The Majority of Peripheral Blood Monoclonal IgM Secreting Cells Are CD5 Negative in Three Patients With Mixed Cryoglobulinemia

By Jean-Louis Pasquale, Caroline Waltzinger, Jean-Louis Kuntz, Anne-Marie Knapp, and Honey Levallouix

The mixed cryoglobulinemia is considered to be a nonmalignant human B-cell proliferation that frequently produces a monoclonal IgM with anti-IgG activity (rheumatoid factor). Using murine monoclonal anti-idiotypic antibodies specific for private minor idiotopes on monoclonal IgM from three patients suffering from nonmalignant mixed cryoglobulinemia, we investigated the presence of the CD5 antigen on the monoclonal IgM producing cells in these patients. It is shown by two-color cytofluorometric analysis that the majority of the peripheral blood monoclonal IgM rheumatoid factor secreting cells is CD5 negative in these three patients. One of the monoclonal rheumatoid factor K variable regions was sequenced at the protein level and belongs to the human VK311 gene as previously described. FIV3.3 is a monoclonal MoAb that was produced as previously described after immunizing mice with purified Fel IgM RF. The majority of the monoclonal anti-idiotopic antibodies that were produced by immunizing a mouse with polyclonal RF reacts with a low proportion of polyclonal RF and with Bul monoclonal RF. Thus, it recognizes a minor idiotope of RF. Fab', of the MoAbs were prepared after digestion with pepsin (Sigma, St Louis, MO) as described and purified over a protein A-affinity column (Bio Rad MAPS II) following the manufacturer's instructions.

Enzyme-linked immunosorbent assays (ELISA). Reactivity of MoAbs with the RFs was established by both direct assay and inhibition of binding assay. Briefly, during the direct assay, plastic microtiter wells (NUNC) were coated with purified RFs or control proteins (10 µg/mL). After blocking the remaining active sites with 1% bovine serum albumine phosphate-buffered saline (BSA-PBS), the purified MoAbs (1 µg/mL) were added and incubated for 1.5 hours at 37°C. After washing with PBS, binding of the MoAbs was measured by adding peroxidase-labeled affinity purified goat anti-mouse IgG (0.5 µg/mL; Jackson Immunoresearch Labs, Bar Harbor, ME) followed by washings of the peroxidase substrate (Sigma).

Inhibition of antigen binding was performed as described. Briefly, microtiter wells were coated with purified human IgG (50 µg/mL). The remaining active sites were blocked with 1% BSA-PBS. Mixtures of the MoAbs (1 µg/mL) and IgG (400 ng/mL) were prepared and incubated for 1 hour at room temperature. These mixtures were then added to the IgG coated microtiter wells. After a 1.5-hour incubation at 37°C and washings with PBS, binding of IgM RF was measured by adding peroxidase-labeled affinity purified goat anti-IgM (Cappell, Malvern, PA) in T-PBS (1% Tween 20, 0.05% BSA, 0.05 M Tris-Cl, pH 7.4) containing hydrogen peroxide and 4-chloro-1-naphthol as a chromogen. The remaining peroxidase activity was measured by using a spectrophotometer. The results were expressed as percentage of inhibition on a relative scale.

The majority of peripheral blood monoclonal IgM secreting cells is CD5 negative.

MATERIALS AND METHODS

Patients. Alt, Fel, and Bul are three unrelated patients suffering from nonmalignant mixed cryoglobulinemia. Clinically there was no evidence of a malignant proliferation after 5 years (Alt), 3 years (Fel), and 6 years (Bul) of follow-up and treatment with repeated plasmapheresis. The three monoclonal IgM K had an anti-IgG activity (rheumatoid factor [RF]).

Monoclonal anti-idiotypic antibodies. Murine monoclonal antibodies (MoAbs) specific for private idiotopes on Alt IgM RF and Fel IgM RF were used in this study. A75 is a murine IgG1 MoAb specific for a light chain associated idiotope on Alt IgM RF. FIV3.3 is a monoclonal IgG1 MoAb that was produced as previously described after immunizing mice with purified Fel IgM RF. The majority of the monoclonal anti-idiotopic antibodies that were produced by immunizing a mouse with polyclonal RF reacts with a low proportion of polyclonal RF and with Bul monoclonal RF. Thus, it recognizes a minor idiotope of RF. Fab', of the MoAbs were prepared after digestion with pepsin (Sigma, St Louis, MO) as described and purified over a protein A-affinity column (Bio Rad MAPS II) following the manufacturer's instructions.

