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Construction and Characterization of 1F5 Chimeric Anti-CD20 Monoclonal Antibodies: An Efficient Splicing by Overlap Extension-polymerase Chain Reaction Technique

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ABSTRACT

Despite the unparalleled success of anti-CD20-targeted immunotherapy, the currently available mAbs are not sufficiently efficacious in the treatment of lymphoma. 1F5 is one of a panel of anti-CD20 mAbs that was used in the B-cell lymphoma serotherapy. Despite the efficacy of murine 1F5 mAbs in lymphoma patients, the 1F5 chimeric antibodies with human effector functionality are yet to be approved and widely used in the treatment of lymphoma. In this study, the conversion of 1F5 mAb from mouse IgG2a to human-mouse chimeric IgG1 was achieved and the chimeric antibody was partially characterized.

We constructed the 1F5 chimeric mouse-human anti-CD20 antibody genes using an efficient Splicing by overlap extension-polymerase chain reaction (SOE-PCR) technique and cloned the chimeric heavy and light genes in pBudCE4.1 mammalian expression vector, followed by purification of the expressed chimeric 1F5 mAbs using affinity chromatography. Our investigation also included the biological properties of purified chimeric antibodies.

The generated 1F5 chimeric mAbs mediate complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) against Raji and Daudi Burkitt's lymphoma cell lines, which were comparable with rituximab and exhibit superior reduction in cell viability *in vitro*, compared to rituximab.

The current study indicated that the generated chimeric 1F5 mAbs has potential CDC and ADCC activity which was comparable with rituximab whereas it exhibits a superior reduction in cell viability, compared to rituximab. Our work contributes to future studies involving *in vivo* biological functions and the application of the 1F5 chimeric antibody.

Keywords: CD20; Cell proliferation; Cytotoxicity tests; Monoclonal antibody

INTRODUCTION

Non-Hodgkin's lymphoma (NHL), a current type of blood cancer occurs following abnormally growth of lymphocytes.[1] Monoclonal antibody (mAb) against B-lymphocyte antigen CD20 has been the most

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successful in the treatment of refractory NHL.[2-4] Despite the unparalleled success of anti-CD20-targeted immunotherapy, the presently available mAbs are only partially effective in lymphoma therapy and there is an essential need to develop new therapeutic mAbs. Rituximab, a chimeric (murine–human) anti-CD20 mAb, was introduced in 1997. It was the first therapeutic anti-CD20 mAb that gained FDA approval for the B cell disorders, including NHL and chronic lymphocytic leukemia (CLL). Subsequently, rituximab has been applied in the treatment of some autoimmune diseases.[5,6] Rituximab is frequently used in lymphoma therapy alone, or combined with chemotherapy, but patients often relapse and become resistant to rituximab. Nowadays, different types of novel CD20 mAbs have been generated.[7,8] Some of these, such as tositumomab, ibritumomab, obinutuzumab (GA 101), and ofatumumab are FDA approved for the treatment of NHL and rheumatoid arthritis such as lupus and multiple sclerosis.[1]

CD20, a non-glycosylated, transmembrane phosphoprotein, is a superior biomarker for immunotherapies targeting B-cell lymphoma and leukemia.[9] CD20 exists on the most malignant and normal B-cells, but it is not present on the differentiated B-cells and hematopoietic stem cells.[10] It is also believed that no shedding or internalization occurs after antibody binding, meaning that CD20 applies various cytotoxic effects after binding to anti-CD20 mAbs.[11] After ligation with CD20-expressing cells, antibodies can induce several mechanisms of cell death such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), direct programmed cell death (PCD), and cell growth inhibition.[8,12,13] Based on the cell death mechanisms, CD20 mAbs are classified into two different types. Type I comprising rituximab-like antibodies can activate complement, however, they have a poor ability at inducing PCD. Type II comprising tositumomab-like mAbs are not effective inactivation of complement, but usually tend to induce strong PCD.[14-16]

According to our increasing knowledge of the limitations and action mechanism of rituximab and other CD20 mAbs, there is an essential need to develop new therapeutic mAbs.[1] Our group has recently embarked on a series of researches aimed at providing anti-CD20 mAbs with improved efficacy by genetic engineering methods or developing superior novel anti-CD20 mAbs.[17] One of our objectives to improve the efficacy and reduce the immunogenicity of mouse 1F5 anti-CD20 mAbs through the production of human-mouse 1F5 chimeric mAbs is described in the present study. 1F5, a mouse IgG2a antibody, is a monoclonal antibody against the CD20. In lymphoma patients, earlier studies have shown that clinical responses were obtained; using high doses of murine 1F5 anti-CD20 mAbs.[18] Despite the efficacy of murine 1F5 mAbs in lymphoma patients, the 1F5 chimeric antibodies with human effector functionality have not been approved yet and are not widely used in the treatment of lymphoma. In the present study, we describe the use of splicing by overlap extension-polymerase chain reaction (SOE-PCR) for generating mouse-human 1F5 chimeric anti-CD20 genes. SOE-PCR is an approach to recombine DNA molecules without the use of restriction enzymes and ligase.[19] Owing to the high J-region similarity between mouse and human immunoglobulin genes, SOE-PCR allows the fusion of an original mouse variable antibody domain to the human constant domain without the addition of complementary tags.[20]

Here we describe the construction and expression of a chimeric 1F5 anti-CD20 antibody in Chinese hamster ovary (CHO)-K1 cells. Also *in vitro* characterization of 1F5 chimeric mAbs having a human constant region was examined. In the previous study, we reported several difficulties with the screening methods and the cloning of 1F5 chimeric heavy-chain genes due to a predicted hairpin cluster in the coding sequence resulting in resistance to DNA polymerase during both PCR and sequencing methods.[21] Despite difficulties, the 1F5 chimeric anti-CD20 antibody was successfully constructed and purified and its biological properties investigated. The 1F5 chimeric antibodies maintain their bioreactivity to human CD20 and offer the potential for therapeutic efficacy via CDC and ADCC which is similar to rituximab, whereas it exhibits a superior reduction in cell viability, compared to rituximab. It is prospected that produced 1F5 chimeric antibodies have a long life and slow clearance compare to murine 1F5 monoclonal antibodies.

