Monoclonal Antibodies Specific for Human IgM Fc Receptor Inhibit Ligand-binding Activity

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A panel of six different murine hybridoma clones secreting IgG monoclonal antibodies (MAbs) specific for the human IgM Fc receptor (FcμR) was generated. All MAbs specifically precipitated a major protein of ~60 kDa from membrane lysates of FcμR-bearing, but not FcμR-negative, cells as did IgM-ligands. Pre-incubation of membrane lysate of FcμR-bearing cells with these MAbs completely removed the ~60 kDa IgM-reactive protein. By using recombinant human/mouse chimeric FcμR proteins, the epitope recognized by HM7 and HM10 MAbs was mapped to the Ig-like domain of human FcμR, whereas the other MAbs recognized the stalk region. Pre-incubation of FcμR⁺ cells with the Ig-like domain-specific MAbs, but not with others, markedly inhibited subsequent IgM-ligand binding. A similar, but much weaker, inhibition was also observed when the incubation order was reversed. When FcμR⁺ cells were simultaneously incubated with both IgM-ligands and MAbs, HM7 MAb efficiently competed with IgM for FcμR binding. Unlike control Jurkat cells, FcμR-bearing cells were resistant to apoptosis induced by agonistic IgM anti-Fas MAb (CH11); however, addition of the HM7 MAb inhibited the interaction of the Fc portion of CH11 MAb with FcμR, thereby promoting apoptosis of FcμR-bearing Jurkat cells. The variable regions of the HM7 MAb were composed of Ighv14-3, Ighd1-2, and Ighj2 for the γ2b heavy chain and Igk3-4 and Igkj2 for the κ light chain. These findings suggest that HM7 MAb efficiently blocks the ligand-binding activity of FcμR.

Introduction

RECEPTORS FOR THE FC REGION OF IMMUNOGLOBULIN (Ig) molecules (FcRs) are expressed by many different cell types in the immune system, and their interaction with Ig-ligands initiates a broad spectrum of effector functions, including phagocytosis of antibody-coated microbes, antibody-dependent cell-mediated cytotoxicity, release of inflammatory mediators, and regulation of B cell responses. These diverse functions of FcRs depend upon their Ig-ligands and cellular distribution. FcRs are thus considered as central mediators of antibody-triggered responses, coupling the innate and adaptive immune responses, in effector cell activation.^(1,2) FcRs for switched Ig isotypes (i.e., FcγRs, FcεRs, and FcαR) have been extensively characterized at both protein and genetic levels, but FcμR long defied genetic identification, despite extensive biochemical evidence of IgM-binding proteins since decades ago.^(3–5) We have successfully identified a cDNA encoding an authentic FcμR from cDNA libraries of human B-lineage cells using a functional cloning strategy.⁽⁶⁾ FcμR is a transmembrane sialoglycoprotein of ~60 kDa, with a single Ig-like domain that has homology with two other IgM-binding receptors, the polymeric Ig receptor (pIgR) and FcR for IgA and IgM (Fcα/μR), but con-

fers exclusive Fcμ binding specificity. Unlike other FcRs, FcμR is selectively expressed by lymphocytes: B, T, and NK cells in humans^(6,7) and only by B cells in mice.^(8–10) *FCMR* is a single copy gene located on chromosome 1q32.2, adjacent to the above IgM-binding receptors *PIGR* and *FCAMR*. Upon IgM binding, FcμR is rapidly internalized to the lysosome compartment.⁽¹¹⁾ Enhanced levels of both membrane-bound and soluble forms of FcμR are observed in patients with chronic lymphocytic leukemia (CLL).^(12–14) Recent studies of *Fcmr* ablation in mice revealed its critical role in IgM homeostasis and humoral immune responses.^(9,10,15)

In the present study, a panel of six different MAbs against human FcμR has been generated and two MAbs, HM7 and HM10, are described in detail with emphasis on their ability to block the interaction of IgM-ligand with FcμR.

