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Development of Novel Mouse Monoclonal Antibodies Against Human CD19

Shinji Yamada,[1] Mika K. Kaneko,[1] Yusuke Sayama,[1] Teizo Asano,[1] Masato Sano,[1] Miyuki Yanaka,[1] Takuro Nakamura,[1] Saki Okamoto,[1] Saori Handa,[1] Yu Komatsu,[1] Yoshimi Nakamura,[1] Yoshikazu Furusawa,[1] Junko Takei,[1] and Yukinari Kato[1,2]

CD19 is a type I transmembrane glycoprotein belonging to the immunoglobulin superfamily. It is expressed in normal and neoplastic B cells, and it modulates the threshold of B cell activation for amplifying B cell receptor signaling. Blinatumomab (a CD3–CD19-bispecific T cell-engaging antibody) and tisagenlecleucel (genetically modified T cells that express a CD19 chimeric antigen receptor [CART-19]) provide significant benefits for patients with CD19-positive relapsed or refractory B cell malignancies. In this study, we first employed the Cell-Based Immunization and Screening (CBIS) method to produce anti-CD19 monoclonal antibodies using CD19-overexpressing cells for both immunization and screening. One established clone—C_{19}Mab-1—proved to be useful in flow cytometry assays against lymphoma cell lines, such as BALL-1, P30/OHK, and Raji. Second, the extracellular domain of CD19 was immunized into mice, and enzyme-linked immunosorbent assays were performed for the first screening. One established clone—C_{19}Mab-3—was determined to be useful for Western blotting and immunohistochemical analysis. Due to their complementary utility, a combination of C_{19}Mab-1 (established using CBIS) and C_{19}Mab-3 (established using conventional method) could be useful for the pathological analysis of CD19.

Keywords: CD19, hybridoma production, monoclonal antibody

Introduction

CD19 (B-LYMPHOCYTE SURFACE ANTIGEN B4), a type I transmembrane glycoprotein of 95 kDa, belongs to the immunoglobulin (Ig) superfamily and comprises two extracellular C2-set Ig-like domains, a transmembrane domain, and a cytoplasmic tail.[1] CD19 is expressed in normal and neoplastic B cells as well as in follicular dendritic cells; it functions coordinately with several cell surface receptors, such as complement receptor CD21, tetraspanin membrane protein CD81, and CD225.[2] These receptors form a cell surface complex with the B cell receptor (BCR) that modulates the threshold of B cell activation for amplifying BCR signaling.[3,4] A previous report has clarified that B cells from CD19-KO mice are hyporesponsive to BCR stimulation *in vitro* and show relatively modest immune responses *in vivo*.[5] In addition, another study has revealed that deletion of the cytoplasmic domain of CD19 in transgenic mice resulted in abnormal B cell development, modest mitogen responses, minimal serum Ig levels, and attenuated humoral immunity, indicating that specific signals through the cytoplasmic domain of CD19 are prerequisites for B lymphocyte function.[6]

CD19 is a biomarker for lymphoma and an immunotherapy target for B cell lymphoproliferative diseases because of its restricted expression on most stages of B cells (except for plasma cells).[7] Blinatumomab (Blincyto) and tisagenlecleucel (Kymriah) represent recent breakthroughs in CD19-targeted therapy. Blinatumomab is a CD3–CD19-bispecific T cell-engaging antibody; it comprises two single-chain variable fragments (scFv) and binds to both CD19 on B cells and CD3 on T cells.[8] Blinatumomab is approved for treating relapsed and refractory pediatric B cell acute lymphoblastic leukemia (B-ALL). Similarly, considerable effects have been achieved by administration of tisagenlecleucel, such as genetically modified T cells that express a CD19-specific antigen receptor (CART-19). Tisagenlecleucel is approved for the treatment of children and young adults with B-ALL that is refractory or at a second or later relapse.[9] Treatment with the anti-CD19 antibody–drug conjugate coltuximab ravtansine (SAR3419) resulted in moderate clinical responses in pretreated patients with relapsed/refractory diffuse large B cell lymphoma.[10] Fc-engineered anti-CD19 monoclonal antibodies that enhance the killing function of effector cells have also shown potential as candidate therapies.[11,12]