MATERIALS AND METHODS

Ethics

This study was approved by the ethical committee of Kermanshah University of Medical Sciences (Ethics code: IR.KUMS.REC.1396.503).

Cell Lines

The 1F5 hybridoma cell line producing the murine IgG2a anti-CD20 antibody was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The Raji and Daudi (Burkitt's lymphoma) and Jurkat (T lymphoblastic lymphoma) cell lines were prepared from the National Cell Bank of Iran (NCBI, Tehran, Iran). RPMI-1640 medium (Antibiotic-free) enriched with 2 mM L-glutamine (Gibco, Grand Island, NY) and 10% fetal bovine serum (FBS) was used for maintaining the cells in log-phase growth at 37 ºC in a 5% CO_2 incubator. Chinese hamster ovary (CHO)-K1 cells were purchased from the Iranian Biological Resource Centre (Tehran, Iran) and grown in DMEM/F-12 (Gibco, Grand Island, NY) with 10% FBS at 37 ºC with 5% CO_2.

mRNA Purification and cDNA Synthesis

mRNAs were isolated from human peripheral blood mononuclear cells (PBMC) and 1F5 hybridoma cells (ATCC HB-9645) using oligo $(dT)_{25}$-coated magnetic beads (Dynabeads mRNA direct purification kit; Life Technologies, Carlsbad, CA) based on the instructions of the manufacturer. Subsequently, the mRNA was converted to cDNA in a reaction mixture including 1 µL of beads/mRNA complex (200 ng mRNA), 1×RT buffer (Invitrogen, Waltham, MA), 5 mM MgCl2, 1 mM deoxynucleotide triphosphates (dNTPs) (SinaClone Bioscience Co. Tehran, Iran), 10 mM *Dithiothreitol* (DTT), 10 U/µL SuperScript III Reverse Transcriptase (Invitrogen, Waltham, MA), and 2 U/µL RNase inhibitor (Thermo Fisher Scientific, Waltham, MA). The bead-bound oligo (dT) was used as a primer. The conditions were 50°C, 50 minutes, followed by 85°C, 5 minutes. The isolated mRNA and cDNA were analyzed by a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Amplification of the Mouse V_H and V_L and the Human C_H and C_L Genes

The cDNAs of 1F5 variable gamma heavy (V_H) and variable kappa light chains (V_L) were amplified using two sets of primers. V_HF and J_HR, Forward and the reverse primers, were used for amplifying the *1F5* V_H gene (GeneBank AY058907.1). The *1F5* V_L gene (GeneBank AY058906.1) was amplified; using V_KF and J_KR primers. The primer sequences are mentioned in Table 1. Specific forward primers V_HF and V_KF were designed based on each of the sequences of the *1F5* V_H and V_L genes. The J-region reverse primers J_HR and J_KR were made, which contained complementary sequences to the immunoglobulin J-regions for the heavy and light chains.

Human IgG1 constant heavy-chain (C_H) and constant kappa light-chain (C_L) genes were amplified from human PBMC cDNA with two sets of primers the forward J_HF and reverse IgG_HR primers for the *IgG1* C_H gene, and the forward J_KF and reverse IgG_KR primers for the *IgG1* C_L gene. The primer sequences are mentioned in Table 1. The J-region forward primers J_HF and J_KF were made, which contained complementary sequence to the J-regions, for heavy and kappa chains. The class-specific reverse primers IgG_HR and IgG_kR were similar to those described previously.[20]

In all cases, PCR reactions were set up containing 5 µL of10× *Pfx* Amplification buffer (Invitrogen), 1 µL of $MgSO_4$(50 mM), 2 µL of dNTPs (10 mM), 1 µL of the forward and reverse primers (10 µM), 2 µL of cDNA as template, 0.4 µL (1 U) Platinum *Pfx* DNA polymerase (Invitrogen), and water up to 50 µL. The PCR was performed as follows: 1 cycle at 94°C, 5 minutes; 35 cycles of denaturation at 94°C, 45 seconds; annealing at 55°C (V_H, V_L, and C_L), or 60°C (C_H), 45 seconds; extension at 68°C, 1 minute; and a final cycle of 15 minutes at 68°C. The amplified products of V_H, V_L, C_H, and C_L genes were analyzed using agarose gel electrophoresis to confirm successful amplification of individual genes, followed by Purification with a Qiaquick gel extraction kit (Qiagen, Valencia, CA).