Materials and Methods

FcμR-specific MAbs

Six different hybridoma clones secreting IgG MAbs specific for human FcμR (HM2 [γ3κ]; HM3, HM6, and HM7 [γ2bκ]; HM10 and HM14 [γ1κ]) were developed by hybridization of Ag8.653 plasmacytoma line with regional

lymph node cells from BALB/c mice hyper-immunized with the BW5147 mouse thymoma line stably expressing human Fc μ R. These MABs were selected based on their restricted reactivity with Fc μ R-bearing cells but not with pIgR-expressing FT-29, Fc α / μ R⁺, or control BW5147 cells; the characterization of one of the MABs (HM14) was previously described.⁽⁶⁾ The secreted IgG MABs were purified from the culture supernatants of single cell-derived hybridoma clones that were grown in media containing IgG-depleted fetal bovine sera (FBS) by protein G-coupled affinity columns. Some aliquots of the purified IgG MABs and highly purified, human myeloma IgM protein were labeled with biotin. Protein concentration was determined by absorbance at 280 nm with an extinction coefficient of 1.4 as 1 mg/mL for both IgG and IgM.

Immunofluorescence analysis

To examine if receptor-specific MABs block the IgM-ligand binding to Fc μ R, flow cytometric analysis was performed. Briefly, a mixture of BW5147 cells stably expressing both human Fc μ R and green fluorescein protein (Fc μ R⁺/GFP⁺) and wild-type control BW5147 cells was sequentially incubated with either anti-Fc μ R MAB or IgM at 10 μ g/mL, then washed and incubated with either biotin-labeled IgM (4 μ g/mL, $\sim$ 4 nM) or anti-Fc μ R MAB (1 μ g/mL, $\sim$ 6 nM), respectively. The bound biotin-IgM or -MABs were detected by the addition of phycoerythrin-labeled streptavidin (PE-SA, Southern Biotechnology Associates, Birmingham, AL) as previously described.⁽⁶⁾ Alternatively, cells were simultaneously incubated with biotin IgM in the presence of various concentrations of anti-Fc μ R MABs, washed, and developed with PE-SA. For epitope mapping, cells stably expressing a recombinant human/mouse chimeric Fc μ R protein (see below) were incubated with receptor-specific MABs, washed, and developed with PE-labeled goat anti-mouse Ig antibody (Southern Biotechnology Associates). Stained cells were analyzed using an Accuri C6 flow cytometer and flow cytometric data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Cell surface biotinylation and immunoprecipitation analysis

Cell surface proteins were labeled with sulfo-NHS-LC-biotin (Thermo Fisher Scientific, Pierce, Rockford, IL) as previously described.⁽⁶⁾ Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of immunoprecipitated materials from lysates of biotinylated plasma membrane proteins was essentially the same as that described previously.⁽⁶⁾

Chimeric Fc μ R

The Ig-like domain of human and mouse Fc μ Rs was swapped by an overlapping polymerase chain reaction (PCR) strategy.⁽¹⁶⁾ In brief, the cDNA corresponding to the Ig-like domain of each Fc μ R was amplified by PCR using PrimeStar HS DNA polymerase (Takara, Mountain View, CA) and a set of primers: (1) O-481F (5'-gaattcTAGAAGGGACAATGGA CTTC-3') and O-469R (5'-gttctgggtattCACTGTGGACA TTCAGGGTG-3') with human Fc μ R cDNA⁽⁶⁾ as template and (2) O-479F (5'-gaattcagggaaccttgacttttgg-3') and O-471R (5'-ATGGCTCGTATTcattatggacattcagggtg-3') with mouse Fc μ R cDNA.⁽⁹⁾ The cDNA corresponding to the remaining regions of Fc μ R was amplified using a set of primers: (3) O-468F (5'-

