Type II mixed cryoglobulinemia as an oligo rather than a mono B-cell disorder: evidence from GeneScan and MALDI-TOF analyses

V. De Re¹, S. De Vita², D. Sansonno³, D. Gasparotto¹, M. P. Simula¹, F. A. Tucci¹, A. Marzotto¹, M. Fabris², A. Gloghini⁴, A. Carbone⁴,⁵, F. Dammacco³ and M. Boiocchi¹

Objective. To identify and characterize rheumatoid factor (RF)-producing B-cells and cryoprecipitate immunoglobulin (Ig) M in hepatitis C virus (HCV)-positive patients.

Methods. We purified and characterized, by peptide mass fingerprinting integrated with an NCBI IgBlast data bank search, the IgM component of cryoprecipitate and analysed the VDJ pattern of bone marrow B-cells by gene scan analysis of 17 HCV-positive patients with type II mixed-cryoglobulinemia.

Results. IgM purified from all of the patients presented an RF specificity. In three of these patients a high and predominant B-cell clone (≥30%) was found in the bone marrow. B-cell-receptor sequences were determined and immunophenotyping of these clones was performed. Peptide masses originating after tryptic digestion of the B-cell-receptor combinatorial regions and those originating by tryptic digestion of the cryoprecipitated IgM from the same patient were comparable. In the remaining patients an oligoclonal/polyclonal pattern was found. However, in some of these patients we were able to find peptides that matched with the B-cell-receptor sequences of overexpanded B cells, indicating that, even in the absence of a clear monoclonal expansion, a fraction of total cryoprecipitated IgM may derive from overexpanded B-cell clones found in patients’ bone marrow.

Conclusions. In the majority of mixed cryoglobulinemia-HCV-positive patients, both in the serum and in B cells from the bone marrow, an oligoclonal pattern is the main molecular picture. When a monoclonal B-cell clone is found, its B-cell-receptor shows an antigen-binding fragment identical to that of cryoprecipitable RF-IgM. Phenotypically, B cells are CD20-positive but CD5-negative, suggesting that the B-1 B-cell subset is not likely to produce high-affinity IgM-RF molecules.

Key words: Cryoglobulinemia, Immunoglobulins, Molecular biology, Rheumatoid factor, Viral disease.

The term cryoglobulinemia refers to the presence in the serum of one (monoclonal cryoglobulinemia) or more (mixed cryoglobulinemia, MC) immunoglobulins (Ig), which precipitate at temperatures below 37°C. This is an in vitro phenomenon; the real mechanism(s) of cryoprecipitation remains obscure. Cryoglobulins are classified according to Brouet’s criteria [1]. MC consists of polyclonal Ig G and either polyclonal (type III; MC-III) or monoclonal (type II; MC-II) IgM rheumatoid factors (RFs).

MC-II is frequently associated with development of serious vascular, renal and neurological lesions; B-cell clonal expansions are also a frequent laboratory finding. Oligoclonal or monoclonal B-cell expansions are significant features of liver, peripheral blood and bone marrow, and are associated with clinical manifestations and laboratory data [2]. MC-II has been associated with higher risk of non-Hodgkin’s lymphoma (NHL) [3] and, although more controversial, with cirrhosis [4]. Ectopic lymphoid follicles generally express IgM and the limited number of family genes used to construct the antigen-binding fragment (Fab) suggest the possibility that chronic antigenic stimulation contributes to B-cell expansion [5].

