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Epitope Mapping of an Anti-CD20 Monoclonal Antibody (C_{20}Mab-60) Using Enzyme-Linked Immunosorbent Assay

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CD20 is a glycosylated transmembrane protein and is expressed on normal B cells and B cell malignancies. Therapeutic antibodies against CD20 are developed and used in clinic. The understanding of antibody–antigen binding by revealing the epitope is essential for future application to antibody technology. Previously, we developed an anti-human CD20 monoclonal antibody, C_{20}Mab-60 (IgG_{2a}, kappa), using the Cell-Based Immunization and Screening (CBIS). C_{20}Mab-60 can be used for flow cytometry, Western blot, and immunohistochemical analyses. In this study, we examined the critical epitope of C_{20}Mab-60 using enzyme-linked immunosorbent assay (ELISA) with synthesized peptides. We performed ELISA with deletion mutants, and C_{20}Mab-60 reacted to the 160–179 amino acids sequence of CD20. Next, we analyzed the reaction to 20 point mutants, and C_{20}Mab-60 did not recognize the alanine-substituted peptides of N171A, P172A, S173A, and E174A. The results indicate that the binding epitope of C_{20}Mab-60, developed by CBIS, includes Asn171, Pro172, Ser173, and Glu174 of CD20.

Keywords: CD20, C_{20}Mab-60, epitope mapping, monoclonal antibody, enzyme-linked immunosorbent assay

Introduction

CD20 is a glycosylated transmembrane protein and comprises four hydrophobic membrane domains, two extracellular loops (~7 and 44 amino acids), and three intracellular regions including N- and C-terminus.[1] The intracellular region of CD20 is phosphorylated upon antibody binding, thereby mediating cellular signaling.[2] CD20 is expressed on normal B cells from pre-B to mature B cell development, but not detected in early pro-B cells and terminally differentiated plasma cells.[3] CD20 is also detected in human B cell malignancies including non-Hodgkin lymphoma and B-lymphoblastic leukemia/lymphoma.[4] Since CD20 is not expressed in early pro-B cells, anti-CD20 therapy is thought to be effective to these malignancies without affecting early lineage of normal B cells.

CD20 was first defined by the murine monoclonal antibody (mAb) tositumomab.[5] Rituximab, a chimeric anti-CD20 mAb, was developed and approved for treatment of the B cell malignancies.[6–9] Rituximab destroys both CD20 expressed normal B cell and malignancies through complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC).[10] Rituximab is also approved for autoimmune diseases including rheumatoid arthritis.[11] Although rituximab has dramatically improved outcomes for patients with CD20-positive malignancies and autoimmune diseases for two decades, the responses are not universal and resistance can develop.[10]

Through recombinant DNA technology, second and third generation anti-CD20 mAbs were developed.[12] Among these, ofatumumab and obinutuzumab have been approved for clinical treatment of B cell malignancies, such as chronic lymphoid leukemia and rituximab refractory follicular lymphoma.[13,14] Obinutuzumab was developed to potentiate the CDC activity and overcome resistance to rituximab.[15] Therefore, the development of anti-CD20 antibody and the understanding of its molecular actions are thought to be important.

In our previous study, we established an anti-human CD20 mAb (C_{20}Mab-60) by using a Cell-Based Immunization and Screening method. C_{20}Mab-60 is useful not only for flow cytometry, but also for Western blot and

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Table 1. Identification of C_{20}Mab-60 Epitope Using Synthesized Peptides (Deletion Mutant) by Enzyme-Linked Immunosorbent Assay

Peptides	*Sequence*	*C_{20}Mab-60*
71_90	MIPAGIYAPISVTVWYPLWG	–
140_159	NIKISHFLKMESLNFIRAHT	–
150_169	ESLNFIRAHTPYINIYNSEP	–
160_179	PYINIYNSEPANPSEKNSPS	+++
170_189	ANPSEKNSPSTQYSYSIQSL	–
180_190	TQYSYSIQSL	–

+++, OD655 ≧ 0.5; ++, 0.3 ≦ OD655 < 0.5; +, 0.1 ≦ OD655 < 0.3; –, OD655 < 0.1.

immunohistochemical analyses.[16] To clarify further characteristics of C_{20}Mab-60, we performed epitope mapping using enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

CD20 peptides

CD20 peptides (accession No.: NM_152866), including 6 deletion mutants (Table 1) and 20 point mutants (Table 2), were synthesized by utilizing PEPScreen (Sigma-Aldrich Corp., St. Louis, MO). Three cysteine residues were replaced by serine: C81S, C167S, and C183S.