ATGTCCACAGTGaataccagaaccattctgg-3') and O-474R (5'-gcggccgctcattggcatgaagatctg-3') with mouse Fc μ R cDNA and (4) O-480F (5'-atgtccataatgAATACGAGCCATCATGGGA AG-3') and O-482R (5'-gcggccgcTCAGGCAGGAACATT GATGTAG-3') with human Fc μ R cDNA. (The upper and lower case letters correspond to the human and mouse Fc μ R cDNA sequences, respectively; the underlines indicate the overlapping complementary sequences; and the italics indicate *Eco*RI or *Not*I sites.) Each PCR product with the expected size (402, 396, 919, and 820 bp for primer sets 1 to 4, respectively) was gel-purified. An equimolar mixture of products 1 and 3 as well as products 2 and 4 was used as overlapping template DNAs in the second PCR with a pair of primers of O-481F/O-474R and O-479F/O-482R to amplify respectively the chimeric Fc μ R consisting of the Ig-like domain of human origin and the remaining stalk/transmembrane and cytoplasmic regions of mouse origin (HMM-type) and of the reverse (MHH type). The amplified PCR products with the expected size (1297 bp for HMM and 1192 bp for MHH) were gel-purified, subcloned into the ZeroBlunt TOPO vector (Invitrogen, Carlsbad, CA), and digested with *Eco*RI and *Not*I restriction enzymes, before ligation of an *Eco*RI/*Not*I insert DNA fragment into the pMX-PIE retroviral expression vector.⁽⁶⁾ After confirming the correct sequences of the resultant HMM and MHH Fc μ R cDNAs on both DNA strands, they were transfected into the Plat-E packaging cell line before transduction into BW5147 cells as previously described.⁽⁶⁾

Apoptosis assay

The apoptosis assay was essentially the same as that described previously.⁽⁶⁾ In brief, Fc μ R⁺/GFP⁺ or GFP⁺ Jurkat cells (5×10^5 cells/mL) were cultured for 15 to 20 h in the absence or presence of the agonistic IgM anti-Fas MAB (CH11 clone, 10 ng/mL; Millipore, Billerica, MA), along with various concentrations of anti-Fc μ R MAB (HM7 or HM3). After washing, cells were stained with 7-aminoactinomycin D (7-AAD) and allophycocyanin (APC)-labeled annexin V for detecting apoptotic cells according to the manufacturer's recommendation (BD, Biosciences, San Jose, CA).

Sequencing of Ig heavy and κ light chain variable regions

Nucleotide sequence of variable regions of HM7 MAB was determined by reverse transcription PCR (RT-PCR). Briefly, total RNA was isolated from the HM7 hybridoma clone producing an Fc μ R-blocking MAB of IgG2b κ isotype and was converted to first-strand cDNA using SuperScript II reverse transcriptase (Invitrogen) and a primer O-454R (5'-ggacagggmtccakagtcc-3') or O-456R (5'-gttggtgcagcatcagc-3') corresponding with the constant region of murine γ heavy and κ light chains, respectively. The resultant first-strand cDNAs were used as template DNAs for amplification of cDNA encoding Ig heavy (VH) and light chain (Vk) variable regions with a set of primers: (1) O-453F (5'-aggtsmarctg cagsagtewgg-3', in which S = C or G, M = A or C, R = A or G, and W = A or T)⁽¹⁷⁾ and O-454R for the Ig VH; and (2) O-455F (5'-ccagatgtgtgatgaccagactcca-3')⁽¹⁸⁾ and O-456R for the Ig Vk region. The amplified products of the expected size were subcloned into the ZeroBlunt TOPO vector before sequencing analysis and then with the IMGT/V-Quest program.⁽¹⁹⁾

Results

FcμR-blocking MAbs, HM7, and HM10

A panel of six different MAbs (HM2 [$\gamma 3\kappa$ isotype], HM3, HM6 and HM7 [$\gamma 2\text{b}\kappa$], HM10 and HM14 [$\gamma 1\kappa$]) was generated from mice hyper-immunized with the mouse thymoma cell line BW5147 stably expressing human FcμR. The hybridomas were selected based on their reactivity by flow cytometric analysis with FcμR⁺ BW5147 cells and other FcμR-bearing cells (i.e., phorbol myristate acetate-activated 697 pre-B cell line and CLL cells), but not with control or