Eighty per cent of the monoclonal IgMs found in hepatitis C virus positive patients with a type II mixed cryoglobulinemia- (HCV-MC) patients share a major complementary region named WA. This WA crossing idiotyp has been long recognized to be associated with a high degree of RF activity. These antibodies almost invariably express an IgH variable heavy (VH) chain derived from the VL-69 germline gene and an IgK variable light chain (VL) derived from the V3-20 germline gene [6]. Approximately 25% of B-NHLs in patients with HCV type II cryoglobulinemia appeared to arise from WA cross-reactive B cells, and about 70% of light chain of Ig are from V3-20 or V3-15 germline genes [7, 8]. Other VH/VK gene combinations frequently found in MC-associated NHLs were V3-7/V3-15 and V4-59/V3-20 [9]. All this evidence suggests that the malignant cells could derive from the RF-producing B-cells that sustain of the MC syndrome. However, a direct link between clonal B-cell proliferation and production of the IgM component of the cryoprecipitate still remains to be demonstrated.

Clinical and epidemiological evidence and laboratory investigations have established the pathogenic role of HCV in MC-II. Furthermore, HCV is the most prevalent hepatotropic virus in

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humans, causing hepatitis, cirrhosis and hepatocellular carcinoma. The prevalence of anti-HCV antibodies in patients with MC-II in different populations ranges between 90 and 100% and the viral genome concentration is much higher (about 1000-fold) in the cryoprecipitate than in the supernatant. In the general population, the worldwide prevalence of HCV is high (5.6% in Italy [10]), but only a subset of HCV-infected individuals develop MC-II, with a higher prevalence in southern Europe (34% in Italy [3], 20% in France [11] and less than 5% in the United States [12]). Although HCV infects both sexes equally, females have a higher risk of MC (63–71%); moreover, association with some HLA class II molecules has also been reported [13, 14]. The clinical manifestation of MC-II appears to be a late event in HCV infection, being more frequent in patients with extensive liver damage [15]. Previous studies hypothesized that the B-cell B-1/CD5þ population and the RF-IgM component of cryoprecipitate, we demonstrating the relationship between the monoclonal B-cell processes are still controversial [17, 19]. With the aim of involving the innate (CD5þ) B cells in the lymphoproliferative disorders [16], and the origin of cells that would produce RF antibodies [16], and a diagnosis of type II MC syndrome, all attending the Division of Rheumatology-DPMS, University of Udine and the Department of Biomedical Sciences and Human Oncology, University of Bari, were studied. The subject’s consent was obtained according to the Declaration of Helsinki, and the experimental design conforms to ethical standards. Patient evaluation, including diagnosis and laboratory values, is reported in Table 1. All patients were negative for human immunodeficiency virus (HIV) and hepatitis B surface antigen (HBsAg). Four patients had a concomitant B-cell NHL: two lymphoplasmacytic lymphoma (patients 1 and 3), one extranodal (gastric) marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) (patient 10) and one diffuse large B-cell lymphoma (patient 17). The last two patients had no bone marrow involvement. In the remaining cases, no evidence of malignant lymphoproliferative disorders was found by physical examination, thorax and abdomen computed tomography (CT), or by bone marrow biopsy.

**GeneScan analysis of clonal B cells and determination of the Ig heavy and light chain families**

Genomic DNA was prepared from bone marrow mononuclear cells using the Genomix kit (Talent, Trieste, Italy). Polymerase chain reactions (PCRs) for the VDJ GeneScanning technique (GS) were performed using the previously reported VDJ FR3 PCR condition, with the exception of a forward primer labelled with 5-carboxyfluorescein (FAM) fluorochrome in the second PCR round [20]. The PCR product and the internal size standard GeneScan 400 HD (ABI-PE Applied Biosystems) were added to deionized formamide, then subjected to capillary electrophoresis on an ABI PRISM 3100. Data were elaborated with 3100 GeneScan 3.7 Software. The distribution of the peaks ranged between 65 and 130 bp.

Detection of Ig heavy family gene segments (VH) was performed using a modified VDJ FR1 PCR protocol [21] in which a second multiplexing PCR, VH3, VH4, VH5, VH6 primers and the JH region were added. VH1 to VH4, and VH3 to VH6 primers were each labelled with a different fluorescent dye: FAM, HEX, NED or ROX. PCR products were added to the internal GeneScan 500 LIZ size standard (ABI-PE Applied Biosystems). This mixture was subjected to capillary electrophoresis (range 310–360 bp).