Recently, we developed a Cell-Based Immunization and Screening (CBIS) method to produce useful monoclonal antibodies (mAbs) against several membrane proteins.[13–16] In this study, we generated two novel anti-CD19 mAbs—C_{19}Mab-1

[1]Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Japan.
[2]New Industry Creation Hatchery Center, Tohoku University, Sendai, Japan.

and C_{19}Mab-3—using CBIS and a conventional method (a soluble protein-based method), respectively. We further compared their utility in flow cytometry, Western blotting, and immunohistochemical analyses.

Materials and Methods

Cell lines

CHO-K1, LN229, and P3U1 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Lymphoma-derived cell lines P30/OHK, BALL-1, and Raji were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer at Tohoku University (Miyagi, Japan). CD19-overexpressed CHO-K1 cells (CHO/CD19) and LN229 cells (LN229/CD19) were produced by transfecting pCAG/CD19-RAP-MAP into CHO-K1 and LN229 cells using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Inc., Berkeley, CA) and a Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA), respectively. CD19 extracellular domain-producing LN229 cells (LN229/CD19ec) were prepared by transfecting pCAG/PA-CD19ec-RAP-MAP into LN229 cells using the Neon transfection system. The PA tag system (PA12: GVAMPGAEDDVV vs. clone: NZ-1), RAP tag system (RAP12: DMVNPGLEDRIE vs. clone: PMab-2), and MAP tag system (MAP12: GDGMV PPGIEDK vs. clone: PMab-1) were previously established at our laboratory.[17–19] The stable transfectants of CHO/CD19 were established using a cell sorter (SH800; Sony Corp., Tokyo, Japan). The stable cell lines of LN229/CD19 and LN229/CD19ec were established by limiting dilution.

LN229, LN229/CD19, and LN229/CD19ec were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque, Inc., Kyoto, Japan), and CHO-K1, CHO/CD19, P3U1, P30/OHK, BALL-1, and Raji cells were cultured in RPMI 1640 medium (Nacalai Tesque, Inc.) at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin B (Nacalai Tesque, Inc.).

Animals and human tissues

Female BALB/c mice (6 weeks old) were purchased from CLEA Japan (Tokyo, Japan) and kept under specific pathogen-free conditions. The Animal Care and Use Committee of Tohoku University approved all animal experiments described in this study. We used tonsil tissues from a patient with oropharyngeal squamous cell carcinoma who had undergone surgery at the Sendai Medical Center. Informed consent for sample procurement and subsequent data analyses was obtained from the patient or the patient's guardian.

Production of mouse monoclonal antibodies against human CD19

Hybridomas were produced as follows. LN229/CD19 cells (1×10^8 cells/dose), or soluble CD19 (100 μg/dose), were intraperitoneally administered into the BALB/c mice. Imject Alum (Thermo Fisher Scientific, Inc.) was mixed with an immunogen at initial immunization as an adjuvant. After three booster immunizations at 1-week intervals, the final injection was intraperitoneally administered 2 days before harvesting the spleen cells. These spleen cells were then fused with P3U1 cells using PEG1500 (Roche Diagnostics, Indianapolis, IN). The resulting hybridomas were grown in RPMI medium supplemented with 10% FBS, hypoxanthine, aminopterin, thymidine selection medium supplement (Thermo Fisher Scientific, Inc.), and 5% BriClone hybridoma cloning medium (QED Bioscience, Inc., San Diego, CA). Subsequently, 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin B (Nacalai Tesque, Inc.) were added to the medium. Hybridomas were selected on the basis of the reactivity of culture supernatants against cell-bound or soluble CD19 by flow cytometry or enzyme-linked immunosorbent assay (ELISA), respectively. mAbs were purified from the supernatants of hybridomas and cultured in hybridoma-SFM medium (Thermo Fisher Scientific, Inc.) using protein G Sepharose 4 Fast Flow (GE Healthcare UK Ltd., Buckinghamshire, England).