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µg/mL, 1 hour at 37°C) followed by washings of the peroxidase substrate (Sigma).

The effect of the anti-id MoAb on RF binding was expressed as a percentage of the initial RF activity, i.e.,
\[
\% \text{ inhibition} = 100 - \frac{\text{binding activity with MoAb}}{\text{control}} \times 100
\]

The control was a mixture of the supernatant and an unrelated MoAb of the same subclass. Each assay was done in duplicate.

**Cytofluorometric analysis of cells.** About 2 ✕ 10^3 peripheral blood mononuclear cells were incubated 15 minutes at 4°C with purified Fab', fragments of FITC 3,3 (10 µg/mL) or A75 (10 µg/mL) or BII 2.1 (10 µg/mL), then centrifuged and washed twice in FACS wash (PBS; 3% heat inactivated fetal calf serum; 0.1% sodium azide; 30 mmol/L Hepes). The presence of the anti-idiotopes was shown by a fluorescein conjugated Fab', fragment of rabbit anti-mouse IgG (Fab', fragment specific; Jackson) 15 minutes at 4°C.

For the double staining experiments, the cells were then washed in FACS wash, saturated with normal purified murine IgG, then incubated with a murine, monoclonal biotin labeled anti-human CD5 (Becton Dickinson, Mountain View, CA) 15 minutes at 4°C. Cells were then washed twice in FACS wash and identified by CD5 (Becton Dickinson, Mountain View, CA) 15 minutes at 4°C. The patients' PBLs contained 70% to 81% of CD5 positive cells, and 4% to 7% of non-RF Ig.

Peripheral blood mononuclear cells contain membrane idiototype positive cells. As previously shown, patients' peripheral blood contains the monoclonal Ig secreting cells during mixed cryoglobulinemia. Using the anti-idiotypic MoAbs, we show that membrane idiotype positive cells are consistently detectable, either at a low frequency (0.7% to 1%) in two patients' peripheral mononuclear cells (Alt and Fel, Fig 1), or at a higher frequency (Bul, 3% to 4%).

**RESULTS AND DISCUSSION**

A75, FITC 3,3 and BII 2.1 are anti-idiotypic antibodies specific for idiotopes on Alt, Fel, and Bul IgM RFs. Table 1 shows the results of the ELISA (direct and inhibition of binding) obtained with the Fab'2 of the MoAbs. MoAbs A75 and FITC 3,3 do not recognize cross-reactive determinants on monoclonal IgM RFs, and they do not react with pooled polyclonal IgM or IgG. Both are able to inhibit the binding of their respective IgM RF with solid phase IgG. BII 2.1 recognizes Bul IgM RF even though it was produced against unrelated polyclonal RF. Purified Fab', fragments of A75, FITC 3,3 and BII 2.1 were subsequently used for the cytofluorometric analysis as they were considered to be specific markers of Alt, Fel, and Bul IgM RF, respectively. For our purpose, the use of anti-cross-reactive idiotypic reagents was unsuitable because these antibodies frequently react with a low proportion of non-RF Ig.

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The majority of the idiotype positive cells is CD5 negative. Using a two-color cytofluorometric analysis, we show that the majority of the Alt, Fel, and Bul idiotope positive cells do not contain a significant amount of the CD5 membrane marker (Fig 2A and B for Alt and Fel cells). The mean CD5 fluorescence intensity of the idiotope positive cells was 20 ± 3% lower than the mean CD5 fluorescence intensity of the CD5 positive cells. The patients' PBLs contained 70% and 81% of CD5 positive cells, and 9% to 12% of B cells (membrane Ig positive cells). By comparison, CD5 positive B CLL cells express 10 to 15 times more CD5 fluorescence.