Fcα/μR⁺ BW5147 cells and pIgR-bearing FT-29 cell line. These MAbs also did not react with myeloid cells or the corresponding cell lines expressing FcγRs, FcεRI, and/or FcαR, consistent with the results with HM14 MAb.⁽⁶⁾ To determine the biochemical nature of the proteins recognized by these MAbs, SDS-PAGE analysis of biotin-labeled cell surface proteins was performed. A major protein with an M_r of ~60 kDa was specifically precipitated with all MAbs, as well as with IgM-ligands from membrane lysates of FcμR⁺ BW5147 cells but not from those of control BW5147 cells (Fig. 1A). The sequential immunoprecipitation analysis with

FIG. 1. Biochemical characterization of proteins reactive with anti-FcμR MAbs and their epitope mapping. **(A)** SDS-PAGE analysis of biotinylated cell surface proteins. FcμR⁺/GFP⁺ or control BW5147 cells were surface biotinylated, quenched, and incubated with the indicated FcμR-specific MAbs (HM14, HM10, HM2, HM7, and HM3), isotype-matched control MAbs (IgG1κ, IgG3κ, and IgG2bκ) or mouse myeloma IgMκ (TEPC183), before washing and solubilization in 1% NP-40 lysis buffer containing protease inhibitors. The MAb- or IgM-bound cell surface proteins were captured by addition of beads coupled with rat anti-mouse κ MAb (187.1 clone) and resolved on SDS-10% PAGE under reducing and non-reducing conditions (not shown), followed by transfer onto membranes, blotting with horseradish peroxidase-coupled SA, and visualization with ECL. Essentially the same results were obtained with the HM6 MAb (not shown). These experiments were performed at least three times. M_r is shown in kDa. **(B)** Epitope mapping analysis. BW5147 cells stably expressing a chimeric FcμR protein made by swapping of the Ig-like domain of human (H) or mouse (M) origin, MHH- or HMM-type FcμR, were incubated with the indicated receptor-specific MAbs (thick black lines) or isotype-matched control MAbs (thin gray lines), before developing with PE-labeled goat anti-mouse IgG antibodies. Stained cells were analyzed by flow cytometry. The acronym MHH indicates the Ig-like domain of mouse origin and the remaining stalk/transmembrane and cytoplasmic regions of human origin, and the HMM indicates the reverse. The flow cytometric analyses were performed three times.

MAbs and IgM-ligands revealed that pre-incubation of the membrane lysates of Fc μ R⁺ cells with MAbs completely removed the ~60 kDa IgM-reactive protein, whereas the reverse did not efficiently remove the ~60 kDa MAb-reactive protein. These findings confirm the Fc μ R specificity of these MAbs and suggest that these MAbs are better than IgM-ligands for the detection of Fc μ R.

To map the epitope recognized by these MAbs, we made recombinant human and mouse Fc μ R fusion proteins by swapping Ig-like domains, as those MAbs did not cross-react with mouse Fc μ R. The acronym HMM indicates the Fc μ R

consisting of the Ig-like domain of human origin and the remaining stalk/transmembrane and cytoplasmic regions of mouse origin, and MHH indicates the reverse. Both HM7 and HM10 MAbs reacted with the HMM-type Fc μ R, but not with the MHH-type Fc μ R (Fig. 1B), indicating that these MAbs recognize the Ig-like domain of human Fc μ R. By contrast, the other four MAbs exhibited an opposite reaction pattern, reactive with the MHH-type Fc μ R but not with the HMM-type Fc μ R, indicating that these MAbs recognize the stalk region of human Fc μ R.