Flow cytometry

Cells were harvested by briefly exposing them to 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc.). After washing with 0.1% bovine serum albumin/phosphate-buffered saline (PBS), the cells were treated with the hybridoma supernatant or 10 μg/mL of purified antibodies for 30 minutes at 4°C and subsequently with Alexa Fluor 488-conjugated anti-mouse immunoglobulin G (IgG, 1:2000; Cell Signaling Technology, Inc., Danvers, MA). Fluorescence data were collected using SA3800 Cell Analyzers (Sony Corp.).

Enzyme-linked immunosorbent assay

Soluble CD19 protein, purified from the supernatant of LN229/CD19ec, was immobilized on Nunc MaxiSorp 96-well immunoplates (Thermo Fisher Scientific, Inc.) at 1 μg/mL for 30 minutes at 37°C. After blocking with the SuperBlock T20 (PBS) blocking buffer (Thermo Fisher Scientific, Inc.), the plates were incubated with the hybridoma culture supernatant, followed by a 1:2000 dilution of peroxidase-conjugated anti-mouse IgG (Agilent Technologies, Inc., Santa Clara, CA). The enzymatic reaction was conducted using 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific, Inc.). Optical density (OD) was measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc., Berkeley, CA).

Western blotting

Cell lysates (10 μg) were boiled in sodium dodecyl sulfate sample buffer (Nacalai Tesque, Inc.), and the proteins were electrophoresed on 5%–20% polyacrylamide gels (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and transferred onto polyvinylidene fluoride membranes (Merck KGaA, Darmstadt, Germany). After blocking with 4% skim milk (Nacalai Tesque, Inc.), the membranes were incubated with 10 μg/mL anti-CD19 (clone C_{19}Mab-1 and C_{19}Mab-3) or 1 μg/mL anti-RAP tag (clone PMab-2). Anti-β-actin mAb (clone AC-15; Sigma-Aldrich Corp., St. Louis, MO) was used with 1 μg/mL as an internal control. The membranes were then reacted with peroxidase-conjugated anti-mouse IgG (Agilent Technologies Inc.; 1:1000 dilution) and developed using ImmunoStar LD (FUJIFILM Wako Pure Chemical Corporation) using a Sayaca-Imager (DRC Co. Ltd., Tokyo, Japan).

Immunohistochemistry

Cell blocks were produced using iPGell (Genostaff Co., Ltd., Tokyo, Japan) and processed into 4-μm paraffin-embedded cell

sections, which were directly autoclaved in citrate buffer (pH 6.0; Nichirei Biosciences, Inc., Tokyo, Japan) for 20 minutes. Tissue sections of 4-μm thickness were directly autoclaved in EnVision FLEX Target Retrieval Solution, High pH (Agilent Technologies, Inc.), for 20 minutes. These sections were blocked using the SuperBlock T20 (PBS) blocking buffer (Thermo Fisher Scientific, Inc.) and incubated with 1 μg/mL (cell sections) or 10 μg/mL (tissue sections) of anti-CD19 mAbs (clone C_{19}Mab-1 and C_{19}Mab-3) for 1 hour at room temperature. Peroxidase-conjugated anti-mouse IgG was then applied using an Envision+ Kit (Agilent Technologies, Inc.) for 30 minutes. Color was developed using 3, 3′-diaminobenzidine tetrahydrochloride (Agilent Technologies, Inc.) for 2 minutes and counterstaining was performed using hematoxylin (FUJIFILM Wako Pure Chemical Corporation).

Determination of binding affinity

CHO/CD19 and P30/OHK were suspended in 100 μL of serially diluted anti-CD19, followed by addition of Alexa Fluor 488-conjugated anti-mouse IgG (1:200; Cell Signaling Technology, Inc.). Fluorescence data were collected using the EC800 Cell Analyzer (Sony Corp.). The dissociation constant (K_D) was obtained by fitting the binding isotherms to built-in, one-site binding models in GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA).