Detection of clonal Ig kappa chain family gene segments (VK) was performed according to previously reported protocols [21]. The range of PCR products was 120–180, 190–210 and 260–300 bp, respectively, for VK1/6/VK7-JK, VK3-JK and VK2/VK4/VK7-JK.

**Table 1. Patient characteristics**

<table>
<thead>
<tr>
<th>Age/sex</th>
<th>Diagnosis*</th>
<th>Bone marrow B-cell pattern</th>
<th>HCV genotype</th>
<th>Cryoglobulins (%)</th>
<th>Rheumatoid factor (IU/ml)</th>
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<td>MC-II and lymphoplasmacytic 1 lymphoma</td>
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<td>2a/2c</td>
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*Bone marrow was not involved in lymphoma in patients 10 and 17.

**Materials and methods**

**Patients**

Seventeen HCV-infected patients with HCV RNA in the serum and a diagnosis of type II MC syndrome, all attending the Division of Rheumatology-DPMS, University of Udine and the Department of Biomedical Sciences and Human Oncology, University of Bari, were studied. The subject’s consent was obtained according to the Declaration of Helsinki, and the experimental design conforms to ethical standards. Patient evaluation, including diagnosis and laboratory values, is reported in Table 1. All patients were negative for human immunodeficiency virus (HIV) and hepatitis B surface antigen (HBsAg). Four patients had a concomitant B-cell NHL: two lymphoplasmacytic lymphoma (patients 1 and 3), one extranodal (gastric) marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) (patient 10) and one diffuse large B-cell lymphoma (patient 17). The last two patients had no bone marrow involvement. In the remaining cases, no evidence of malignant lymphoproliferative disorders was found by physical examination, thorax and abdomen computed tomography (CT), or by bone marrow biopsy.

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**VH and VK PCR sequencing**

PCR sequencing of VH and VK region genes was performed as previously reported [5].
Estimation of B-cell clone frequency

ABI PRISM 3100 GeneScan 3.7 Software can calculate the peak area of a single fragment. The B-cell clone percentage was calculated as:

\[
\text{% clonal B-cell peak area} = \frac{A}{A + B} \times 100
\]

where \(A\) is the peak area of monoclonal B cells and \(B\) is the sum of all peaks in the 65–130 bp FR3 range.

Separation and purification of cryoprecipitated IgM

Cryoprecipitate was obtained by placing serum in a 4°C refrigerator for 74 h, and then centrifuging at 4°C, 4000 rpm for 15 min.

To inactivate HCV virions, cryoprecipitate was suspended in a mixture of solvent and detergent [1% tri(a-butyl)phosphosphate (TNBP) and 1% Triton X-100] at 37°C for at least 4 h.

Monoclonal IgM was purified from cryoprecipitate by gel filtration fractionation on a HiLoad Superdex 200 h 26/60 column (Amersham Pharmacia Biotech, Milan, Italy) in acetate buffer at pH 4.0, followed by IgM-affinity chromatography (Pharmacia). The column was run with a 20 mM sodium phosphate and 0.5 mM potassium sulphate pH 7.5 (binding buffer), then with 20 mM sodium phosphate pH 7.5 in isopropanol gradient buffer (elution buffer). Chromatographic fractions were then subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). To entirely exclude any residual IgG from purified IgM fractions, protein G sepharose (Pharmacia Biotech, Uppsala, Sweden) binding overnight in 20 mM sodium phosphate, pH 7.0, was performed.