Chimerization of the *Human–mouse 1F5* Genes by SOE-PCR

A polymerase chain reaction was carried out with SOE-PCR to create the full-length human-mouse chimeric heavy-chain (V_H-C_H) and light-chain (V_L-C_L) genes as described previously[20] with some modifications. SOE-PCR is an approach to recombine DNA molecules without the use of restriction enzymes and ligase.[19] Owing to the high J-region similarity between mouse and human *immunoglobulin* genes, SOE-PCR allows the fusion of an original mouse variable antibody domain to the human constant domain without the addition of complementary tags.[20] Chimerization of mouse 1F5 variable genes to the human *IgG1 constant* genes was performed; using immunoglobulin J-region similarity. The J-region primers, J_KR for the amplification of the 1F5 V_L

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domain and J_KF for the amplification of IgG1 C_L domain, are reverse complementary, similar to those reported by Jones and Barnard,[20] which allows subsequent fusion of the sequences by SOE. However, we designed new J-region primers (J_HR and J_HF) for the amplification of 1F5 V_H and IgG1 C_H domains.

To allow generation of the chimeric heavy-chain sequence by SOE-PCR, V_H and C_H fragments should ideally overlap by at least 15-bp. Because the overall sequence similarity of 1F5 and IgG1 heavy-chain J-regions was 10-bp (ggggccaagg), new J_HF and J_HR primers were designed to increase the overlap sequences. The reverse primer, J_HR: 5'CCTTGGCCCCAGTAGTCAAAG3', was designed, containing the antisense of the 1F5 V_H 5' end at its 3' end and the J-region sequence similarity at its 5' end (showed by the underlined nucleotides). The forward primer, J_HF: 5'GACTACTGGGGCCAAGGGAC3', was designed, containing the 17-bp of the J_HR 5' end, reverse complimentary, at its 5' end (showed by the underlined nucleotides), but the primers included non-complementary sequences at their 3' end.

Using the new primers, overlap with the 1F5 V_H 3' end was created at the 5' start of the human C_H domain by the J-region sequence similarity, resulting in an increased overlap sequence by 17-bp. Moreover, the improved reverse primer J_HR allows more specific isolation of V_H gene from 1F5 hybridoma cDNA than previously reported[20] for the isolation of each variable gene.

SOE-PCR was then carried out using the primers V_HF and IgG_HR for the creation of chimeric heavy-chain (V_H-C_H) and the primers V_kF and IgG_kR for the light chain (V_L-C_L) sequences. PCR was performed using 5 µL of10× *Pfx* Amplification buffer, 1 µL of the suitable forward and reverse primers,2 µL of DNA templates (20 ng/µL), 2 µL dNTPs,1 µL $MgSO_4$, 0.4 µL (1 U) Platinum *Pfx* DNA polymerase, and water up to 50 µL. PCR was performed as follows: 1 cycle at 94 C, 5 minutes; 35 cycles (94 C, 45 seconds, 60°C [V_H-C_H] or 55°C [V_L-C_L] for 45 seconds, 68°C for 1 minute 30 seconds), and a final extension for 15 minutes at 68°C. The full-length chimeric products were verified by agarose gel electrophoresis and isolated from the agarose gels using the Qiaquick gel extraction kit.

Construction of Plasmids Encoding Chimeric 1F5 Antibodies

The heavy and light chain chimeric genes constructed by SOE-PCR were each subcloned into the pCR-Blunt II-TOPO vector using a Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA) and *E.coli* DH5α (NCBI, Tehran, Iran) based on the instructions of the manufacturer. The resulting colonies were screened by the colony PCR and digestion with a restriction enzyme followed by agarose gel electrophoresis. The orientations of chimeric genes in selected clones were confirmed by PCR using TOPO vector primers and specific primers for the insert. Plasmid DNAs from positive clones were then extracted with a QIA prep spin miniprep kit (Qiagen) and sequenced; using ABI 3730xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA). The resulting TOPO plasmids containing 1F5 chimeric heavy-chain gene or light-chain gene were named TOPO-HC or TOPO-LC, respectively.

The chimeric heavy-chain gene was isolated from the TOPO-HC vector through digestion with KpnI and XhoI and purified from agarose gel. The obtained fragment (6 ng/µL) was ligated into 20 ng/µLpBudCE4.1 mammalian expression vector (Invitrogen), pre-treated with KpnI and XhoI, under EF-1αpromoter. The new plasmid with the 1F5 chimeric heavy gene was named pBudCE4.1-HC.

Subsequently, the chimeric light-chain gene was isolated from the TOPO-LC vector through digestion with HindIII and XbaI, and ligated at the concentration of 10 ng/mL into the plasmid pBudCE4.1-HC (10 ng/µL), pretreated with HindIII and XbaI, under CMV promoter. The resulting plasmid then contained both the 1F5 chimeric heavy gene and chimeric light gene. The new plasmid was named pBudCE4.1-HC-LC. Plasmid pBudCE4.1 has the ability for the simultaneous expression of two genes in mammalian cell lines. The vector contains two multiple cloning sites each under the control of two independent promoters, cytomegalovirus (CMV) and elongation factor 1α-subunit (EF-1α). The vector includes c-myc/polyhistidine (His) tags with *Zeocin resistance* gene. The recombinant plasmids, pBudCE4.1-HC and pBudCE4.1-HC-LC, were transformed into DH5α cells (chemically competent) following standard protocols[22] and plated on Low Salt LB plates containing Zeocin (25 µg/mL). The resulting colonies were screened by the colony PCR and restriction enzyme digestion. Positive clones were then purified and sequenced from Plasmids.