Results

Production of anti-CD19 mAbs using CBIS

In the first trial, we immunized two mice with LN229/CD19 cells and performed flow cytometry. Hybridoma supernatants of 1056 wells were screened against CHO/CD19 cells and CHO-K1 cells, and three wells (3/1056; 0.3%) that were positive against CHO/CD19 cells and negative against CHO-K1 cells were selected. Although we performed Western blotting and immunohistochemical analyses using CHO/CD19 cells and CHO-K1 cells, none of these three wells produced specific signals against CD19. After performing limiting dilution of the three wells that showed specific signals in flow cytometric analyses, one clone—C_{19}Mab-1 (IgG_1, kappa)—was established (Supplementary Table S1).

Production of anti-CD19 mAbs using a soluble protein-based method

In the next trial, we immunized two mice with soluble CD19 (LN229/CD19ec) and performed ELISA. The culture supernatants of 958 wells were screened against LN229/CD19ec, and 18 wells (18/958; 1.9%) that showed a positive reaction (OD >0.1) were selected. We then performed immunohistochemical analyses using CHO/CD19 cells and CHO-K1 cells. Of these 18 wells, 7 (38.9%) were positive against CHO/CD19 cells and negative against CHO-K1 cells. We further performed flow cytometry using CHO/CD19 cells and CHO-K1 cells and found that two wells were positive against CHO/CD19 cells and negative against CHO-K1 cells. After performing limiting dilution, one clone—C_{19}Mab-3 (IgG_1, kappa)—was established (Supplementary Table S1).

Flow cytometric analysis against lymphoma cell lines

C_{19}Mab-1 reacted with CHO/CD19 cells, but did not react with CHO-K1 cells, suggesting that C_{19}Mab-1 is specific

