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A Flow Cytometry-Based Strategy to Identify and Express IgM from VH1-69⁺ Clonal Peripheral B Cells

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Abstract

Pathologic rheumatoid factor (RF) levels are hallmarks of several human diseases. Production of monoclonal RF in vitro is essential for studies of the antigenic specificities of RF, as well as for a dissection of the mechanisms of aberrant RF⁺ B cell activation. We have expanded upon previous methods to develop a flow cytometry-based method to efficiently clone monoclonal antibodies (mAbs) from humans with expansions of RF-like, immunoglobulin heavy chain variable region (IgVH) 1-69 gene segment-containing B cells. The cloned variable regions are expressed as IgM and produced during culture at concentrations between 5–20 µg/ml. Using this system, we show that clonal Igs from patients with HCV-related mixed cryoglobulinemia, when expressed as IgM, have RF activity. We anticipate that this system will be useful for the cloning and expression of mAbs partially encoded by VH1-69 and for determination of the reactivity patterns of polyspecific, low-affinity IgMs of human pathogenic importance.

Keywords

IgM; Rheumatoid factor; flow cytometry; cloning; expression; mixed cryoglobulinemia

1. Introduction

A high level of circulating IgM rheumatoid factor (RF) is a feature of several human autoimmune diseases, such as rheumatoid arthritis, Sjögren's syndrome, systemic sclerosis, and hepatitis C virus-associated mixed cryoglobulinemia (HCV MC). By definition, RF has reactivity towards IgG Fc; however, Fc specificities vary with disease process and RF mutational status (Bonagura et al., 1998). In vitro production of monoclonal RF has traditionally involved heterohybridomas (Brown et al., 1990) or EBV-transformed lymphocytes (Steinitz et al., 1980); however, both of these are highly selective, low-efficiency processes (Laffly and Sodoyer, 2005). A protocol for the efficient transformation of human memory B cells has more recently been described (Traggiai et al., 2004), but this procedure is limited to memory B cells that can be activated by CpG and transformed by EBV. In HCV MC, however, the clonal B cells are often CD21^{low/-} (Charles et al., 2008),

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and are resistant to EBV infection in vitro. A relatively nonbiased system for cloning of IgVH from singly-sorted B cells and expression as human IgG₁ has been well-described (Wardemann et al., 2003; Tiller et al., 2008). This system is not restricted to particular B cell populations and does not require prior B cell stimulation. However, expression of IgM RF as an IgG₁ poses several difficulties for downstream specificity analyses. First, avidity may be lost upon conversion from a decavalent IgM to a bivalent IgG. Second, the expressed IgG₁ RF could form immune complexes due to the presence of the antigen-binding domain and its target antigen in the same molecule. Third, heavy chain constant region domain swapping may affect affinity, specificity and V-region structure (reviewed in (Torres and Casadevall, 2008)). Motivated by these considerations, we have built upon this previous system to develop a flow cytometry-based method to clone RF-like Ig from humans with expansions of VH1-69⁺ B cells and express it as IgM in high yield, in order to more accurately assess the reactivities of the RF-like IgM towards putative antigens.

In HCV MC, pathologic RF is typically monoclonal IgM κ of the cross-reactive WA idiotype, which is frequently encoded by VH1-69 and V κ 3-20 gene segments (Silverman et al., 1988; Gorevic and Frangione, 1991; Knight et al., 1993). We have previously reported that HCV MC is associated with a clonal expansion of modestly hypermutated IgM⁺ κ ⁺ memory B cells that express Ig encoded by VH1-69 and V κ 3-20 gene segments. The G6 mAb, which recognizes the VH1-69 gene product (Potter et al., 1999), has previously been used to identify these clonally-expanded B cells in HCV MC patients (Carbonari et al., 2005). We have singly-sorted G6⁺ B cells by FACS and performed nonbiased IgVH and IgV κ RT-PCR as previously described (Wardemann et al., 2003); sequencing confirmed the overwhelming majority of these cells to be VH1-69⁺/JH4⁺/V κ 3-20⁺. We next performed a third round of VH1-69/JH4-specific and V κ 3-20-specific PCR to correct for 5' IgV mutations introduced by the unbiased first and second stage PCR primers. IgVH and IgV κ were then ligated into Igu and Igk expression vectors. We then co-transfected these constructs into 293T cells expressing human J chain. After 6 days of culture, supernatants typically contained 5–20 μ g/ml IgM, which was demonstrated to have RF activity by ELISA.

2. Materials and Methods

2.1 Patients

The studies were approved by the Institutional Review Boards at The Rockefeller University Hospital (RUH) and New York Presbyterian Hospital (NYPH). Volunteers were recruited through the RUH outpatient clinic and the hepatology clinic at NYPH. All donors gave written informed consent according to the principles of Helsinki before enrollment. We enrolled HCV[−] donors and HCV RNA⁺ subjects with symptoms of MC.

2.2 Blood collection and processing

Blood was collected into ACD tubes before processing. PBMCs were purified by centrifugation (800 $\times$ g) over lymphocyte separation medium (ICN). The mononuclear cell layer was washed three times in RPMI supplemented with 1% HEPES and 2% FBS. Cells were resuspended at a final concentration of 10⁷/ml in RPMI 1640 supplemented with 0.5% HEPES, 20% FBS and 7.5% DMSO for cryopreservation. Cells were slowly frozen to -80° C and maintained at -150° C. Viable cell recovery was typically >90% as judged by incorporation of an amine-reactive violet dye (LIVE/DEAD Fixable Dead Cell Stain, Invitrogen). Tissue culture reagents were purchased from Life Technologies (Invitrogen).