Enzyme-linked immunosorbent assay

Synthesized CD20 peptides (Tables 1 and 2) were immobilized on Nunc Maxisorp 96-well immunoplates (Thermo Fisher Scientific, Inc., Waltham, MA) at a concentration of 10 μg/mL for 30 minutes at 37°C. After washing with phosphate-buffered saline containing 0.05% Tween20 (PBST; Nacalai Tesque, Inc., Kyoto, Japan), wells were blocked with 1% bovine serum albumin-containing PBST for 30 minutes at 37°C. The plates were incubated with C_{20}Mab-60 (1 μg/mL), followed by a peroxidase-conjugated anti-mouse immunoglobulins (1:2000 diluted; Agilent Technologies, Inc., Santa Clara, CA). Enzymatic reactions were performed using the ELISA POD Substrate TMB Kit (Nacalai Tesque, Inc.). Optical density was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

Table 2. Identification of C_{20}Mab-60 Epitope Using Synthesized Peptides by Enzyme-Linked Immunosorbent Assay

Peptides	*Sequence*	*C_{20}Mab-60*
P160A	AYINIYNSEPANPSEKNSPS	+++
Y161A	PAINIYNSEPANPSEKNSPS	+++
I162A	PYANIYNSEPANPSEKNSPS	+++
N163A	PYIAIYNSEPANPSEKNSPS	+++
I164A	PYINAYNSEPANPSEKNSPS	+++
Y165A	PYINIANSEPANPSEKNSPS	+++
N166A	PYINIYASEPANPSEKNSPS	+++
S167A	PYINIYNAEPANPSEKNSPS	+++
E168A	PYINIYNSAPANPSEKNSPS	+++
P169A	PYINIYNSEAANPSEKNSPS	+++
A170G	PYINIYNSEPGNPSEKNSPS	++
N171A	PYINIYNSEPAAPSEKNSPS	–
P172A	PYINIYNSEPANASEKNSPS	–
S173A	PYINIYNSEPANPAEKNSPS	–
E174A	PYINIYNSEPANPSAKNSPS	–
K175A	PYINIYNSEPANPSEANSPS	+++
N176A	PYINIYNSEPANPSEKASPS	+++
S177A	PYINIYNSEPANPSEKNAPS	+++
P178A	PYINIYNSEPANPSEKNSAS	+++
S179A	PYINIYNSEPANPSEKNSPA	+++

+++, OD655 ≧ 0.5; ++, 0.3 ≦ OD655 < 0.5; +, 0.1 ≦ OD655 < 0.3; –, OD655 < 0.1.

Results

Epitope mapping of C_{20}Mab-60 using deletion mutants

To determine the binding epitope of C_{20}Mab-60, we synthesized 6 peptides comprising 71–90 amino acids (aa), 140–159 aa, 150–169 aa, 160–179 aa, 170–189 aa, and 180–189 aa of the CD20 protein sequence (Table 1). The results demonstrated that C_{20}Mab-60 reacted with the 160–179 aa sequence ($_{160}$-PYINIYNSEPANPSEKNSPS-$_{179}$) of CD20 (Fig. 1A). We summarized the results in Figure 1B.

Epitope mapping of C_{20}Mab-60 using point mutants

Then, we synthesized 20 alanine-substituted CD20 peptides of 160–179 aa (Table 2). C_{20}Mab-60 showed reaction