To determine if any of these Fc μ R-specific MAbs can inhibit the interaction of Fc μ R with IgM-ligands, a mixture of Fc μ R⁺/GFP⁺ and control cells was sequentially incubated with receptor-specific MAbs and then with biotin-labeled IgM (plus PE-SA) before flow cytometric analysis. Pre-incubation with the four stalk region-specific MAbs did not affect the subsequent IgM binding to Fc μ R⁺/GFP⁺ cells, but HM7 and HM10, the two Ig-like domain-specific MAbs, could inhibit the IgM binding almost completely (Fig. 2A). Similar, but much weaker, inhibition by these two MAbs was also observed with the reverse order of incubation—that is, cells were first incubated with IgM and then with biotin-labeled MAbs (Fig. 2B). When cells were simultaneously incubated with ~4 nM biotin-IgM in the presence of the two-blocking MAbs (5–75 nM), the HM7 MAb efficiently inhibited the binding of IgM to Fc μ R⁺/GFP⁺ cells in a dose-dependent manner, but the HM10 MAb did so only at the highest concentration (Fig. 2C). These findings suggest that two of the Fc μ R-specific MAbs (HM7 and HM10) recognize an epitope near the site for IgM-ligand binding and that the HM7 MAb efficiently blocks the ligand binding activity of Fc μ R.

HM7 MAb allows Fc μ R-bearing Jurkat cells to become apoptotic with IgM anti-Fas MAb treatment

In previous studies by our group and others, apoptosis-prone Jurkat cells stably expressing Fc μ R were shown to be protected from Fas/CD95-mediated apoptosis when ligated with an agonistic IgM anti-Fas MAb (CH11 clone), but not

FIG. 2. Binding characteristics of Fc μ R-specific MAbs. Effects of pre-incubation of anti-Fc μ R MAbs (A) or IgM (B). A mixture of Fc μ R⁺/GFP⁺ and control BW5147 cells was first incubated with: (A) the indicated anti-Fc μ R MAb (10 μ g/mL) (+) or PBS (-) at 4°C for 20 min, washed, and then with biotin-IgM (2 μ g/mL) or (B) IgM (10 μ g/mL) (+) or PBS (-), washed, and then with the indicated, biotin-anti-Fc μ R MAbs (2 μ g/mL), before developing with PE-SA. Stained cells were analyzed by flow cytometry. Horizontal lines indicating the peak fluorescence in PBS-treated cells are included for comparison. (C) Competition of IgM-ligand and anti-Fc μ R MAbs in binding to Fc μ R. Similar mixture of cells was simultaneously incubated with biotin-IgM (2 μ g/mL) and various concentrations of anti-Fc μ R MAbs, washed, and then with SA-PE. The % IgM binding was determined by: $100 \times [\text{mean fluorescence intensity (MFI) of IgM binding by Fc}\mu\text{R}^+/\text{GFP}^+ \text{ cells with anti-Fc}\mu\text{R MAb}] \div [\text{MFI of IgM binding by Fc}\mu\text{R}^+/\text{GFP}^+ \text{ cells in PBS}] \div [\text{MFI of IgM binding by control cells in PBS}]$. These experiments were performed at least twice.

when ligated with an agonistic IgG anti-Fas MAb or Fas ligand.^(6,7,20,21) Since this apoptotic inhibition is a well-defined readout of the FcμR ligation, we employed this as a functional assay to determine whether the HM7 MAb, which blocks FcμR ligand binding activity, can inhibit the FcμR-mediated protection from apoptosis induced by the IgM anti-Fas MAb. As expected, the addition of the IgM anti-Fas MAb (10 pM) induced robust apoptosis of control GFP⁺ Jurkat cells, as determined by early and late apoptotic dyes Annexin V and 7-AAD, respectively (Fig. 3A). By contrast, FcμR⁺/GFP⁺ Jurkat cells were protected, consistent with the previous findings.^(6,7,20,21) HM7 MAb alone did not induce apoptosis in either of the cell types; however, its inclusion in the assay resulted in an increase in the IgM anti-Fas MAb-mediated apoptosis of FcμR⁺/GFP⁺ cells in a dose-dependent manner (Fig. 3B). This observation suggests that the HM7 MAb blocks the binding of the Fc portion of the IgM anti-Fas MAb to FcμR, thus preventing the anti-apoptotic activity of FcμR⁺ cells in the IgM Fas MAb-mediated apoptosis assay.