2.3 Flow cytometry

For immunophenotypic analysis, cells were stained with Live/Dead Aqua (Invitrogen) and the indicated Abs at room temperature in PBS supplemented with 2% (w/v) BSA (Fraction V; Fisher Biotech, Fair Lawn, NJ) and 0.02% NaN₃. All antibodies and reagents were obtained from BD Biosciences, except for G6 (provided by R. Jefferis) and biotinylated anti-Fc receptor-like 4 (FCRL4) F(ab')₂ (provided by Götz Ehrhardt). Anti-CD20-PerCp/Cy5.5 (clone L27), IgM-FITC (clone G20-127), kappa-PE (clone G20-193), CD27-Alexa 700 (clone M-T271), and CD21-APC (clone B-ly4) were used. G6 was conjugated to biotin using EZ-Link Micro Sulfo-NHSBiotinylation Kit (Pierce), and to Alexa Fluor 594 using Invitrogen's Alexa Fluor® 594 Antibody Labeling Kit. Cells were incubated with primary and secondary (anti-FITC Alexa 488, streptavidin-APC/Cy7) reagents for 30 min each, and were washed 3 times with incubation buffer. Stained cells were fixed in PBS/2% paraformaldehyde and analysis was performed within one hour on an LSRII flow cytometer equipped with 405, 488, 561, and 640 nm lasers (BD Immunocytometry Systems). Lymphocytes were identified by forward and side-angle light scatter characteristics.

2.4 Single-cell sorting and RT-PCR

PBMCs were stained as above, except staining was performed in the absence of NaN₃, and cells were not fixed in paraformaldehyde. G6⁺ CD20⁺ B cells were singly-sorted either on a MoFlow (DAKO Cytomation) or FACSaria II (BD) cell sorter equipped with 355, 405, 488, 561, and 640 nm lasers. Single cell unbiased Ig RT-PCR using IgVH and IgVL family-specific primers was performed by a previously-described technique (Wardemann et al., 2003; Tiller et al., 2008) using random hexamer priming, Superscript III reverse transcriptase (Invitrogen), Pfu DNA polymerase (Invitrogen), and modified IgH 3' antisense primers containing an XhoI site (Table 1). As negative controls for RF assays, IgVH and IgVL were also amplified from CBH5 hybridoma cDNA (provided by S. Foung) or mGO IgG₁ expression vectors (provided by M. Nussenzweig) and cloned as below. After incubation with Taq polymerase at 72° C for 10 min, products were TA cloned into pCR2.1-TOPO (Invitrogen) and were sent for sequencing. Ig variable region analysis was performed using Joinsolver (Souto-Carneiro et al., 2004). For VH1-69⁺/Vκ3-20⁺ clones, an additional round of PCR (94° C 30 sec, 55° C 30 sec., 68° C 1 min, 35 cycles) was then performed using VH1-69- and Vκ3-20-specific primers, in order to correct 5' mutations introduced by primers during the previous two PCR rounds. We sequenced the resulting products, and verified that nucleotides 1–18 and 1–33 corresponded to germline VH1-69 and Vκ3-20 sequences, respectively, and that no mutations beyond the 5' VH1-69 and Vκ3-20 primers had been introduced during this third round of PCR.

2.5 IgM cloning

Human B cell RNA was reverse-transcribed using random hexamers and the Superscript III First-Strand Synthesis System (Invitrogen). IgCμ1-4 was amplified by nested PCR (94° C 30 sec, 57° C 30 sec., 68° C 90 sec, 35 cycles each round) using XhoI-containing sense and EagI-containing antisense primers (Table 2) for the second round. An internal IgCμ4 AgeI site was subsequently abolished with overlapping PCR using primers containing a silent mutation at T111. For IgM expression vector construction, XhoI/EagI-digested product was ligated into an IgG₁ expression vector (Tiller et al., 2008) from which Cγ was removed by XhoI/EagI digestion. For subsequent IgM cloning, AgeI/XhoI-digested IgVH products were ligated into the similarly-digested IgM expression vector. Igκ vector construction was carried out as previously described (Tiller et al., 2008).

For production of human J chain-secreting 293T cells, J chain was first amplified from human B cell cDNA using sense (10565) and antisense (10566) primers (Table 3) in one round of PCR (94° C 30 sec, 57° C 30 sec., 68° C 45 sec, 35 cycles). After PCR of Kozak

and signal sequences from the IgM expression vector (primers 10736 and 10860), Kozak/signal sequence and J chain product was generated using overlapping PCR with 5' HindIII sense (11094) and 3' XhoI antisense (10738) primers (94° C 30 sec, 57° C 30 sec., 68° C 45 sec, 35 cycles). After digestion with HindIII/XhoI, the product was ligated into pcDNA3.1 Zeo (+) (Invitrogen). Plasmid was transfected into 293T cells using Lipofectamine 2000 (Invitrogen). Individual cells were plated by limiting dilution in 96-well dishes, and following Zeocin selection, were expanded into 12-well dishes. Supernatants from each well were screened for IgJ production by ELISA. Cells with the highest level of IgJ secretion were subsequently used for IgM expression.