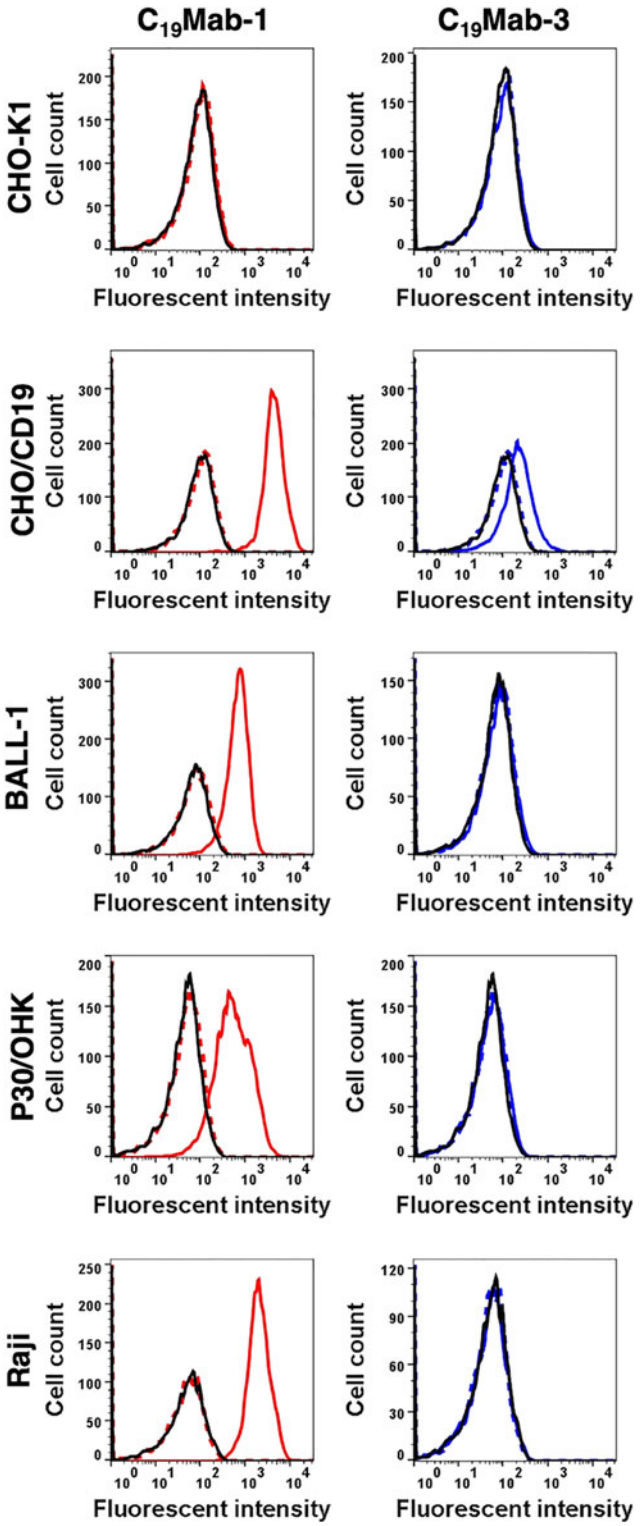

FIG. 1. Flow cytometric analysis of C_{19}Mabs. Cells were treated with 10 µg/mL C_{19}Mab-1 (red line) or C_{19}Mab-3 (blue line), followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. Black line, fluorescence-activated cell sorting buffer; dashed line, mouse IgG_1 isotype control (10 µg/mL). IgG, immunoglobulin G.

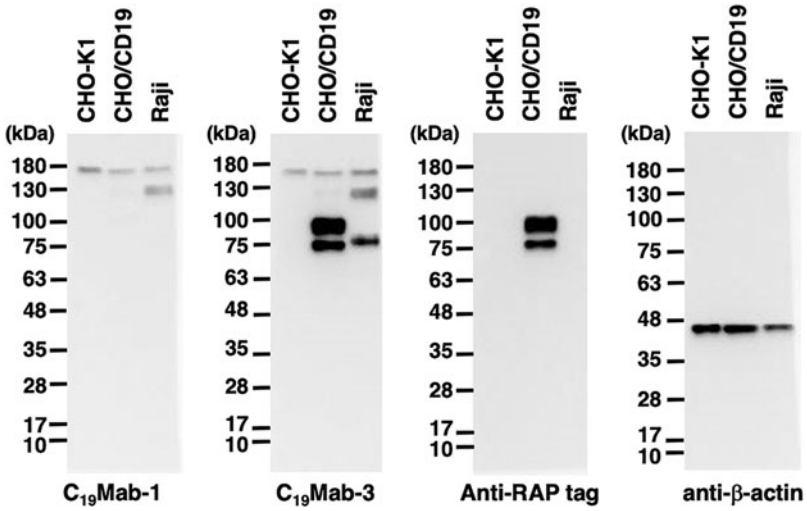

FIG. 2. Western blotting analysis using C_{19}Mabs. Cell lysates (10 μg) were electrophoresed under reducing conditions on 5%–20% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. After blocking, the membrane was incubated with C_{19}Mab-1 (10 μg/mL), C_{19}Mab-3 (10 μg/mL), anti-RAP tag (1 μg/mL), and anti-actin (1 μg/mL) and then with peroxidase-conjugated secondary antibodies.

for CD19 (Fig. 1). C_{19}Mab-1 also detected endogenous CD19 on human lymphoma cell lines BALL-1, P30/OHK, and Raji, indicating that C_{19}Mab-1 is useful for detecting endogenous CD19 by flow cytometry. Although C_{19}Mab-3 reacted with CHO/CD19 cells weakly, it could not detect the endogenous CD19 of human lymphoma cell lines in flow cytometry (Fig. 1).

Subsequently, we performed a kinetic analysis of the interaction of C_{19}Mab-1 with CHO/CD19 or P30/OHK cells using flow cytometry. The dissociation constants K_D of C_{19}Mab-1 were determined to be 4.0×10^{-9} M against CHO/CD19 and 1.5×10^{-8} M against P30/OHK, indicating that C_{19}Mab-1 possesses moderate affinity for these cells (Supplementary Fig. S1).