IgM heavy and light chain bands were separated on 12% SDS-PAGE, excised from the gel and destained with ammonium bicarbonate 25 mM in 50% acetonitrile. In-gel trypsin digestion was performed and peptides were extracted with 1% trifluoroacetic acid (TFA) and then subjected to ZipTip clean-up (Millipore s.p.a., Milano, Italy). MALDI-TOF mass spectrometry was carried out on a Voyager-De-Pro Biospectrometry Workstation mass spectrometer (Applied Biosystems Inc.). Proteins from both heavy and light chain samples were identified from the resulting peptide masses by peptide mass fingerprinting. The Mascot search engine was used, with 100 parts per million mass tolerance error. Identified peptides were searched for the most similar VH and VL germline protein with the IgBlast protein database (http://www.ncbi.nlm.nih.gov/igblast/).

BCR and IgM sequence alignment

Peptides shared between monoclonal BCR and IgM-RF were searched, matching the masses of peptides theoretically determined by a virtual tryptic digestion of VH and VL sequences from B-cell DNA (http://us.expasy.org/tools/dna.html) and the masses of peptides obtained after the tryptic digestion of heavy and light IgM chains.

In the remaining samples, with an oligo/polyclonal pattern, peptides identified after IgM in-gel trypsin digestion and MALDI-TOF analysis were matched with sequences reported on NCBI IgBlast GenBank (http://www.ncbi.nlm.nih.gov/igblast/) to search the most similar germline corresponding to VH and VK genes. In five samples taken at random (patients 8, 9, 10, 13, 14), we matched the masses obtained with peptides from IgM with those obtained from BCR of multiple B-cell clones of the same patient.

Rheumatoid factor activity

RF activity was measured on serum sample by nephelometry (in which a level >20U/ml was considered positive). RF activity of IgMs purified from cryoprecipitate was tested by enzyme-linked immunosorbent assay (ELISA) using the IgM Rheumatoid Factor ELISA Kit (Sanquin, Central Laboratory of the Netherlands Red Cross, Amsterdam, The Netherlands). Each sample was tested in triplicate using 100 ng/well of IgM (1 mg/ml) as primary. RF activity (IU/mg) was calculated by plotting the net average absorbance against a standard dilution curve.

Immunophenotype

Immunophenotyping and lineage assignment of lymphoma cells in patients 1 and 3 was performed with conventional methods, by immunohistochemistry on bone marrow biopsy (patient 1) or by flow cytometry on bone marrow aspirate (patient 3).

The phenotype of bone marrow cells from patient 2 was determined by flow cytometry.

Results

Ig gene amplification and analysis of somatic mutations

A monoclonal B-cell pattern from bone marrow DNA was found in three HCV-positive patients by VDJ FR3 GS analysis (Table 1, samples 1–3, Fig. 1). Clonal B cells represent 70, 29 and 36.6%, respectively, of all B cells present in the bone marrow of samples 1, 2 and 3; the remaining cases were oligo- or polyclonal (Fig. 1).

Single VH and VK PCR products from monoclonal samples were purified from agarase gel, cloned, and multiple clones were screened. Complete sequences were submitted to NCBI GenBank (AF301518, AY703067, AY703068, AY704914, AY703069, AY703066). The consensus Ig VH and VK germline counterparts were determined as VH1-2/VK3-15, VH1-69/VK3-20 and VH3 7/VK3-15 (Table 2). Somatic mutations present in both the VH and the VK sequences, compared with the most similar germline gene, were highlighted in grey type in the corresponding peptides (Table 2).

A clonal B-cell pattern was found in the gastric MALT sample (case 10). Its consensus Ig VH chain sequence presents a somatic mutation that introduces a stop codon, and it consequently cannot be translated to a protein (data not shown). The amount of neoplastic skin biopsy from sample 17 wasn’t enough to be analysed by VDJ PCR.

MALDI-TOF analysis of cryoprecipitated IgM

Purified IgM from the cryoprecipitate of patients 1 to 6 (Table 1) is shown in Fig. 2. Peptide masses from tryptic digestion of cryoprecipitated IgM, compared with the corresponding virtual ones obtained from translated BCR VH and VL sequences from an autologous patient, indicated a protein identity in all three samples with a B-cell monoclonal pattern (Table 2). This finding included segments with somatic mutations as resulting after comparison with the most similar to VH and VK germline gene. Mutations are shown in grey type in Table 2.