2.6 IgM expression

293T cells were grown in 150 mm plates (Falcon, Becton Dickinson) in Dulbecco's modified eagle medium (DMEM) supplemented with ultralow IgG fetal calf serum (Invitrogen) and 1 mM sodium pyruvate (Invitrogen). Transient transfections were performed on exponentially growing 293T cells at 80% confluency. For each plate, equal amounts (12.5 µg) of IgVHµ and IgVk vector DNA were added to 1.5 ml 150 mM NaCl, to which 200 µl branched polyethylenimine (Sigma) was added. After incubation at RT × 10 min., the DNA/polyethylenimine mixture was added to cells that had been washed twice with DMEM, and the cells and DNA mixture were incubated at RT × 1 h. with 25 ml DMEM supplemented with 1 mM sodium pyruvate and 1% Nutridoma-SP (Roche). Supernatants were harvested on days 2, 4, and 6; fresh media was added to the cells on days 2 and 4. 150 ml total supernatant (representing two 150 mm plates/Ig) was concentrated with Amicon Ultra-15 centrifugal filter units with a 100 kDa molecular weight cutoff (Millipore). After the total volume was reduced to 2 ml, five washes with 12 ml PBS were performed (volume was reduced by centrifugation to 2 ml each time) and 0.05% sodium azide added to a total volume of 2 ml.

2.7 Human IgM ELISA

Nunc MaxiSorp plates were coated overnight at 4° C with capture goat polyclonal anti-human Igµ antibody (Bethyl Labs). After blocking with 1% BSA for 2 h at RT, antibody dilutions were added for 1 h at RT. Bound IgM was detected by addition of HRP-conjugated goat anti-human Igµ and, after stopping the reaction with 1 N HCl, A₄₅₀ was measured with a FLUOstar Omega microplate reader (BMG Laboratories). Human myeloma IgM (Jackson ImmunoResearch) was used to generate a standard curve.

2.8 Gel Filtration

500 µg of sample was equilibrated in 20 mM sodium phosphate buffer, pH 7.0 and applied to a HiPrep 16/60 Sephacryl S-300 HR column using an AKTA Explorer chromatography system (GE Healthcare Life Sciences). Two column volumes of buffer were run through the column at 0.25 ml/min, A₂₈₀ nm was measured in the eluate, and 3 ml fractions were collected. Data were analyzed using Unicorn software (GE Healthcare Life Sciences).

2.9 SDS-PAGE and western blotting

For molecular weight estimation of intact IgM, samples were separated on tandem NativePage Novex 3–12% Bis-Tris polyacrylamide gels (Invitrogen) at 150 V for 2 h under non-reducing conditions as previously described (Vorauer-Uhl et al., 2010). The protein marker contained bovine IgM pentamers with a molecular weight of 1048 kDa and hexamers with a molecular weight of 1236 kDa. LDS sample buffer containing Li-dodecylsulfate (NuPAGE LDS 4× sample buffer, pH 8.5, Invitrogen) was used for IgM-SDS-PAGE. Either 7.5 µl of transfected cell culture supernatant or gel filtration eluate, or 100 ng of concentrated IgM mAb were loaded per lane. For controls, IgMλ purified from a

human with multiple myeloma (Jackson ImmunoResearch) was loaded at 1 µg/lane. One gel was stained for total protein using Sypro Ruby, and the other was analyzed for IgM content using western blotting.

In order to detect J chain, samples were run under reducing conditions on Novex 4–20% Bis-Tris polyacrylamide gels (Invitrogen) using a Benchmark protein ladder (Invitrogen). Samples were loaded as above, except 100 ng of human IgM was loaded.

2.10 Sypro Ruby Staining

Gels were fixed with 50% methanol and 7% acetic acid twice for 30 min. and then stained with 80 ml Sypro Ruby (Invitrogen) overnight. They were then washed for 30 min. with 10% methanol and 7% acetic acid, and rinsed with water for 10 min. Gels were imaged with a Typhoon 9400 variable mode imager (GE Healthcare Life Sciences) using an excitation laser of 457 nm and an emission filter of 610 nm with a band pass of 30 nm. Photomultiplier tube voltage and pixel size were set on 600 V and 200 µm, respectively.

2.11 Western Blot Analysis

Gels were electroblotted onto a nitrocellulose membrane (GE Life Sciences) for 1.5 h at 300 mA. Blots were blocked overnight with 5% dry milk in PBS/0.05% Tween. For detection of IgM, peroxidase-coupled anti-human Igµ, diluted 1:5000 (Bethyl) was then applied for 1 h. For detection of J chain, 1 µg/ml anti-human J chain (clone Mc 19-9, US Biological) was applied for 1 h, after which the blot was washed and incubated with HRP-conjugated rabbit anti-mouse IgG (1:5000, Pierce) for 1 h. Blots were then washed three times with PBS/0.05% Tween for 20 minutes, developed with ECL Super Signal West Pico (Pierce), and exposed to autoradiograph film (Kodak) for 5–30 sec (for IgM detection) or 1 h (for J chain detection).

2.12 RF assay

For determination of reactivities towards IgG isotypes, 96-well Nunc MaxiSorp plates were coated with 1 µg/well human myeloma IgG₁λ, IgG₂λ, IgG₃λ, or IgG₄λ (Sigma), and incubated at RT for 1 h. After washing three times, plates were incubated with 1% BSA/PBS for 1 h at RT. Serial dilutions of IgM mAbs cloned from HCV⁺MC⁺ subject LDU 125 or sera isolated from the same patient were then added for 1 h at RT. After washing, HRP-labeled goat anti-human Igµ (Bethyl) was added for 1 h.

