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Research paper

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 and 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.

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), 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 IgG$_1$ 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 IgG$_1$ 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 IgG$_1$ 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.

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In HCV MC, pathologic RF is typically monoclonal IgMκ of the cross-reactive WA idiotype, which is frequently encoded by VH1-69 and Vκ3-20 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 $IgM^+\kappa^+$ memory B cells that express Ig encoded by VH1-69 and Vκ3-20 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 IgVκ RT-PCR as previously described (Wardemann et al., 2003); sequencing confirmed the overwhelming majority of these cells to be $VH1\text{-}69^+/JH4^+/V\kappa3\text{-}20^+$. We next performed a third round of VH1-69/JH4-specific and Vκ3-20-specific PCR to correct for 5′ IgV mutations introduced by the unbiased first and second stage PCR primers. IgVH and IgVκ were then ligated into Igμ and Igκ 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 μg/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 10^7/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% NaN_3. 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-NHS-Biotinylation 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 1 h 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 NaN_3, 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 IgG_1 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 $VH1\text{-}69^+/V\kappa3\text{-}20^+$ clones, an additional round of PCR (94 °C 30 s, 55 °C 30 s., 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 s, 57 °C 30 s., 68 °C 90 s, 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 IgG_1 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