Western blotting analyses

C_{19}Mab-3 detected bands of ~75 and 100 kDa in lysates of CHO/CD19 cells, although C_{19}Mab-1 did not detect any specific signal at the expected molecular size (Fig. 2). The difference between the 75- and 100-kDa bands might be because of post-translational modification(s). In Raji cells, signals between 75 and 100 kDa were detected using C_{19}Mab-3, indicating that C_{19}Mab-3 specifically detects endogenous CD19 in Western blotting.

Immunohistochemical analyses

Immunohistochemical analyses revealed that C_{19}Mab-1 did not react with CHO-K1 (Fig. 3A) or CHO/CD19 cells (Fig. 3B). Conversely, C_{19}Mab-3 stained CHO/CD19 cells strongly (Fig. 3D), whereas it did not react with CHO-K1 cells (Fig. 3C), indicating that C_{19}Mab-3 is specific for CD19. No signal was observed for the negative control (Fig. 3E, F).

We further performed immunohistochemical analysis in human tonsil tissues using C_{19}Mab-1 and C_{19}Mab-3. As shown in Figure 4A and B, C_{19}Mab-1 did not stain the lymphocytes in the tonsil tissue, whereas C_{19}Mab-3 stained the lymphocytes in the cortex and lymphoid follicles and in the region of the paracortex (Fig. 4C, D). No signal was observed for the negative control in a serial section (Fig. 4E, F).

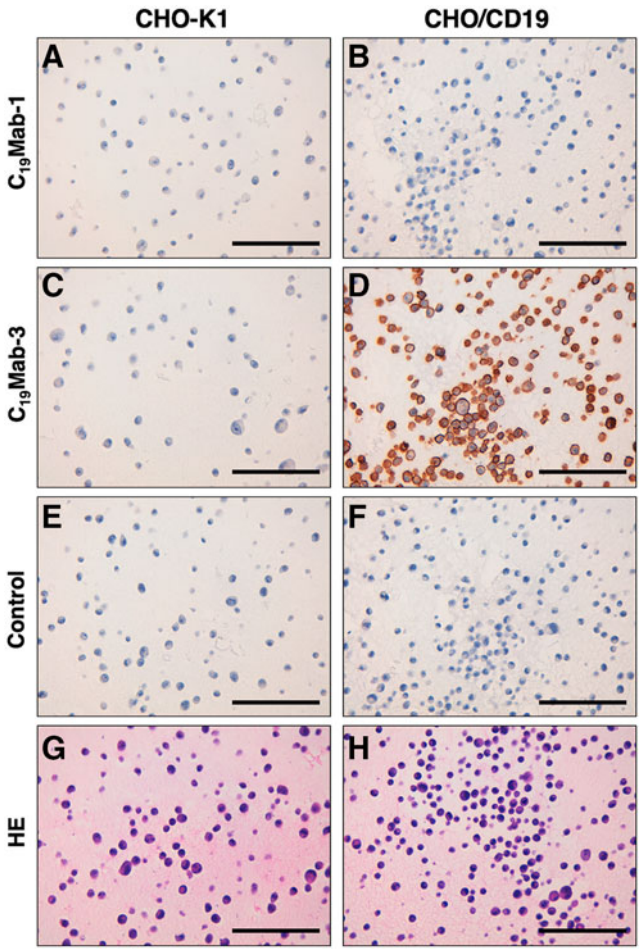

FIG. 3. Immunohistochemical analysis of C_{19}Mabs against CD19-expressing CHO-K1 cells. After antigen retrieval, sections were incubated with 1 μg/mL of C_{19}Mab-1 **(A, B)**, C_{19}Mab-3 **(C, D)**, and blocking buffer **(E, F)**, followed by treatment with peroxidase-conjugated secondary antibodies. Sections were counterstained with hematoxylin. **(G, H)** HE staining. Scale bar = 100 μm. HE, hematoxylin and eosin.