To determine if mAb G6 recognized the cloned IgM, microwells were coated with anti-human IgM and, after blocking, 0.5–10 µg/ml LDU 125 A4 IgM (partially encoded by VH1-69) or mGO (partially encoded by VH4-39) were added, followed by detection with G6 mAb (1:5000), and after washing, HRP-conjugated rabbit anti-mouse IgG₁ (1:5000, Pierce).

All assays were developed with TMB (BioFX Laboratories). After stopping reactions with 1 N HCl, A₄₅₀ was measured.

2.13 Data Analysis and Statistics

Data were analyzed using Prism (GraphPad Software). Results are presented as means +/– standard deviation. GenBank accession numbers for the cloned Ig sequences are GQ903484–GQ903547.

3. Results

3.1 The G6 mAb can be used to identify an expanded IgM⁺κ⁺ B cell population

We immunophenotyped PBMCs from HCV⁻ donors and HCV⁺MC⁺ subjects, using G6 to detect B cells expressing the VH1-69 gene product. Results for representative HCV⁻ (LDU 148) and HCV⁺MC⁺ (ECH 559) subjects are shown in Figure 1. Lymphocytes were identified by forward (FSC) and side light scatter (SSC) characteristics, and dead cells were excluded with a membrane-impermeant fluorescent dye. In contrast to the HCV⁻ individual, in whom <3% of B cells were G6⁺, 41% of CD20⁺ B cells in the HCV⁺MC⁺ patient stained with G6. Further analysis revealed that the G6⁺ cells in the HCV⁺MC⁺ subject were predominantly IgM⁺ and Igκ⁺ (Fig. 1A). When we examined total B cells and the G6⁺ and G6⁻ B cell subsets from this patient, we found that IgM⁺CD27⁺ memory B cells were overrepresented in the G6⁺ fraction. Moreover, consistent with our earlier observations (Charles and Dustin, 2009), (Charles et al., submitted), a preponderance of the G6⁺ B cells from this HCV⁺MC⁺ patient were IgM⁺κ⁺ and CD21^{low}, and the majority of the CD21^{low} B cells were CD27⁺ (Fig. 1B).

3.2 Cloning and expression of RF-like IgM

In order to clone RF-like Ig from HCV MC patients, we used FACS to isolate B cells expressing the VH1-69 gene product. The sorting strategy for a representative HCV⁺MC⁺ patient (LDU 125) is shown in Figure 2. After gating on live lymphocytes as described above, FSC and SSC doublets were excluded, and G6⁺ CD20⁺ B cell subsets were identified (Fig. 2A). G6⁺ and G6⁻ B cell subsets may be subsequently sorted on the basis of CD27 and CD21 staining (Fig. 2B) or FCRL4 staining (not shown) for either Ig RT-PCR or cell function analysis. For Ig cloning, cells were singly-sorted into individual wells of a 96-well microplate containing lysis buffer and RNase inhibitors (Wardemann et al., 2003). Unbiased IgV RT-PCR was then performed as described in the Materials and Methods. Since clinical tests had revealed subject LDU 125 to have elevated serum RF, a serum monoclonal IgMκ gammopathy, and an expansion of IgM⁺κ⁺ peripheral B cells, we performed IgM- and Igκ-specific PCR (primers listed in Table 1). After the first and second rounds of PCR, 58% and 52% of the wells had size-appropriate products for IgVμ and IgVκ, respectively. Paired IgH and IgL products from the same individual cells were TA-cloned and sent for sequencing (Table 3). A majority of clones A4-H10 had very similar IgVH and IgVL, with the same complementarity determining region 3 (CDR3) sequences, whereas there was considerably more variability among clones NA6-NH5. Note that G6⁺ cells were sorted into one plate (wells A1-H12) based upon expression of FCRL4, and were sorted into another plate (wells NA1-NH12) based upon absence of FCRL4 expression. Overall, 26/32 clones were VH1-69; 24 of these were also DH3-22/JH4⁺, and 25/26 were Vκ3-20⁺. Three clones were VH3-07⁺, and one clone each was VH3-11⁺, VH3-48⁺, or VH3-72⁺. Consistent with our earlier report (Charles et al., 2008), the majority (25/26) of the VH1-69⁺ clones had IgVH mutations; with four exceptions, all clones had 3 or fewer non-synonymous mutations. All of the 25 Vκ3-20 clones were mutated, with 21 of these having 3 or fewer non-synonymous mutations.

We performed a third round of VH1-69- and Vκ3-20- specific PCR on selected cloned individual cells in order to correct for non-germline mutations introduced by first and second round 5' primers. We cloned the resultant IgVH and IgVκ into Igμ and Igκ expression vectors. We sequenced these products to verify that nucleotides 1–18 and 1–33 were germline VH1-69 and Vκ3-20 sequences, respectively, and that no mutations beyond the 5' VH1-69 and Vκ3-20 primers had been introduced during this third round of PCR. We then transiently transfected paired IgH and IgL into J chain-secreting 293T cells, as described in Materials and Methods.