% inhibition = 100 - \frac{\text{binding activity with MoAb}}{\text{control}} \times 100

Table 1. Reactivity of the Three Anti-Idiotypic MoAbs (Fab') With Their Respective Antigens

<table>
<thead>
<tr>
<th>Protein Type</th>
<th>A75</th>
<th>FITC 3,3</th>
<th>BII 2.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alt IgM RF</td>
<td>1.470</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Fel IgM RF</td>
<td>0.000</td>
<td>1.368</td>
<td>1.265</td>
</tr>
<tr>
<td>Bul IgM RF</td>
<td>0.000</td>
<td>0.008</td>
<td>0.000</td>
</tr>
<tr>
<td>Sie IgM RF</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Po IgM RF</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Lef IgM RF</td>
<td>0.000</td>
<td>0.031</td>
<td>0.000</td>
</tr>
<tr>
<td>Dul IgM RF</td>
<td>0.011</td>
<td>0.016</td>
<td>0.000</td>
</tr>
<tr>
<td>Pil IgM RF</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Li IgM</td>
<td>0.000</td>
<td>0.007</td>
<td>0.000</td>
</tr>
<tr>
<td>Ba IgM</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Normal IgM</td>
<td>0.008</td>
<td>0.010</td>
<td>0.000</td>
</tr>
<tr>
<td>Normal IgG</td>
<td>0.008</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>BSA</td>
<td>0.000</td>
<td>0.012</td>
<td>0.004</td>
</tr>
</tbody>
</table>

The direct assays and the inhibition of binding assays were performed as described in Materials and Methods.
MIXED CRYOGLOBULINEMIA SECRETING CELLS

Fig 1. Cytofluorometric analysis of Alt and Fel patients' peripheral blood mononuclear cells with Fab'2 fragments of MoAb A75 (A and C) and FIV 3.3 (B and D). Antibody binding was shown by a fluorescein conjugated Fab'2 rabbit anti-mouse Fab'2 IgG. The fluorescence background in presence or absence of an unrelated Fab'2 mouse MoAb of IgGl class was consistently lower than 0.2%.

Because the majority of peripheral blood CD5 positive cells are T cells, we verified in the absence of T cells the results of the two-color cytofluorometric analysis of the patients' PBL. We sorted the idiotope positive cells from Bul PBL, and labeled the sorted cells with the anti-CD5 antibodies. The results, given in Fig 3, clearly show by two-color analysis that the idiotope positive cells are mainly CD5 negative.

Alt IgM RF uses a VK III light chain sequence. One could argue that the three monoclonal IgM RFs studied in these patients might be peculiar in using an unusual set of variable region genes. Two of these three monoclonal RFs (Alt and Fel) express a cross-reactive idiotope that is present in approximately 30% of the monoclonal IgM RF seen in mixed cryoglobulinemia, and is recognized by a murine MoAb F V 1,1 (unpublished results). Moreover, we performed the protein sequence of Alt IgM RF VK to compare this sequence with known human IgM RF VK primary sequences. Table 2 shows that Alt VK belongs to the VK III family with almost 90% homology with the prototype sequences Les IgM RF and Pom IgM RF (VK IIIa sub subgroup). Thus, at least for the VK use, Alt IgM RF does not appear to be peculiar among the human monoclonal IgM RFs. The germ line gene (VK 328) encoding the Les K chain has been cloned and sequenced from malignant B-cell CLL cells.15 However, the Les B-cell CLL was recently reported as being distinct from classical B-cell CLL: the “Les” is a CD5 negative CLL and is subjected to intraclonal diversity in its expressed VH genes.16 Even though Alt IgM RF is idiotypically stable over a prolonged period of time, the existence of such an intraclonal diversity in a CD5 negative B cell CLL deserves further evaluation at a genetic level of the homogeneity of the expressed Ig genes of mixed cryoglobulinemia cells.

Despite the fact that the classical malignant B cell CLL cells and IgM RF secreting cells from mixed cryoglobulinemia preferentially use similar VK regions, and that both the classical malignant B-cell CLL cells and the nonmalignant mixed cryoglobulinemia cells are apparently not affected by Ig variable region mutations, these two diseases differ at least by the clinical evolution and the CD5 expression of the proliferating cells. Is there any relationship between these two differences? One can propose different explanations: (1) The significance of the CD5...
antigen on B cells is still under debate (see ref. 20 for review): it is unclear, at least in humans, if CD5 B cells belong to a separate lineage. The difference in CD5 expression between B-cell CLL cells and B cells from mixed cryoglobulinemia could then reflect either a different B cell origin (although they use similar VK genes), or a simple differentiation or activation step at which the cells are "frozen" (one being a CD5 negative and secreting step, the other a CD5 positive and nonsecreting one). (2) The presence of the CD5 antigen on the B-cell CLL could be related to the malignant process itself. Under these circumstances, knowledge of the genetic control of the CD5 expression in B cells could shed light on the mechanisms of B-cell malignancy.

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