Generation of Stable Cell Lines Expressing Chimeric 1F5 Antibodies

CHO-K1 cells were transfected with plasmid pBudCE4.1-HC-LC by using Lipofectamine 2000 (Invitrogen) based on the instructions of the manufacturer. In brief, the day before transfection CHO-K1 cells were injected into 6-well plates at a concentration of 3×10^5 cells/well and grown in DMEM/F-12 medium-enriched with 5% FBS. Before transfection, linearization of DNA plasmid was performed by NheI restriction enzyme. On the day of transfection, when the cells were90–95% confluent, they were rinsed with medium without serum. For each well, 4 µg of plasmid DNA in 250 µL of medium (without serum) was mixed with 10 µL Lipofectamine in 250 µL of medium (without serum). After 30 minutes, the DNA/Lipofectamine complexes were added dropwise onto the cells and incubated at 37°C with 5% CO_2 for 18 hours, whereupon the transfection solution was exchanged with a fresh, complete medium. After 48 hours, a fresh medium with 0.5 mg/mL Zeocin was added for the selection of CHO-K1 cells expressing the chimeric constructs. Medium containing Zeocin was changed after every 3–4 days for three weeks. Stable CHO-K1 clones producing chimeric anti-CD20 mAbs were identified by a cell-based enzyme-linked immunosorbent assay (cell-based ELISA).

A limiting dilution step was followed for clonal selection, and one cell per well was seeded in 96-well plates and incubated at 37 °C with 5% CO_2 for 14 days. Isolated clones were expanded and screened by ELISA (cell-based) for the production of chimeric 1F5 mAbs. The stable cell lines having high levels of antibody were selected and their binding affinity for CD20 was tested. Finally, one stable clone named ch1F5-CHO, the best chimeric 1F5 expression clone was obtained.

Purification of Recombinant Chimeric 1F5 Antibodies

Sufficient quantities of chimeric mAbs were generated for use in purification and experimental testing. Culture supernatant (1 Litre) of ch1F5-CHO cells expressing the chimeric mAbs was harvested and centrifuged (15 minutes, 4000×g). The pH of the supernatant was adjusted to 8 with 1M KOH or 1M H_3PO_4 and applied to a HIS-Select Nickel Affinity chromatography column (Sigma-Aldrich, St Louis, MO). Purification was done based on the manufacturer's protocol. The antibody concentration was estimated by a NanoDrop 2000c spectrophotometer. Chimeric 1F5 mAbs were shown to be highly purified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western Blot Analysis

Western blot analysis was performed as described previously.[17] Briefly, Raji or Jurkat cells were lysed and the lysate supernatants were separated by 10% none-reducing SDS-PAGE,[23] and then transferred to a polyvinylidene difluoride membrane.[24] Purified chimeric 1F5 mAbs or rituximab (2 µg/mL) were used as primary antibodies; and horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Razi Biotech, Iran) was used as secondary antibodies at a dilution of 1/500.

Whole-cell Enzyme-linked Immunosorbent Assay

A cell-based ELISA as previously described [17] was used to screen the supernatant of transfected cells to screen positive colonies, detect, and compare the binding affinity of generated chimeric 1F5 mAbs with intact 1F5 mAbs. In these assays, Raji and Jurkat cells (negative control) were used. Primary antibodies were culture filtrate, generated chimeric 1F5 mAbs, or 1F5 mAbs. HRP-conjugated goat anti-human IgG or anti-mouse IgG (Razi Biotech, Iran) were used as secondary antibodies at a dilution of 1/1000. All measurements were tested in triplicate, and each experiment was performed at least three times.

Immunocytochemistry Assay

The binding specificity of generated chimeric 1F5 mAbs was also investigated by an indirect immunocytochemistry assay as previously explained in detail.[17] In brief, Raji or Jurkat cells (negative control) were used and spread on the microscope slides. Purified Chimeric 1F5 anti-CD20 mAbs (10 µg/mL) were used as primary antibodies. Goat anti-human IgG conjugated with fluorescein isothiocyanate (FITC) or HRP (Razi Biotech, Iran) were used as secondary antibodies at a dilution of 1/500. Stained cells with FITC-goat anti-human IgG were observed using an inverted fluorescence microscope (Nikon). Stained cells with HRP-goat anti-human IgG were observed against controls with a Nikon microscope. All measurements were tested in triplicate, and each experiment was performed at least three times.

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Cell Viability Assay

The human-mouse 1F5 chimeric mAbs effect on the viability of Burkitt's lymphoma cells versus the rituximab impact was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) colorimetric assay. Briefly, 50000 cells (Daudi or Raji) were injected into each well of a 96-well cell culture plate. Whereupon, 10 µL of each antibody dilution, was added to each well containing 90 µL medium. The antibody concentration was set at 100, 10, 1, and 0.1 µg/mL in the mixture of 100 µL/well. 72 hours after treatment, MTT (0.5 mg/mL) was added and incubated for 3 hours at 37°C. Then, the plates were centrifuged at 2000 g for 5 minutes, and the medium was removed and formazan crystals were dissolved in 100 µL DMSO. The absorbance of wells was determined at 570 nm using a 96 well microplate reader (Bio-Rad Laboratories Inc. Philadelphia, PA). The following equation was used to estimate the cell viability percentage: Cell viability (%)=[A_{570} (sample)/ A_{570} (control)] ×100. The effects of the antibodies on cell viability were expressed as IC50, which is the concentration of antibody that reduces the absorbance value of treated cells by 50% concerning the untreated cells (control). All measurements were tested in triplicate, and each experiment was performed at least three times.