IgM yields after 6 days of culture typically ranged from 5–20 µg/ml of supernatant. Before and after concentration via centrifugal filtration through 100 kDa molecular weight cutoff membranes, we calculated IgM concentration by ELISA. We then used gel filtration chromatography to verify that the size of the cloned, concentrated IgM approximated that of IgM isolated from human serum. Figure 3A shows that the elution peaks of human IgM, human IgG, and bovine serum albumin corresponded to 37–42 ml, 57–67 ml, and 67–75. respectively. The elution profile of cloned CBH5 IgM revealed major peaks at 32–37 ml and 37–42 ml, as well as smaller peaks at 62–72 ml and 78–82 ml. We concluded that the 37–42 ml peak correlated with the expected size of human pentameric IgM, and that the 32–37 ml peak represented a higher-order IgM aggregate.

We further confirmed the size of the cloned IgM using non-reducing SDS-PAGE (Figure 4 A, B). IgM corresponding to the 1048 kDa bovine pentamer molecular weight marker was detected in the cell culture supernatants (lanes 2–3), and this IgM could be concentrated via centrifugal filtration with 100 kDa cutoff membranes (lanes 4–6). Interestingly, size exclusion fractions 12 and 13 did not show an apparent size difference (lanes 8–9), suggesting that higher-order IgM structures are reversibly concentration- and solvent-dependent. Surprisingly, we did not detect size differences in IgM produced from 293T cells with and without human J chain. However, there were minor clone-dependent migration pattern differences irrespective of the transfected cell line, and the human IgM λ obtained from a commercial source ran at an appreciably lower level between 720–1048 kDa. We did not detect monomeric IgM in cell culture supernatants or concentrated IgM.

Samples were then subjected to reducing SDS-PAGE, followed by either staining with Sypro Ruby or electroblotting and probing with anti-human J chain. In one sample, we detected a band between 25–30 kDa, which is consistent with the previously-demonstrated retarded migration pattern for human J chain (Symersky et al., 2000; Schrohenloher et al., 1973). This band was discernable in a concentrated IgM that had been produced in 293T cells transfected with J chain, but not in the other samples (Figure 4 C, D).

3.3 IgM expressed from G6⁺ B cells has RF activity

We assessed whether mAbs cloned and expressed from G6⁺ B cells possessed RF activity, as measured by their abilities to bind human IgG₁, IgG₂, IgG₃, or IgG₄. Data for three clones from patient LDU 125 and the RF negative, VH1-69-encoded control (CBH5) are shown in Figure 5A–D. Clones E3, E5, and H5 demonstrated substantial binding to IgG₁, with decreased avidities for IgG₂ and IgG₄, and markedly decreased avidities for IgG₃, consistent with earlier reports regarding isotype specificities of certain RF subclasses (Normansell, 1975; Bonagura et al., 1998). The IgG isotype specificity pattern of these mAbs mirrored that seen in the serum of subject LDU 125 (Fig. 5E), suggesting that the cloned mAbs are a physiological reflection of circulating RF found in this patient in vivo. Finally, we used ELISA to confirm that the cloned IgM could be recognized by the G6 mAb (Fig. 5F). G6 bound to the VH1-69-encoded LDU 125 A4 clone, but not to the VH4-39-encoded mGO clone.

4. Discussion

We have developed a system to preferentially clone VH1-69-encoded mAbs as IgM from singly-sorted B cells. In particular, the expression of IgM RF⁺ B cells' mAb as IgM, instead of IgG₁, offers the potential advantage of an improved physiologic assessment of these mAbs' reactivities. Modestly hypermutated RF typically has low to moderate affinity for its target antigen, IgG Fc, and the decavalent stoichiometry of pentameric IgM confers a substantial increase in avidity. Supporting this, it has been shown that the binding of a low-affinity mouse IgG_{2a} anti-RBC autoantibody is enhanced > 1000 fold when expressed as an

IgM, compared to IgG_{2a}, isotype (Fossati-Jimack et al., 1999). Thus, our method may prove to be most beneficial in the determination of the reactivity patterns of polyspecific, low-affinity IgMs. Our procedure can easily be adapted for the analysis of RF expressing non-G6 cross-reactive epitopes, synthesis of germline IgVH and IgVL mAbs (described in (Tiller et al., 2008)), cloning of mAbs from class-switched B cells, or expression of VH1-69-encoded mAbs as IgG₁.

One area where this technique may be most immediately useful is in the accurate definition of the IgM RF that is observed in HCV⁺MC⁺ patients. We have previously observed that these patients have clonal expansions of memory B cells expressing modestly somatically hypermutated IgM (Charles et al., 2008) and that some of these clones have CDR3 sequences identical to RF IgMs isolated from patients with mucosa-associated lymphoid tissue neoplasms (Bende et al., 2005). We are currently utilizing our expression system to examine the binding of IgM RF to IgG₁ and non-IgG₁ antigens, and to determine whether somatic hypermutation modulates these interactions. Knowledge gained from such experiments could possibly be applied to therapeutics capable of blocking antigen-RF complexes.

The selective cloning of VH1-69-encoded mAbs may be particularly useful in the identification of effective anti-viral humoral responses. VH1-69-encoded Abs are enriched in anti-influenza (Corti et al.; Sui et al., 2009) and anti-HIV (Huang et al., 2004) responses. Moreover, CBH5, which is partially encoded by VH1-69, is a potent cross-neutralizing anti-HCV mAb (Chan et al., 2001; Keck et al., 2007). The distinct hydrophobic CDR-H2 loop encoded by VH1-69 may confer antiviral activity by binding to hydrophobic viral targets (Sui et al., 2009).