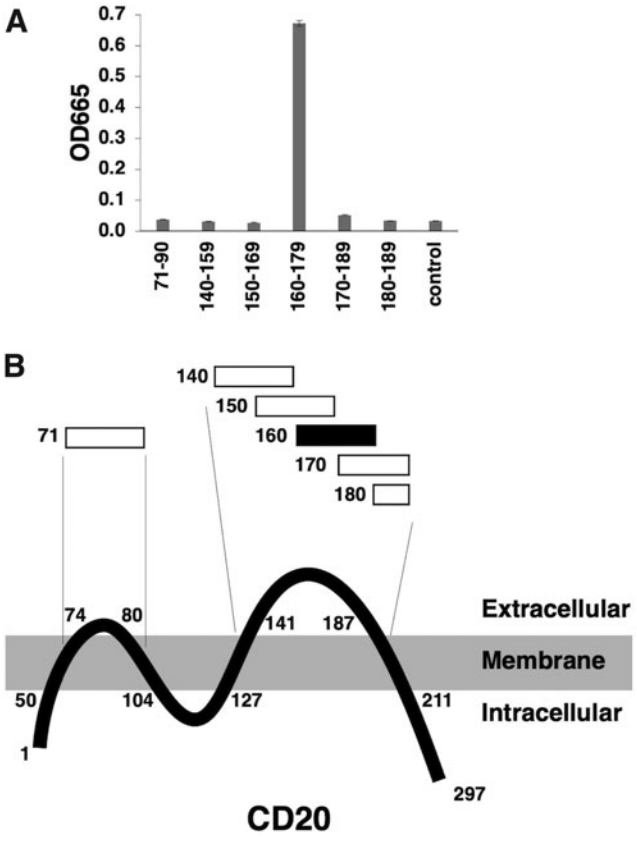

FIG. 1. Determination of the C_{20}Mab-60 epitope for CD20 by ELISA using deletion mutants. **(A)** Synthesized peptides of CD20 were immobilized on immunoplates. The plates were incubated with C_{20}Mab-60 (1 μg/mL), followed by incubation with peroxidase-conjugated anti-mouse immunoglobulins. **(B)** Schematic illustration of CD20 and the C_{20}Mab-60 epitope. Black bar indicates the peptide recognized by C_{20}Mab-60. ELISA, enzyme-linked immunosorbent assay.

with P160A, Y161A, I162A, N163A, I164A, Y165A, N166A, S167A, E168A, P169A, A170G, K175A, N176A, S177A, P178A, S179A, and wild-type 160–179 aa (Fig. 2A). In contrast, C_{20}Mab-60 exhibited no reaction with N171A, P172A, S173A, and E174A (Fig. 2A), indicating that Asn171, Pro172, Ser173, and Glu174 are included in the binding epitope of C_{20}Mab-60. We summarized the results in Figure 2B.

Discussion

In this study, we investigated the binding epitope of C_{20}Mab-60 using synthesized peptides and determined $_{171}$-NPSE-$_{174}$ as an epitope (Fig. 2B). Peptide scanning is a simple and useful technique for determining a linear epitope. However, peptide structure is unfolded and different from native proteins. X-ray crystallography is the most convincing strategy to determine the epitope, however, this method requires difficult techniques for crystallization of antibody–antigen complex and costs a lot.[17]

In a previous study, we developed two novel epitope mapping methods, such as REMAP method to insert RIEDL tag[18–22] and HisMAP method to insert five His tags[23] into targeted proteins. These methods are useful for determining both linear and conformational epitopes. We have investigated the epitope of C_{20}Mab-60 using HisMAP method, and the epitope is identical to this study.[23] Therefore, this strategy is useful to determine the critical epitope. Further studies are needed to confirm whether the strategy is applicable to other antibodies.

Rituximab recognizes a separated epitope of $_{170}$-ANPS-$_{173}$ and $_{182}$-YCYSI-$_{186}$ within the extracellular domain of CD20.[24] Especially, $_{170}$-ANPSEKN-$_{176}$ is recognized by several anti-CD20 antibodies including ocrelizumab, obinutuzumab, tositumomab, and C_{20}Mab-60. In contrast, ofatumumab recognizes a discontinuous epitope of $_{72}$-IPAGIYAPI-$_{80}$ and $_{148}$-KMESLNFIRAHT-$_{159}$. Furthermore, based on their mechanisms, anti-CD20 mAbs are classified into Type I (CDC and ADCC) and Type II (programmed cell death [PCD] and ADCC) mAbs.[12]