Complement-dependent Cytotoxicity

Burkitt's lymphoma cells, Raji and Daudi, were used to test the CDC function of purified 1F5 chimeric mAbs or rituximab. In brief, after washing with PBS, the cells were adjusted to 1×10^6 cells/mL with serum-free RPMI-1640. Subsequently, 70 µL of cell suspension and 10 µL of 1F5 chimeric mAbs or rituximab to a final concentration of 1 µg/mL were added to each tube. After incubation for 10 minutes, 20 µL of normal human serum (NHS, 20%) was added to all tubes. Negative controls included background (cells only or cells plus Ab) and complement cytotoxicity (cells plus complement). The tubes were incubated at 37°C for 2 hours to promote complement-mediated cell cytotoxicity. CDC activity was determined by flow cytometry analysis of stained cells with Annexin V/PI apoptosis detection kit (BioLegend, San Diego, CA) based on the instructions of the manufacturer. Stained cells were analyzed by Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific).[25] FlowJo software version 7.6.1 was used for the analysis of the results. All measurements were tested in triplicate, and each experiment was performed at least three times.

Antibody-dependent Cellular Cytotoxicity

In this assay, fresh PBMCs (effector cells), and Raji and Daudi cells (target cells) were used. ADCC assays were done; using a lactate dehydrogenase (LDH) assay kit (Promega, Madison, USA) based on the protocol of the manufacturer. Briefly, a total of 10,000 target cells/well were incubated at 37°C for 20 h in a U bottom 96-well plate. Then, 1µg/mL chimeric 1F5 or rituximab were added into each well. Then, fresh PBMCs were added into the wells with the effector to target (E:T) ratios of 2:1, 5:1, 10:1, 20:1, 50:1, and 100:1. After, 4h co-incubation, LDH release was assayed at 490 nm. The rate of cellular cytotoxicity was estimated using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) based on the instructions of the manufacturer. Data were analyzed using GraphPad Prism8.0.[26] All measurements were tested in triplicate, and each experiment was performed at least three times.

Sequencing

All DNA was sequenced using ABI 3730xl DNA Analyzer. Standard sequencing and GC-rich template protocols were used for sequencing. Primer sequences are mentioned in Table 1. 1F5 chimeric heavy and light chain genes cloned into the pCR-Blunt II-TOPO or pBudCE4.1 bicistronic expression vector were used as templates for sequencing.

Statistical Analysis

Data are presented as mean±standard deviation (SD). The *p*-values<0.05 were considered significant using One-way ANOVA. SPSS software 19.0 (SPSS, Inc., Chicago, IL) was used for data analysis.

RESULTS

Design of Primers

The new primers for the J-region of heavy chain immunoglobulin were designed to allow more specific amplification of variable heavy gene from 1F5 hybridoma and the amplification of IgG1 constant heavy gene from human blood, which contains an overlap with the 1F5 V_H 3' end at its 5' start. The J-region primers (J_HR and J_HF) for the heavy chain are reverse complementary at the 5' ends of 17 bp, but they

are not reverse complementary at a few nucleotides at the 3' end. The new primers are more specific for the *1F5 V_H* gene, yet the amplified V_H and C_H domains are comprised of the most overlapping sequences at the V_H 3' and C_H5' ends. The J-region primers (J_kR and J_kF) for the kappa chain are reverse complementary, like those described previously.[20] IgG_HR and IgG_kR are class-specific reverse primers for the immunoglobulin G heavy and light chains, respectively.[20] Specific forward primers, V_HF and V_KF, were designed according to each of the sequences of the *1F5 V_H* and *V_L* genes. MFE primer (http://biocompute.bmi.ac.cn/CZlab/MFEprimer-2.0/) was used for specific primer design. Primer sequences for the amplification of mouse variable and human constant genes are mentioned in Table 1.

Table 1: Sequence of each primer and its designated applications

Primer name	Sequence (5'-3')	Use
V_HF	ATGGCCCAGGTGCAACTGCG	Sequencing/PCR
V_KF	ATGGCCCAAATTGTTCTCTCC	Sequencing/PCR
J_HF	GACTACTGGGGCCAAGGGAC	PCR
J_HR	CCTTGGCCCCAGTAGTCAAAG	Sequencing/PCR
J_KF	GGGACCAAGCTGGAAATCAACG	PCR
J_KR	CGTTGATTTCCAGCTTGGTCCC	PCR
IgG_HR	TCATTTACCCGGAGACAGGGAG	Sequencing/PCR
IgG_kR	CTCCCTCTAACACTCTCCCCTG	Sequencing/PCR
I_HF	CTCACACATGCCCACCGTG	Sequencing
M13 Forward (-20)	GTAAAACGACGACGGCCAG	Sequencing/PCR
M13 Reverse	CAGGAAACAGCTATGAC	Sequencing/PCR

Construction of 1F5 Chimeric Heavy and Light Genes

The method for constructing 1F5 chimeric antibody genes involves the following:

Primary PCR amplification of *V_H* and *V_L*, *C_H*, and *C_L* genes from 1F5 hybridoma and human PBMC cDNAs, respectively; (2) Joining of the V_H and C_H, and V_L and C_L sequences via overlap PCR to create chimeric heavy- and chimeric light-chain genes, respectively.

PCR products Analysis in a 1% agarose gel showed two bands for the *V_H* and *V_L* genes at about 350 bp and 325bp, respectively, in consonance with the expected sizes (Figure 1A). The *C_H* and *C_L* genes were observed at 1032bp and 350bp, respectively, in consonance with the expected sizes (Figure 1B). An undesirable 550-bp fragment was generated in addition to the C_L domain. The *V_H*, *V_L*, *C_H*, and *C_L* genes were subsequently gel-purified.