Additionally, the usefulness of our approach may not be limited to patients with rheumatic or viral diseases. VH1-69/Vκ3-20 gene pairs are also increased in frequency among B cell chronic lymphocytic leukemia (CLL) clones (Messmer et al., 2004). Expression of CLL patients' IgM mAbs as IgG₁ suggests that somatic hypermutation abrogates germline BCR reactivity (Herve et al., 2005), and recent evidence suggests that they may recognize nonmuscle myosin heavy chain IIA (Chu et al., 2010). Expression of these mAbs as IgM may reveal additional, or altered antigenic specificities by virtue of Cμ-dependent IgV conformations.

To conclude, we have described a system to clone and express IgM RF from VH1-69⁺ B cells. The expression of monoclonal RF as IgM allows for an assessment of antigenic reactivity that more accurately reflects in vivo disease processes. This methodology may be utilized for the elucidation of the pathogenesis of human rheumatic disease, B cell neoplasia, or for the assessment of effective antimicrobial humoral responses.

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Figure 1. The G6 mAb identifies an expanded clonal B cell population

Flow cytometric analyses of a representative HCV⁻MC⁻ (LDU 148) patient and a symptomatic HCV⁺MC⁺ patient with elevated serum rheumatoid factor (702 IU/ml) (ECH 559) are shown. Lymphocytes were identified by forward and side light scatter characteristics. After exclusion of dead cells, 2.8% and 41% of CD20⁺ B cells from LDU 148 and ECH 559, respectively, were G6⁺. The G6⁺ B cells from ECH 559 are predominantly IgM⁺ and Igκ⁺ (A). Depiction of total B cells and G6⁺ and G6⁻ B cell subsets from subject ECH 559 (B). Compared to total B and G6⁻ B cells, the G6⁺ B cell subset is enriched for IgM⁺CD27⁺ and IgM⁺κ⁺ B cells. The G6⁺ B cell subset is enriched for CD21^{low} B cells, the majority of which are CD27⁺.

Figure 2. Sorting strategy for single-cell Ig RT-PCR

PBMCs from an HCV⁺MC⁺ patient with a monoclonal IgM κ ⁺ B cell expansion (LDU 125) are shown. Lymphocytes were identified by forward and side light scatter characteristics. After exclusion of dead cells and doublets, G6⁺ CD20⁺ B cells were identified (A) and singly-sorted into individual wells of a 96-well microplate. CD27^{+/−}/CD21^{high/low} G6⁺ and G6[−] B cell subsets (B) could additionally be bulk-sorted for downstream functional analyses.

Figure 3. Gel filtration chromatography of expressed IgM

As sizing standards, 500 μ g each of human IgM λ from a patient with multiple myeloma, human IgG $_1$, or bovine serum albumin were separated on a HiPrep 16/60 Sephacryl S-300 HR chromatography column. A_{280} nm of the eluate is depicted as a function of eluate volume. The horizontal lines represent conductivity and pH (A). CBH5 IgM (500 μ g) was similarly applied to the column, and 3 ml elution fractions were collected (B).

Figure 4. SDS-PAGE Analysis of expressed IgM

Samples run on Native PAGE 3–12% Bis-Tris gels and either stained with Sypro Ruby (A) or electroblotted to nitrocellulose and probed with HRP-conjugated anti-human IgM (B). Lane 1: NativeMark molecular weight marker; lane 2: mGO IgM transfectant supernatant (7.5 μ l); lane 3: 1403 IgM transfectant (293T cells without J chain) supernatant (7.5 μ l); lane 4: mGO IgM concentrated with a 100 kDa spin filter (100 ng); lane 5: 1403 IgM (produced in 293T cells without J chain) concentrated with a 100 kDa spin filter (100 ng); lane 6: 1403 IgM concentrated with a 100 kDa spin filter (100 ng); lanes 7–9: fractions 11, 12 and 13, respectively, of gel-filtered 1403 IgM concentrated with a 100 kDa spin filter (7.5 μ l each); lane 10: IgM from a patient with multiple myeloma (1 μ g).

Reduced samples run on 4–20% Bis-Tris gels and stained with either Sypro Ruby (C) or electroblotted to nitrocellulose and probed with mouse anti-human IgJ and HRP-conjugated rabbit anti-mouse IgG (D). Lane 1: Benchmark molecular weight marker; lane 2: 773 IgM transfectant supernatant (7.5 μ l); lane 3: 1403 IgM transfectant (293T cells without J chain) supernatant (7.5 μ l); lane 4: mGO IgM concentrated with a 100 kDa spin column (100 ng); lane 5: 1403 IgM (produced in 293T cells without J chain) concentrated with a 100 kDa spin filter (100 ng); lane 6: 1403 IgM concentrated with a 100 kDa spin filter (100 ng); lanes 7–8: fractions 12 and 13, respectively, of gel-filtered 1403 IgM concentrated with a 100 kDa spin filter (7.5 μ l each); lane 10: IgM from a patient with multiple myeloma (100 ng).

Figure 5. RF ELISA of mAbs

Assessment of activities of cloned IgM towards IgG isotypes. Microwells were coated with 1 μg/well monoclonal human IgG₁λ, IgG₂λ, IgG₃λ, or IgG₄λ. After blocking, serial dilutions of IgM mAbs were added. HRP-labeled goat anti-human Igμ was used as the detection Ab. E3, E5, and H5: IgM mAbs from HCV⁺MC⁺ subject LDU 125; CBH5: RF negative control, VH1-69-encoded IgM mAb (A–D). Comparison of reactivities of LDU 125 serum towards IgG isotypes (E). Recognition of cloned IgM by G6 mAb. Microwells were coated with anti-human IgM and after blocking, 0.5–10 μg/ml LDU 125 A4 IgM or mGO were added, followed by detection with G6 mAb (1:5000) and rabbit anti-mouse IgG₁ (1:5000) (F).