Addition of the non-blocking, isotype-matched HM3 anti-FcμR MAb did not affect the protection of FcμR⁺/GFP⁺ cells from the IgM anti-Fas MAb-induced apoptosis. The IgM anti-Fas MAb-mediated apoptosis of the control vector-only GFP⁺ cells was not affected by the addition of either HM7 or HM3 MAb. These findings demonstrate that the ability of the HM7 MAb to block FcμR ligand binding activity has functional consequences in that it also blocks the inhibitory activity of FcμR in IgM anti-Fas MAb-induced apoptosis of Jurkat cells.

VH and Vκ sequence of the HM7 MAb

Since the HM7 MAb might be a good candidate for immunotherapeutic purposes, we determined the nucleotide sequence of variable regions of the γ2b heavy and κ light chains by RT-PCR. HM7 was composed of Ighv14-3, Ighd1-2, and Ighj2 for the heavy chain and of Igkv3-4 and Igkj2 for the κ light chain (Fig. 4).

FIG. 3. Effect of HM7 MAb on FcμR-mediated protection of Jurkat cells from IgM anti-Fas MAb-induced apoptosis. (A) Representative immunofluorescent profiles. Control GFP⁺ or FcμR⁺/GFP⁺ Jurkat cells were cultured at 37°C for 15 h in the absence (-) or presence (+) of agonistic IgM anti-Fas MAb (CH11 clone) at 10 ng/mL, along with (+) or without (-) HM3 or HM7 MAb at 10 μg/mL. Cells were stained with 7-AAD and APC-labeled annexin V to detect early (7-AAD⁺/annexin V⁻) and late (7-AAD⁺/annexin V⁺) apoptotic cells. Profiles of background apoptosis [IgM α-Fas(-)/MAb(-)], another control [IgM α-Fas(-)/HM3(+)] and other concentrations of HM3 or HM7 MAb with IgM anti-Fas MAb were done but are not shown. (B) Induction of apoptosis in FcμR⁺/GFP⁺ Jurkat cells by addition HM7 MAb. The % apoptosis was determined by: 100 × [% annexin V⁺ cells with IgM α-Fas(+)/MAb(+) - % annexin V⁺ cells with IgM α-Fas(-)/MAb(-)] in FcμR⁺/GFP⁺ cells ÷ [% annexin V⁺ cells with IgM α-Fas(+)/MAb(-) - % annexin V⁺ cells with IgM α-Fas(-)/MAb(-) in GFP⁺ cells]. These experiments were performed twice.

HM7 mAb VH:

AGGTGCAGCTGCAGCAGCTCTGGGGCAGAGCTTGTGAAGCCAGGGGCCTCAGTCAAGTTGTCCTGCACAGCTTCTGGCTT
 ValGlnLeuGlnGlnSerGlyAlaGluLeuValLysProGlyAlaSerValLysLeuSerCysThrAlaSerGlyPh

 CAACATTAAAGACACCTATATGCACTGGGTGAAGGAGAGGTCTGAACAGGGCCTGGAGTGGATTGGAAGGATTGATCCT
 eAsnIleLysAspThrTyrMetHisTrpValLysGluArgSerGluGlnGlyLeuGluTrpIleGlyArgIleAspPro

 GCGAATGGTAATACTAAATATGACCCGAAGTTCCAGGGCAAGGCCACTATAACAGCAGACACATCCTCCAACACAGCCT
 AlaAsnGlyAsnThrLysTyrAspProLysPheGlnGlyLysAlaThrIleThrAlaAspThrSerSerAsnThrAlaT