In the remaining samples, the most probable VH and VK family genes of cryoprecipitate IgM were identified by peptide mass fingerprinting integrated with a NCBI IgBlast GenBank search and reported in Table 3. In some cases (samples 4–17, Table 3), in a single IgM cryoprecipitate it is possible to simultaneously identify peptides specific for more than one VH and VL protein family. Heavy chains were frequently from VH1-69 (10/14), VH4-59 (7/14), VH4-34 (4/14) and VH3-30 (2/14) genes. Light chains were frequently from F3-20 (identified in 12 out of 14 samples) and V3-15 (11/14) families. Due to technical limitations of both the mass spectrometer instrument and the number of Ig sequences reported in the Ig database, not all the peptides constituting the VH and VL chains were identified. To make the relationship between BCR and cryo IgM RF sequences even more convincing, we compared serum cryo IgM with BCR of the most overexpanded B-cell clones identified in the bone marrow of five random patients with MC and without immunocytoma (patients 8, 9, 10, 13, 14). In three out of
fluorescence intensity (lysis of fluorescent VDJ FR3 PCR products. Relative
FIG. 1. Representative electropherograms from GeneScan ana-
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associated lymphoma, share the same combinatory region with the cryoprecipitated RF-IgM. Consequently, for the first time, we demonstrated that overexpanded B-cell clones found in the bone marrow of HCV\(^+\) patients are the (or are consequential subclones of) RF-IgM-producing B cells causing the MC-II syndrome. Note that frequently bone marrow is a site of involvement of NHL in patients with MC [28], and it is one of the sites where post-germinal centre B cells migrate to reside [29].

### TABLE 2. Comparison of theoretically calculated peptide masses of monoclonal B-cell VH and VK sequences and experimental peptide masses from the in-gel digestion of VH and VK cryoprecipitated IgM

<table>
<thead>
<tr>
<th>Patient</th>
<th>Residues</th>
<th>Theoretical sequence of variable chain region</th>
<th>Theoretically calculated mass (Da)</th>
<th>Measured mass (Da)</th>
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<tr>
<td>1</td>
<td>22–50</td>
<td>QAPGQGLEWGMGWINPNSGTTNYAQQRFQGR</td>
<td>3193.49</td>
<td>3192.870</td>
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<tr>
<td></td>
<td>7–21</td>
<td>ASGYTF/YHWVR</td>
<td>1879.05</td>
<td>1879.12</td>
</tr>
<tr>
<td></td>
<td>56–68</td>
<td>DTSINTYMMELS</td>
<td>1528.73</td>
<td>1528.93</td>
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<tr>
<td></td>
<td>71–81</td>
<td>SDDTALYYCAR</td>
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<td>1263.53</td>
</tr>
<tr>
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<td>51–68</td>
<td>VT/VTRDTSTINTYMMELS</td>
<td>2085.06</td>
<td>2085.26</td>
</tr>
<tr>
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<td>Light chain V3-15/J1</td>
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<td>1900.96</td>
<td>1900.73</td>
</tr>
<tr>
<td></td>
<td>1–18</td>
<td>ATLSR</td>
<td>706.35</td>
<td>706.27</td>
</tr>
<tr>
<td></td>
<td>19–24</td>
<td>ASQSVSNLAWYQQPGQAPRLLLJYGASTR</td>
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<td>2301.84</td>
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<tr>
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<td>ATGIPAR</td>
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<td>VEIKR</td>
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<tr>
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<td>83–87</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>25–46</td>
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<td>47–55</td>
<td>LJIYGASTR</td>
<td>993.57</td>
<td>993.73</td>
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**Fig. 2.** SDS-PAGE of purified IgM. Lane 1, load of our standard containing 1 \ensuremath{\mu}g of IgM plus 1 \ensuremath{\mu}g of IgG Lane 2-7, IgM purified from patients 1 to 6, respectively.
<table>
<thead>
<tr>
<th>Patient</th>
<th>VH germline family</th>
<th>Identified peptides</th>
<th>VL germline family</th>
<th>Identified peptides</th>
</tr>
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<td>EIVLTSQSGTTLSPGER</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QAPGQGLEWMGGHIPFGTANY</td>
<td>ASQSLSSSSLYLAWYQQKPGQAPR</td>
<td>LLIYGASTR</td>
</tr>
<tr>
<td></td>
<td>VH4-nd</td>
<td>FQGRVTITADESTSTAYMELSS</td>
<td>ASQSVSSNXLQWYQQKPGQAPR</td>
<td>LLIYGASTR</td>
</tr>
<tr>
<td></td>
<td>VH1-45</td>
<td>SRVTISVDTSK</td>
<td>V3-15</td>
<td>FSGSSGSTDFTLTLISR</td>
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<tr>
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<td></td>
<td>YLHWVR</td>
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<tr>
<td>5</td>
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<td>ATGIPDARFSGSGTGDTLTLISR</td>
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<tr>
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<td>9</td>
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<tr>
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<td>11</td>
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<td>VH4-nd ASGGTFSSYAISWVR</td>
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<td>13</td>
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<td>VH4-nd ASGGTFSSYAISWVR</td>
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</tr>
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<td>V3-20</td>
<td>ASQSVSSNXLQWYQQKPGQAPR</td>
</tr>
</tbody>
</table>