Construction of the 1F5 chimeric heavy gene was performed by SOE-PCR using J-region similarity. To allow generation of the complete sequence of chimeric heavy chain gene, the two sequences, V_H and C_H, should ideally overlap by at least 15 bp. Because the overall sequence similarity of 1F5 and IgG1 heavy-chain J-regions was 10-bp (ggggccaagg), new J_HF and J_HR primers were designed. The reverse primer, J_HR, which was designed, contained the antisense of the 1F5 V_H 5' end at its 3' end and the J-region sequence similarity at its 5' end. The forward primer, J_HF, which was also designed, contained the sense of the 1F5 V_H 3' end at its 5' end and the J-region sequence similarity at its 3' end. The C_HDNA was then amplified using J_HF and IgG_HR primers, and an overlap with the 1F5 V_H 3' end was added to the 5' end of IgG1 C_H domain by the 10 nucleotides of J-region sequence similarity. Consequently, the 5' end of 1gG1 C_H and the 3' end of 1F5 V_H hybridized during the overlap PCR to form a complete 1F5 chimeric heavy-chain gene with a length of about 1,365 bp (Figure 2, Lanes 1 and 2).

Similar to the heavy-chain gene, the 1F5 chimeric light gene was constructed by joining V_L and C_L in an overlap extension PCR; using J-region similarity. The J-region primers J_KF and J_KR were reverse complementary with a 22bp overlap sequence. Upon PCR amplification, a complete 1F5 chimeric light-chain gene of approximately 653 bp was obtained (Figure 2, Lanes 3 and 4). An unknown 550-bp

fragment was generated in addition to the chimeric light gene. Each chimeric heavy and light gene was purified from the gel and then cloned into the pCR-Blunt II-TOPO vector.

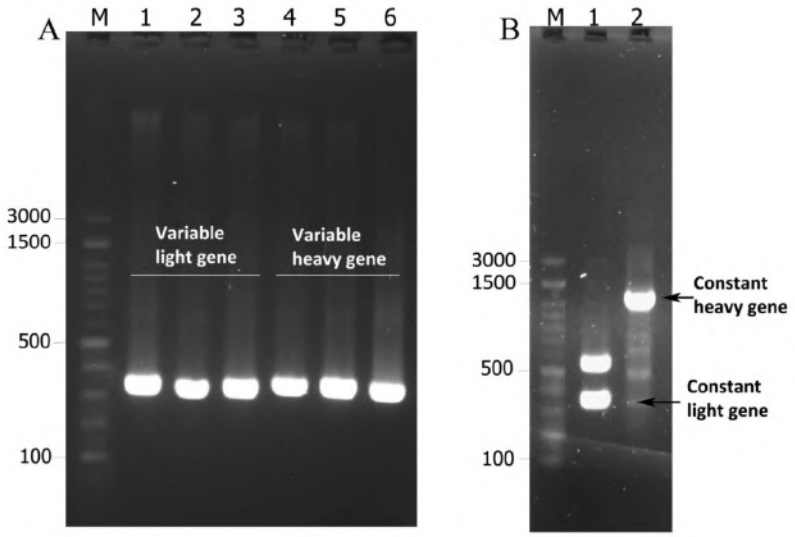

Figure 1. Agarose gel electrophoresis of the amplified mouse variable and human constant genes. (A) Primary PCR amplification of variable genes from 1F5 hybridomas DNA. Lanes 1–3, 1F5 variable light gene (V_L, 325 bp); Lanes 4–6, 1F5 variable heavy gene (V_H, 350 bp). (B) Primary PCR amplification of constant genes from human peripheral blood mononuclear cells (PBMC) cDNAs. Lane 1, human IgG constant light gene (C_L, 350 bp) beside an unknown 500 bp fragment; Lane 2, human IgG constant heavy gene (C_H, 1032 bp). M indicates the molecular weight marker (100 bp DNA ladder, Morganville Scientific).

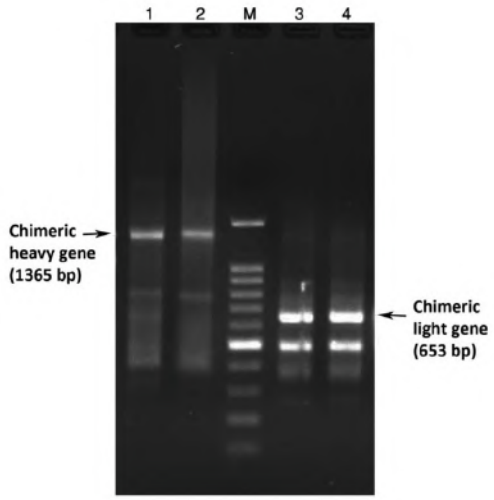

Figure 2. Chimerization of mouse variable and human constant genes by splicing with overlap extension polymerase chain reaction (SOE-PCR). Lanes 1 and 2: Overlap extension PCR; using primers V_HF and IgG_HR yielded the complete 1F5 chimeric heavy-chain sequences (V_H-C_H, 1365 bp). Lanes 3 and 4: Overlap extension PCR; using primers V_LF and IgG_kR yielded the complete 1F5 chimeric light-chain sequences (V_L-C_L, 653 bp) and an unknown 500 bp fragment. M indicates the molecular weight marker (100bp DNA ladder RTU, GeneDirex).