Table 1

Primers used for single cell PCR. Restriction sites are shown in bold.

IgH	Sense		Antisense	
First PCR	5' L-VH1 *	ACAGTGCCCACTCCAGGTGCAG	3' C _H 1 *	GGGAAATTCCTCAGAGGAGACGA
	5' L-VH3 *	AAGGTGTCCAGGTGARGTGCAG		
	5' L-VH4/6 *	CCCAGATGGGTCTGTCCAGGTGCAG		
	5' L-VH5 *	CAAGGAGTCTGTCCGAGGTGCA		
Second PCR	5' Agel VH1/5 *	CTGCA ACCGGT GTACATTCGAGGTGCAGCTGGTGCAG	3' XhoI JH1/2	TGCGAAAGCT TCGAG MACAGTGACCAG
	5' Agel VH3 *	CTGCA ACCGGT GTACATTCGAGGTGCAGCTGGTGGAG	3' XhoI JH3	TGCGAAAGCT TCGAG MACGGTGACCAT
	5' Agel VH4 *	CTGCA ACCGGT GTACATTCGCCAGGTGCAGCTGCAGGAG	3' XhoI JH4/5	TGCGAAAGCT TCGAG ACGGTGACCAG
	5' Agel VH3-23 *	CTGCA ACCGGT GTACATTCGAGGTGCAGCTGTGGAG	3' XhoI JH6	TGCGAAAGCT TCGAG ACGGTGACCGT
	5' Agel VH4-34 *	CTGCA ACCGGT GTACATTCGCCAGGTGCAGCTACAGCAGTG		
Third PCR	5' Agel VH1-69	CTGCA ACCGGT GTACATTCGCCAGGTGCAGCTGGTGCAG	3' XhoI JH4/5	TGCGAAAGCT TCGAG ACGGTGACCAG
Igk	Sense		Antisense	
First PCR	5' L V _K 1/2 *	ATGAGGSTCCCYGCTCAGCTGCTGG	5' C _K 543 *	GTTTCTCTGCTAGTCTGCTTTTGCTCA
	5' L V _K 3 *	CTCTTCTCTCTGCTACTCTGGCTCCCAG		
	5' L V _K 4 *	ATTCTCTGTGTGCTCTGGATCTCTG		
Second PCR	5' Pan V _K *	ATGACCCAGWCTCCABYCWCCCTG	3' C _K 494 *	GTGCTGTCTTGTGCTGTCTGCTC
Third PCR	5' Agel V _K 3-20	TTGTGCTGCA ACCGGT GTACATTCAGAAAATTGTGTGACGCAGTCTCCAGGCACCTG	3' BsiWI Jk1/4 *	GCCACCGT ACG TTTGTGATYTCCACCTTGGTC
			3' BsiWI Jk2 *	GCCACCGT ACG TTTGTGATCTCCAGCTTGGTC

* primers previously reported (Tiller et al., 2008)

Table 2

Primers used for human Ig μ C and J chain PCR. Restriction sites are shown in bold.

Ig μ C	Sense		Antisense	
First PCR	5' C μ CH1-4	GAGTGCATCCGCCCCAACCTT	3' C μ CH1-4	GTAGCAGGTGCCAGCTGTGTCGGA
Second PCR	5' XhoI C μ CH1-4	TGCGAACTCGAGCGGGAGTGCATCC	3' EagI C μ CH1-4	CGGCCGCTAGTAGCAGGTGCCAGC
J Chain				
IgJ	10565	CAAGAAGATGAAAGGATT	10566	TTAGTCAGGATAGCAGGC
Signal peptide	10736	AAGCTTATGGGATGGTCATGTATC	10860	CCTTTCATCTTCTGGGAATGTACACCGTTGC
Overlap PCR	11094	AAGCTTACCATGGGATGGTCATGTATCATC	10738	CTCGAGTTAGTCAGGATAGCA

Table 3
Analysis Heavy and Light Chain Variable Regions Isolated from Singly-Sorted G6⁺ Peripheral B Cells from an HCV⁺MC⁺ Patient