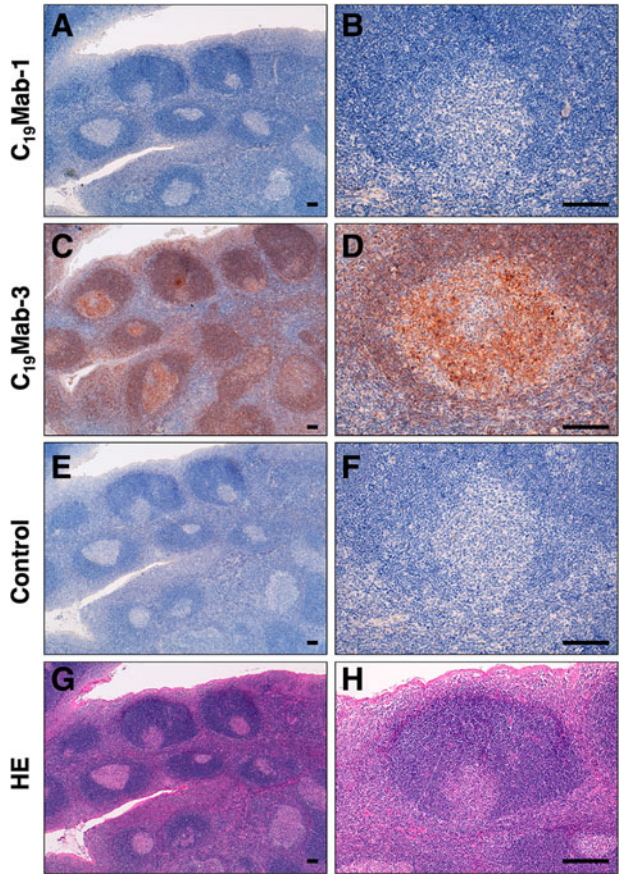

FIG. 4. Immunohistochemical analysis of C_{19}Mabs against human tonsil tissue. After antigen retrieval, sections were incubated with 10 μg/mL of C_{19}Mab-1 **(A, B)**, C_{19}Mab-3 **(C, D)**, and blocking buffer **(E, F)**, followed by treatment with peroxidase-conjugated secondary antibodies. **(G, H)** HE staining. Scale bar = 100 μm.

Discussion

In our first trial of producing anti-CD19 mAbs, we used CBIS to develop specific mAbs against CD19. This method is very convenient because it does not require the purification of membrane proteins in all steps of mAb production.[13–16] Using CBIS, we developed a novel anti-CD19 mAb—C_{19}Mab-1—which is very useful for detection of CD19 in flow cytometric analyses. Unfortunately, C_{19}Mab-1 could not recognize the denatured CD19 protein in Western blotting and immunohistochemistry (IHC) analyses, suggesting that C_{19}Mab-1 binds to a conformational epitope of CD19. In our second trial of producing anti-CD19 mAbs, we developed C_{19}Mab-3 using the purified CD19 ectodomain as an immunogen. Although C_{19}Mab-3 is not suitable for flow cytometry, it is very sensitive to Western blotting and IHC analyses. Taken together, the findings suggest that it is important to select a suitable immunogen and screening method when developing optimal anti-CD19 mAbs.

The CD19 recognition arm of blinatumomab is derived from anti-CD19 mAb B43, which was obtained from a mouse hybridoma.[20,21] A recent study has revealed that B43 binds to CD19 in the middle of the ectodomain and recognizes a conformational epitope, which comprises three loops spanning amino acid residues 97–107, 155–166, and 216–224.[22] Furthermore, a previous report has revealed that several anti-CD19 mAbs (clones AB1, B4, HD37, BU12, F974A2, and SJ25-C1) have exhibited competitive inhibition against B43,[23] indicating that the epitope of B43 is a hotspot for production of useful anti-CD19 mAbs.

