid factor during therapy; therefore, concerning the “immune response”, they were considered as “non-responders” or “partial responders”. These findings indicate the possibility of different effects of IFN on the liver and immune system: in fact, while the disappearance of viral replication is always associated with a normalisation of liver function tests, on the contrary these biochemical parameters could, under therapy, normalise even in presence of viral replication and persistent cryoglobulin production. Similar results had been obtained by several authors (38, 39), and also by us, in patients under IFN therapy for chronic C hepatitis without cryoglobulins, i.e. in patients with normal liver function tests despite circulating levels of HCV-RNA (40).

Despite the disappointing results in terms of virological and immunological responses, most patients improved clinically. In fact, 50% of patients showed complete relief from clinical symptoms and a further fraction (9 cases, 32%) a partial response, while only a minority (5 cases, 17%) did not experience any improvement. It is not easy to explain these results; one possible explanation is the decrease in the viral load during therapy: the reduced viral particles decrease the amount of cold-precipitable immunocomplexes constituted by HCV-RNA/anti-HCV IgG polyclonal antibodies/IgM anti-IgG monoclonal antibodies. However, it is likely that other effects of IFN on the immune system could explain the favourable results of this therapy.

Contrary to our previous results (41, 42), the analysis of clinical or laboratory characteristics associated with the response to therapy showed that the HCV genotype does not seem to be the most important factor. In fact, among the 5 cases with a complete response, two of them (#24 and #26) were carriers of genotype 1b, which is considered to be the most aggressive HCV genotype world-wide. Besides the viral factors, some host factors could be important, such as liver histology (43). Concerning this factor, although no patient affected by cirrhosis showed a sustained response, the low number of such patients (3 cases) does not allow statistical analysis. However, the pretreatment cryocrit level (as well as the other laboratory parameters) was not different between the responders (10.2 ± 8.8%) and non-responders (10.3 ± 16.2%, p = NS).

On the basis of these observations, leukocyte IFN treatment seems to result in
Leukocyte IFN in mixed cryoglobulinemia / C. Mazzaro et al.

a complete recovery from MC, but only in a small number of cases previously treated with recombinant IFN. However, the therapy is able to reduce the clinical signs and symptoms of the disease in most cases. Since our patients were middle-aged, or elderly with long-standing disease, it is likely that a more favourable response rate could be obtained if patients were treated at a younger age. The recovery from viral infection is very important in MC patients since the disease, which could actually be considered a “benign” lymphoproliferative disorder, over the time could evolve toward more aggressive non-Hodgkin’s lymphoma (44, 45). It is likely that the MC can reach a point beyond which the disease is no longer correlated with HCV replication (probably after lymph node involvement), but it is noteworthy that it remains responsive to antiviral therapy for years. This indicates that HCV-positive MC should be treated as soon as possible, before the development of severe chronic liver disease or lymphoproliferative disorders (46, 47).

In conclusion, after a first course of recombinant-IFN, relapsing MC patients can undergo a course of leukocyte-IFN treatment with a reasonable hope of recovering from HCV infection. For non-responders to both courses of IFN, combination therapy with ribavirin + interferon (48) or pegylated interferon alpha plus ribavirin in patients with chronic hepatitis C (41, 42). The high response rate with IFN can be considered a “promising” therapy for the management of MC patients. However, it is important to underline that the use of IFN is associated with significant side effects, and the therapy should be administered with caution, taking into account the patient’s overall health and the potential risks and benefits of the treatment. For further information, refer to the references listed below.

References

35. NORSUIS MI: Advanced Statistics. SPSS/PC. Chicago 1986; SPSS Inc.