Construction and Expression of 1F5 Chimeric Anti-CD20 Antibodies

First, the cloning of the chimeric heavy and light genes into the pCR-Blunt II-TOPO was performed. The TOPO recombinant vectors containing chimeric heavy and light genes were separately transformed into *E. coli* cells. Transformed cells were analyzed for the presence and correct orientation of the chimeric heavy and light genes. Insertion and correct orientation of the chimeric light gene into the TOPO vector was successfully confirmed by colony PCR and restriction enzyme analysis (Figure 3), followed by confirmation of the construct sequence by DNA sequence analysis. However, we run into difficulties when screening and identifying positive clones containing TOPO-HC, probably because the cloned 1F5 chimeric heavy gene is highly GC-rich and forms a stable secondary structure. These structures can be preventive to polymerase during both PCR and sequencing.[21] However, several positive clones were selected by colony PCR; using M13 Reverse (a primer that hybridizes within vector) and IgG_HR (a specific primer), and the correct orientation of the 1F5 chimeric heavy gene was also confirmed (Figure 3). Agarose gel electrophoresis of the isolated chimeric heavy gene from TOPO-HC vector through digestion with *KpnI* and *XhoI* showed a faint DNA band (1,365 bp), despite multiple attempts (Figure. 3). However, this weak band was purified from the agarose gel and used for cloning into the expression vector pBudCE4.1. Under the same conditions, the chimeric light-chain gene (653 bp) was isolated from the TOPO-LC through digestion with HindIII and XbaI, as a strong band (Figure 3).

After the transformation of the plasmid pBudCE4.1-HC with a chimeric heavy gene, the transformed cells were analyzed for the presence of the chimeric heavy gene. The insertion of a chimeric heavy gene into the pBudCE4.1 was confirmed by comparing the sizes of the empty vector (4,595 bp) and the vector containing 1,365 bp sequence (5,960 bp). The chimeric light gene was cloned into the resulting vector pBudCE4.1-HC to construct the pBudCE4.1-HC-LC expression vector. After transformation, the presence of the chimeric light gene was successfully confirmed by colony PCR and

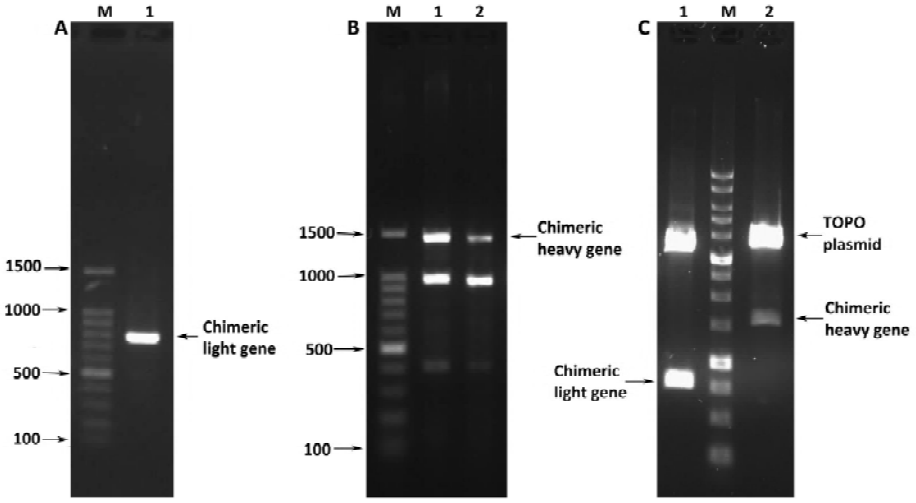

Figure 3. The presence and correct orientation of 1F5 chimeric heavy and light genes in the pCR-Blunt II-TOPO vector was verified by colony PCR and restriction enzyme analysis. (A) Colony PCR for identification of the chimeric light gene (653 bp); using M13 forward and V_KF primers. M: Molecular weight marker (100bp DNA ladder RTU, GeneDirex). (B) Colony PCR for identification of the chimeric heavy gene (1365 bp) with a primer set, M13 reverse, and IgG_HR. M: Molecular weight marker (100bp DNA ladder RTU, GeneDirex) (C) Restriction enzyme digestion of TOPO-LC vector, with HindIII and XbaI releasing the chimeric light gene as a strong band (Lane 1), and restriction enzyme digestion of TOPO-HC vector with KpnI and XhoI releasing the chimeric heavy gene as a faint band (Lane 2), probably due to the formation of secondary structures. M: Molecular weight marker (1Kb DNA ladder RTU, GeneDirex).

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restriction enzyme analysis, followed by DNA sequence analysis. Altogether, the expression vector pBudCE4.1-HC-LC with chimeric heavy- and light-chain genes was successfully constructed as discussed above.

CHO-K1 cells were transfected with the plasmid pBudCE4.1-HC-LCand selected under Zeocin selective pressure for three weeks. Clones expressing the 1F5 chimeric anti-CD20 antibodies were isolated by limiting dilution. Isolated colons were screened by a cell-based ELISA to select the clones expressing the highest levels of 1F5 chimeric mAbs after 14 days. One stable clone called ch1F5-CHO was selected and expanded.1F5 chimeric antibodies were purified from culture supernatants of ch1F5-CHO cells by HIS-Select Nickel Affinity chromatography. Analysis by SDS-PAGE confirmed the purity of the chimeric antibody. Two bands in agreement with the expected molecular weight for the antibody heavy and light chains were observed under reducing conditions (Figure 4). The yield of purified protein was typically about 2.5 mg/L.