 ACCTGCAGCTCAGCAGCCTGACATCTGAGGACACTGCCGTCTATTACTGTGCTAGACTTCATTACTACGACTACAGGGA
 yrLeuGlnLeuSerSerLeuThrSerGluAspThrAlaValTyrTyrCysAlaArgLeuHisTyrTyrAspTyrArgAs

 CTACTGGGGCCCAAGGCACCACTCTCACAGTCTCCCTCAGCCAAAACAACCCCCATCAGTCTATCCACTGGCCCTGGG
 pTyrTrpGlyGlnGlyThrThrLeuThrValSerSerAlaLysThrThrProProSerValTyrProLeuAlaProGly

 TGTGGAGATACAACTGGTTCCTCCGTGACTCTGGGATGCCTGGTCAAGGGCTACTTCCCTGAGTCAGTGACTGTGACTT
 CysGlyAspThrThrGlySerSerValThrLeuGlyCysLeuValLysGlyTyrPheProGluSerValThrValThrT

GGAAGCTCTGGATCCCTGTCC
 rpAsnSerGlySerLeuSer

HM7 mAb Vk:

CCAGATGTGTGATGACCCAGACTCCAGCTTCTTTGGCTGTGTCTCTAGGGCAGAGGGCCACCATCTCCTGCAAGGCCAG
 ValMetThrGlnThrProAlaSerLeuAlaValSerLeuGlyGlnArgAlaThrIleSerCysLysAlaSe

 CCAAAGTTTGTATGATGGTGATAGTTATATGAACGGTACCAACAGAAACCAGGACAGCCACCCAACTCCTCATC
 rGlnSerPheAspTyrAspGlyAspSerTyrMetAsnTrpTyrGlnGlnLysProGlyGlnProProLysLeuLeuIle

 TATGCTGCATCCAATCTAGAATCTGGGATCCAGCCAGGTTTAGTGGCAGTGGGTCTGGGACAGACTTCACCCCTCAACA
 TyrAlaAlaSerAsnLeuGluSerGlyIleProAlaArgPheSerGlySerGlySerGlyThrAspPheThrLeuAsnI

 TCCATCCTGTGGAGGAGGAGGATGCTGCAACCTATTACTGTGTCAGCAAAGTAATGAGGATCCGTACACGTTGGGAGGGG
 leHisProValGluGluGluAspAlaAlaThrTyrTyrCysGlnGlnSerAsnGluAspProTyrThrPheGlyGlyGly

GACCAAGCTGGAAATAAAACGGGCTGATGCTGCACCAAC
 yThrLysLeuGluIleLysArgAlaAspAlaAlaPro

FIG. 4. Nucleotide sequence of VH and Vk regions of HM7 MAb. The nucleotide sequence of VH (upper) and Vk (lower) regions of HM7 MAb and their translated amino acid sequence are indicated. *Upper*, Bold letters highlighted in gray, blue, and yellow correspond to the Ighv14-3, Ighd1-2, and Ighj2, respectively, and regular letters indicate the constant region of the γ 2b heavy chain. The underlines indicate the sites corresponding to primers O-453F and O-454R. *Lower*, Bold letters highlighted in gray and yellow correspond to the Igkv3-4 and Igkj2, respectively, and regular letters indicate the constant region of the κ light chain. The underlines indicate the sites corresponding to primers O-455F and O-456R. The sequencing analysis was conducted with three independent clones. The sequences have been deposited in GenBank under the accession nos. KJ865881 for the VH and KJ865882 for the Vk of HM7 MAb.