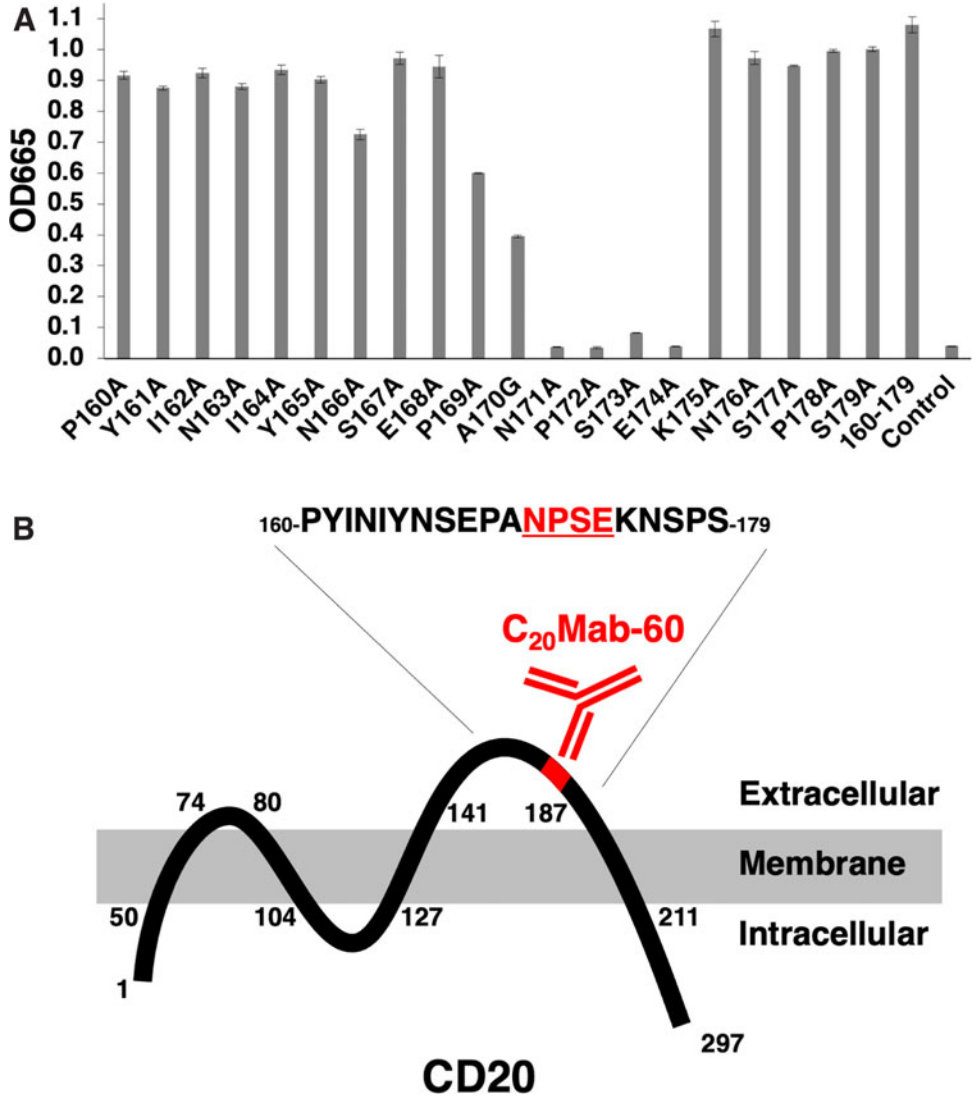

FIG. 2. Determination of the C_{20}Mab-60 epitope for CD20 by ELISA using point mutants. **(A)** Synthesized peptides of CD20 were immobilized on immunoplates. The plates were incubated with C_{20}Mab-60 (1 μg/mL), followed by peroxidase-conjugated anti-mouse immunoglobulins. **(B)** Schematic illustration of CD20 and the C_{20}Mab-60 epitope. The C_{20}Mab-60 epitope of CD20 involves Asn171, Pro172, Ser173, and Glu174.

Type I mAbs, including rituximab and ofatumumab, relocate CD20 into lipid rafts and efficiently activate the complement system.[25] Both Types I and II mAbs possess ADCC. Type II mAbs, including obinutuzumab and tositumomab, induce PCD through a caspase-independent pathway.[26] Therefore, elucidation of the C_{20}Mab-60 action to target is also thought to be important. Furthermore, identification of the complementarity-determining regions of C_{20}Mab-60 is essential to understand the recognition to CD20 and future applications including chimeric antigen receptor T cells technology.

Author Disclosure Statement

No competing financial interests exist.

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