(Continued)
Further, to make the conclusive relationship between the combinatorial regions of BCR and cryo IgM RF even more convincing, we compared the serum cryo IgM with the BCR of the most overexpanded B-cell clones identified in the bone marrow of five random patients with MC and without immunocytoma (patients 8, 9, 10, 13 and 14). In three of five cases (patients 8, 9, 13, Fig. 3), although more than one clone and an intraclonal heterogeneity were present in the same patient’s sample thus complicating the comparison (data not shown), a correlation between cryo IgM RF and one of the most overexpanded B-cell clones was found. We think that B-cell clones producing IgM-RF could be mainly confined to another anatomical site in the remaining two cases (patients 10 and 14). In fact, as previously reported [2], in some cases a monoclonal B-cell population was found only in sites different from the bone marrow, i.e. the liver and the blood. It is interesting, therefore, to note that for case 10, patient with a MC-related MALT lymphoma localized in the stomach but with no bone marrow involvement, none of the cryoprecipitated IgM were similar to the most represented BCR found in the bone marrow. Recently it has been reported that gastric MALT lymphomas frequently express a repertoire of antibodies with RF reactivity which is a typical characteristic of the cryoprecipitable IgM in MC syndrome [30]. However, our data pointed out that this specific lymphoma does not produce Igs (its Ig VH sequence presents a stop codon), a feature that is common to several MALT lymphomas [31, 32].

### Table 3. Continued

<table>
<thead>
<tr>
<th>Patient</th>
<th>VH germline family</th>
<th>Identified peptides</th>
<th>VL germline family</th>
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<td>IYTSGSTNYNPSLKL</td>
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</table>

**Fig. 3.** Peptides shared between the BCR from one of the most over expanded B-cell clones and the cryoprecipitated IgM from the same patient. The coloured sequences represent the Ig (red for VH and blue for VK) of one of the most over expanded B cell clones; peptides reported in black colour show sequences of the cryoprecipitated IgM identified by mass fingerprinting.
polyclonal B-cell pattern in the bone marrow, we were unable to match specific peptides from the VH and VK BCR chains with those of the cryoprecipitated IgM.