As mentioned before, CART-19 (tisagenlecleucel) therapy has shown remarkable potential for treatment of highly refractory B cell malignancies; however, some patients do not respond to CART-19 immunotherapy for several weeks. The acquired resistance of B-ALL to CART-19 is partly based on skipping of CD19 exon2, in which the epitope of CD19 scFv might exist.[24] Therefore, the development of novel anti-CD19 mAbs, which recognize epitopes different from existing Abs, would be valuable for gaining a better understanding of CD19-targeting immunotherapy. In addition, future studies should investigate the epitopes recognized by C_{19}Mab-1 and C_{19}Mab-3.

Author Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Figure S1
Supplementary Table S1

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Address correspondence to:
Yukinari Kato
New Industry Creation Hatchery Center
Tohoku University
2-1 Seiryo-machi, Aoba-ku
Sendai, Miyagi 980-8575
Japan

E-mail: yukinari-k@bea.hi-ho.ne.jp

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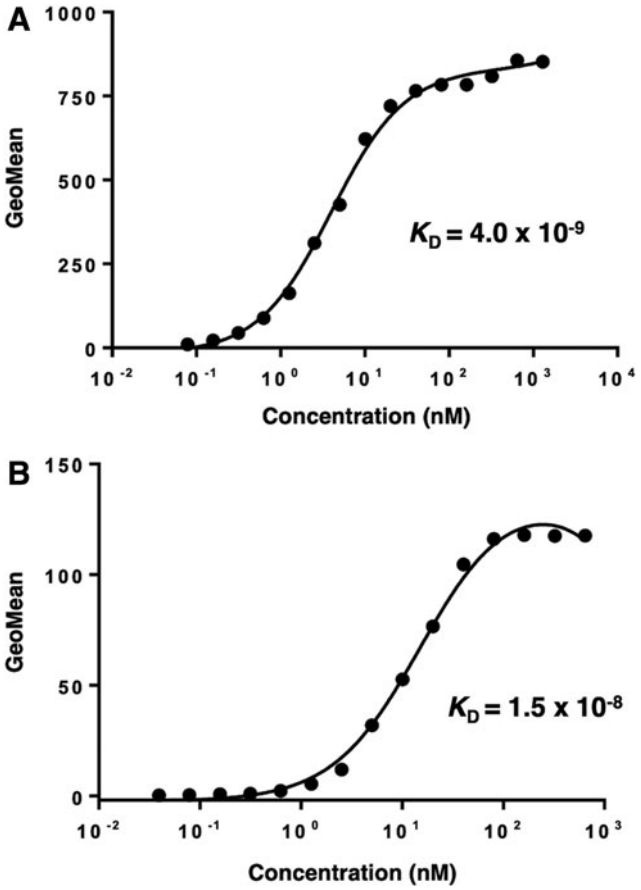
Supplementary Data



SUPPLEMENTARY FIG. S1. Determination of the binding affinity of C_{19}Mab-1 using flow cytometry. CHO/CD19 **(A)** and P30/OHK **(B)** were suspended in 100 μL of serially diluted C_{19}Mab-1 (0.6–10 μg/mL), followed by addition of secondary anti-mouse immunoglobulin G. Fluorescence data were collected using a cell analyzer. GeoMean, geometric mean value.

SUPPLEMENTARY TABLE S1. SUMMARY OF HYBRIDOMA PRODUCTION

Trial	*Immunogen*	*First screening*	*Second screening*	*Third screening*	*Hybridoma clone used in this study*
1	LN229/CD19 (cell-bound CD19)	3/1056 (FCM)[a]	0/3 (IHC)	0/3 (WB)	C_{19}Mab-1 (IgG_1, kappa)
2	LN229/CD19ec (soluble CD19)	18/958 (ELISA)	7/18 (IHC)	2/18 (FCM)	C_{19}Mab-3 (IgG_1, kappa)

Hybridoma supernatants were assayed against CHO-K1 cells and CHO/CD19 cells in FCM, WB, and IHC screening. For ELISA screening, soluble CD19 was used as a solid-phase antigen.

[a]No. of positive wells/no. of tested wells (screening method).

ELISA, enzyme-linked immunosorbent assay; FCM, flow cytometry; WB, Western blotting; IHC, immunohistochemistry; IgG, immunoglobulin G.