Discussion

The present study has focused on the characterization of a panel of six different IgG MAbs reactive with the surface component of human Fc μ R-bearing cells. All MAbs specifically immunoprecipitated a major protein of ~60 kDa from membrane lysates of Fc μ R-bearing cells, but not Fc μ R-negative cells, as did IgM-ligands. Pre-incubation of membrane lysates of Fc μ R⁺ cells with MAbs completely removed the ~60 kDa IgM-reactive protein. By using recombinant human/mouse chimeric Fc μ R proteins, the epitope recog-

nized by two MAbs, HM7 (γ 2b κ) and HM10 (γ 1 κ), was mapped to the Ig-like domain, whereas the other MAbs recognized the stalk region of human Fc μ R. Pre-incubation of Fc μ R⁺ cells with Ig-like domain-specific MAbs, but not with the others, markedly inhibited subsequent IgM-ligand binding. A similar, but much weaker, inhibition was also observed in the reverse order of incubation. When cells were simultaneously incubated with both IgM ligands and MAbs, the HM7 MAb efficiently competed the binding of IgM to Fc μ R-bearing cells. Although Fc μ R-bearing Jurkat cells, unlike control cells, were resistant to apoptosis induced by the

agonistic IgM anti-Fas MAb (CH11), the addition of HM7 MAb inhibited the interaction of the Fc portion of IgM anti-Fas MAb to Fc μ R, thereby allowing the IgM Fas MAb-mediated apoptosis of Fc μ R⁺ cells to occur. The variable regions of the HM7 MAb were defined by cDNA sequencing as VH 14-3, DH 1-2, and JH 2 for the γ 2b heavy chain and as V κ 3-4 and J κ 2 for the κ light chain. HM7 is thus an efficient blocking MAb for Fc μ R.

Fc μ R is the newest member of the FcR family and its cellular distribution is restricted to lymphocytes: B, T, and NK cells in humans^(6,7) and B cells only in mice^(8–10); hence it is quite distinct from the cellular distribution of FcRs for switched Ig isotypes (i.e., Fc γ Rs, Fc ϵ Rs, Fc α R). The precise function of Fc μ R remains elusive but the recent studies of *Fc μ R*-deficient mice have suggested that Fc μ R plays a regulatory role in IgM homeostasis and humoral immune responses.^(9,10,15) The ablation of *Fc μ R* leads to increase in pre-immune serum IgM as well as natural autoantibodies of both IgM and IgG isotypes and to dysregulated antibody responses, especially to suboptimal doses of T cell-independent type 2 antigens. Notably, many abnormalities in *Fc μ R*-deficient mice mirror those observed in mutant mice deficient in IgM secretion, which are able to express surface IgM and other Ig isotypes on B cells and to secrete all other classes of Igs except IgM, suggesting the critical role in normal B cell functions both for secreted IgM and for its interaction with Fc μ R.⁽²²⁾ Although *Fc μ R* deficiency has not yet been identified in humans, it seems likely that the phenotype will be much more complex and profound than the *Fc μ R* deficiency in mice, because human Fc μ R is expressed by additional cell types, namely T and NK cells.

Enhanced levels of both the ~60 kDa membrane-bound Fc μ R and the ~40 kDa soluble Fc μ R were shown in patients with CLL compared with healthy donors.⁽¹²⁾ Specific delivery of toxin-conjugated IgM Fc to the Fc μ R is now being explored for potential treatment for CLL patients.^(11,23) HM7 and/or other MAbs may be another alternative for this purpose. Intriguingly, it has recently been shown that administration of a recombinant fusion protein consisting of human Fc μ R ectodomain and IgG Fc as a soluble Fc μ R ameliorates the myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis in mice, although the mechanism underlying this effect is unclear.⁽²⁴⁾ In this regard, various forms of immune-mediated peripheral neuropathy are known to be associated with monoclonal IgM antibodies against myelin-associated glycoprotein, gangliosides, or related glycolipids.^(25–27) While it is a generally accepted concept that IgM autoantibodies are usually protective but IgG autoantibodies are pathologic in autoimmune disorders, these demyelinating neuropathies are a clear example of disease association with IgM autoantibodies. Another intriguing observation is that patients with Alzheimer's disease have significantly elevated numbers of T cells with autochthonously bound IgM,⁽²⁸⁾ but whether this cytophilic IgM is bound to T cells via the Fc or Fab region remains to be determined.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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