Furthermore, according to a NCBI IgBlast search for similarities, peptides from IgM identified by mass fingerprinting used similarly restricted Ig genes (i.e. V1-69/V3-20, V2-7/V3-15, V4-59/V3-20) as those previously reported for VH and VK sequences of MC-II-associated B-cell NHLs (Table 3) [5, 7, 9]. Thus, although in the latter cases a direct relationship between combinatorial region of BCR and RF-IgM cannot be clearly demonstrated, overall data support that BCR present in overexpanded B-cell clones and cryoprecipitated RF-IgMs share the same origin.

The use of high-resolution proteomic technique such as two-dimensional gel electrophoresis and Fourier transform-ion cyclotron resonance mass spectrometry, they found more than one IgM component associated with polyclonal IgG. Correspondingly, Sène et al. [35] reported that the oligoclonal type appears to be an intermediate stage in the course of type III changing to type II MC. Secondarily, the use of MALDI-TOF spectrometry allows identification of specific sequences common to several RF-IgM (e.g. the ASQYSSSLAWYQQ, LLIYGASTR and EIVMTQSPATLSVSPGER sequences, Table 3), which is not possible with conventional anti-idiotype determination. Identification of common peptides could be useful for the characterization of the IgM structure motif(s) involved in RF activity.

By GeneScan analysis, clonal B-cell populations were respectively 70, 29 and 37% of B cells in the three monoclonal cases (Fig. 1). Since, by fluorescence-activated cell sorter (FACS) analysis, B cells were 8 and 53% of all bone marrow cells in samples 2 and 3, the clonal B cells present in the bone marrow were calculated as 2.39% (29% of 8%) and 19.61% (37% of 53%) of all cells in samples 2 and 3, respectively. CD19/CD5+ B cells were 1 and 8%, respectively, in these two cases. Then, both these values were lower than the percentage of monoclonal B cells; moreover, the immunophenotype of sample 1 was CD20+. CD5 expression because of lymphoproliferative evolution. In these latter cases, the loss of CD5 antigen could favour lymphoproliferation [36]. Further studies are, however, necessary to elucidate this point.

In addition, almost all B cells from monoclonal RF-IgM+ samples (sample 1 immunophenotype CD20+, sample 2, 8%CD19+, 7%CD20+, sample 3, 53%CD19+, 52%CD20+) were found to be CD20+. Moreover, in two independent series, the frequency of CD20+ B cells in HCV-positive patients with mono/oligo clonal B-cell expansions was: in the first series 16.6 ± 11.7% against the 12.1 ± 7.6% found in patients with a polyclonal B-cell pattern, and in the second series 11.82 ± 9.54% in patients with a monoclonal B-cell pattern and 6.33 ± 6.33% in patients with a polyclonal pattern (the difference in CD20 frequencies was significant, P < 0.005, data not shown) (D. Sansonno and S. De Vita, unpublished data). These data are in agreement with the finding that new therapy with anti-CD20 could be effective in MC-II by reducing the RF-producing B-cell component [36–38].

In conclusion, data reported herein confirm, with different approaches (GeneScan and MALDI-TOF analyses) from those previously reported (in gel VDJ PCR analysis) [39], the prevalence of oligoclonalities in MC patients. We demonstrated that B-cell monoclonality in the bone marrow and the cryoprecipitated RF-IgM share the same Ig combinatorial regions, thus indicating that prevalent B-cell clones present in the bone marrow are responsible for, or are a consequential clone that is responsible for, the cryoprecipitable IgM. In addition, we here give evidence that monoclonal B cells linked to RF+ activity are CD5– and CD20+.

Acknowledgements

We thank Professor A. Colombatti for support in the use of the MALDI-TOF mass spectrometer, Dr Federico Sacchetti for the great technical support and Luigina Mei for editorial assistance.

This study was supported in part by ‘Associazione Italiana per la Ricerca sul Cancro’ (AIRC), by a grant from the Regione Autonoma Friuli-Venezia-Giulia, FSN2003-ministero della salute, and Società Italiana di Reumatologia.

The authors have declared no conflicts of interest.

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