Clone	V _H mutations	aa Δ	V _H	D _H	J _H	Heavy Chain CDR3 Sequence	V _K mutations	aa Δ	V _K	J _K	Light Chain CDR3 Sequence
A4	1 (0.35%)	0	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	12 (4.65%)	8	3-20*01	1*01	CQYASSRTFGQG
A6	4 (1.57%)	2	1-69*01	3-22*01	4*02	CARGPDTSGFYFYFYWGQG	6 (2.33%)	5	3-20*01	2*01	CQYGSPPYTFGQG
A7	2 (0.69%)	1	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	3 (1.16%)	2	3-20*01	2*01	CQYGSPPYTFGQG
B1	2 (0.69%)	1	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	3 (1.16%)	3	3-20*01	2*01	CQYGSPPYTFGQG
B4	2 (0.69%)	1	1-69*01	3-22*01	4*02	CARGDYDSSGYYHDAFDIWGQG	3 (1.16%)	3	3-20*01	2*01	CQYGSPPYTFGQG
B6	1 (0.39)	1	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	2 (0.87%)	2	3-20*01	2*01	CQYGSPPYTFGQG
B11	2 (0.69%)	1	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	1 (0.44%)	1	3-20*01	2*01	CQYGSPPYTFGQG
C1	1 (0.35%)	1	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	2 (0.87%)	1	3-20*01	2*01	CQYGSPPYTFGQG
D4	2 (0.69%)	1	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	2 (0.87%)	2	3-20*01	2*01	CQYGSPPYTFGQG
D6	1 (0.35%)	0	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	1 (0.44%)	1	3-20*01	2*01	CQYGSPPYTFGQG
D12	15 (5.21%)	10	1-69*01	3-22*01	4*02	CAREFNVDSSGYYYFYWGQG	2 (0.87%)	2	3-20*01	2*01	CQYGSPPYTFGQG
E3	4 (1.39%)	3	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	2 (0.78%)	2	3-20*01	2*01	CQYGSPPYTFGQG
E5	2 (0.69%)	1	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	2 (0.78%)	1	3-20*01	2*01	CQYGSPPYTFGQG
F6	1 (0.35%)	0	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	5 (1.94%)	2	3-20*01	2*01	CQYGSPPYTFGQG
F10	1 (0.35%)	0	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	2 (0.78%)	2	3-20*01	2*01	CQYGSPPYTFGQG
G4	4 (1.39%)	3	1-69*01	6-13*01	4*02	CAREGIAAANPFDYWGGQ	2 (0.78%)	2	3-20*01	2*01	CQYGSPPYTFGQG
G7	1 (0.35%)	1	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	2 (0.87%)	2	3-20*01	2*01	CQYGSPPYTFGQG
H5	0 (0.00%)	0	1-69*01	3-22*01	4*02	CARGPETSRYFYFYWGQG	2 (0.78%)	2	3-20*01	2*01	CQYGSPPYTFGQG
H9	1 (0.35%)	0	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	1 (0.44%)	1	3-20*01	2*01	CQYGSPPYTFGQG
H10	2 (0.69%)	0	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	2 (0.87%)	2	3-20*01	2*01	CQYGSPPYTFGQG
NA6	1 (0.03%)	0	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	4 (1.64%)	2	3-20*01	2*01	CQYGSPPYTFGQG
NB2	19 (6.60%)	11	1-69*01	1-01*01	4*02	CAREFNFDITGYFYFLYWGGQ	13 (4.87%)	8	3-20*01	1*01	CQYASSRTFGQG
NB5	13 (4.51%)	10	3-48*01	3-22*01	4*02	CARDLRYYYDRSGPLPPSDYWGGQ	3 (1.45%)	2	3-15*01	2*01	CQYDNWPPYTFGQG
NB10	11 (3.82%)	5	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	7 (3.08%)	6	3-20*01	2*01	CQYGSPPYTFGQG
NC9	2 (0.69%)	1	3-07*01ns1	3-22*01	3*02	CARGDYDSSGYYHDAFDIWGQG	4 (1.64%)	3	1-8*01	1*01	CQYTYTYPRTFGQG
NC11	1 (0.35%)	0	1-69*01	3-22*01	4*02	CARGPDSSGYYYFYWGQG	1 (0.44%)	1	3-20*01	2*01	CQYGSPPYTFGQG
ND2	16 (6.13%)	10	3-72*01	3-09*01	4*02	CARADILTGYSIDYWGGQ	3 (1.45%)	2	1-16*01 ns1	5*01	CQYTYTYPIITFGQG

Clone	V _H mutations	aa Δ	V _H	D _H	J _H	Heavy Chain CDR3 Sequence	V _K mutations	aa Δ	V _K	J _K	Light Chain CDR3 Sequence
ND9	7 (2.43%)	3	1-69*01	3-22*01	4*02	CARGPDSSAYYFYWGQG	7 (3.08%)	3	3-20*01	2*01	CQQYGSSPYTFGQG
ND11	17 (6.61%)	11	3-11*01	6-19*01	3*01	CARGADYSSGWTEVAFDLWGQG	1 (0.44%)	1	4-01*01	4*01	CHQYYR:TPLTFGQG
NE3	6 (3.98%)	3	3-07*01ns1	3-22*01	3*02	CARGHYDSDGGYYHDAFDSWGQ	12 (5.29%)	2	1-8*01	1*01	CQQYYTYTYPRTFGQG
NE7	11 (3.82%)	5	1-69*01	5-05*01	4*02	CVREGFPD:TAMVNPFDYWGQG	2 (0.87%)	0	3-11*01	1*01	CQQRSNWPRTFGQG
NH5	2 (0.69%)	1	3-07*01ns1	3-22*01	3*02	CARGDYDSSGYHDAFDIWGQG	13 (5.33%)	7	1-8*01	1*01	CQQYYTYTYPRTFGQG

Clones A4-H10 and NA6-NH5 were isolated from G6⁺ FCRL4⁺ and G6⁺ FCRL4⁺ B cells, respectively, sorted from subject LDU 125.
V_H= Ig heavy chain variable region; aa Δ= amino acid changes in IgVH; D_H= Ig heavy chain diversity region; J_H= Ig heavy chain joining region; CDR3= complementarity determining region 3; V_K= Ig κ variable region; aa Δ= amino acid changes in IgVκ; J_K= Ig κ joining region.