Binding Activity of Chimeric 1F5 Antibody

The relative CD20 binding specificity of the produced chimeric 1F5 was detected by immunocytochemistry (ICC), ELISA (cell-based), and western blot; using CD20-expressing Raji cells and control Jurkat cells ($CD20^-$). For ICC, 1F5 chimeric constructs were added to Raji cells and stained with FITC- or HRP-conjugated goat anti-human IgG. The assay was analyzed with fluorescence or inverted microscopes. Fluorescence or brown color was seen on the Raji cells, but not seen on the Jurkat cell surface ($CD20^-$), indicating that chimeric 1F5 bind to CD20 specifically (Figure 5A and B). Additionally, we designed a cell-based ELISA to detect the binding affinity of 1F5 chimeric constructs. For cell-based ELISA, various concentrations (1-40 µg/mL) of chimeric 1F5 or intact 1F5 mAb were used. Analysis of reactivity with CD20 revealed that the generated chimeric 1F5 mAbs retained the CD20 binding activity and reacted to the CD20 with high affinity. However, the affinity of the produced chimeric 1F5 was slightly lower than that of the 1F5 mAbs (Figure 5C).

Non-reducing western blot also confirmed the specific binding of chimeric 1F5 to CD20at 33-kDa (Figure 6). The native epitopes of CD20 can probably be detected by the chimeric 1F5 under non-reducing conditions.[17]

1F5 Chimeric mAbs Significantly Reduces Cell Viability Compared to Rituximab

The effects of chimeric 1F5 antibodies on cell viability compared to rituximab were evaluated by an MTT assay. The maximum inhibition occurred at ≥ 1µg/mL (Figure 7). The percent of cell viability in cells treated with chimeric 1F5 was dramatically lower than cells treated with rituximab at lower than 100 µg/mL of antibody (p<0.001) (Figure 7). The IC50 of 1F5 chimeric mAbs on cell viability was 1 µg/mL. The effects of the chimeric 1F5 construct on cell viability were further compared with rituximab. The percent of cell viability induced by chimeric 1F5 (IC50=1 µg/mL) was considerably lower than rituximab (IC50=10 µg/mL), which showed the strong ability of 1F5 chimeric antibodies to reduce cell viability.

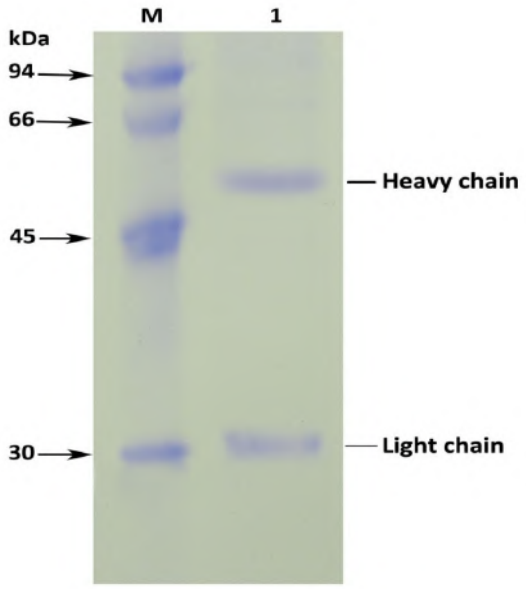

Figure 4. SDS-PAGE of the purified 1F5 chimeric antibodies under reducing conditions using 12.5% acrylamide separating gel. Staining was performed by Coomassie Blue R250. Lane 1, the two bands represent the antibody heavy and light chains. Lane M shows the protein markers.

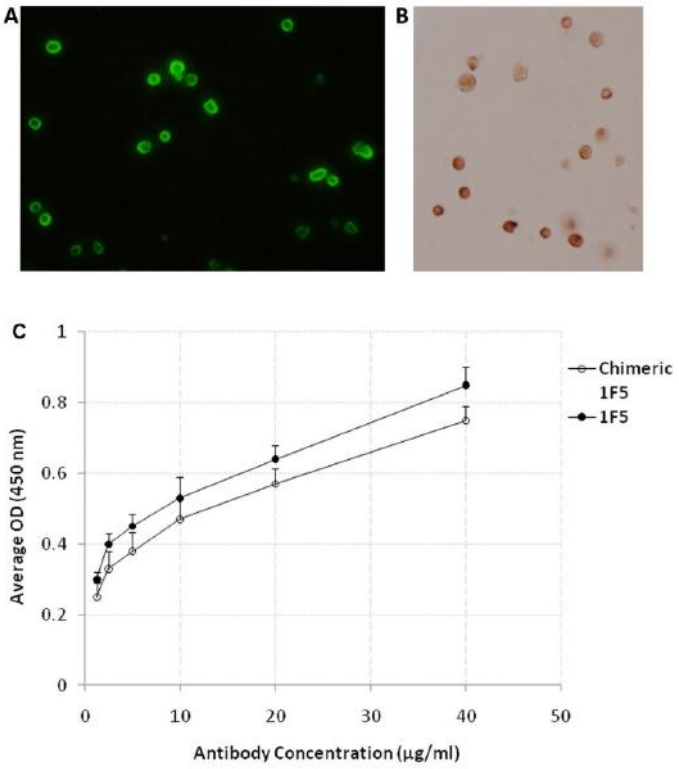

Figure 5. The binding specificity of the chimeric 1F5 to the CD20 was detected by immunocytochemistry (ICC) and whole-cell ELISA using CD20-expressing Raji cells. (A) ICC of chimeric 1F5 under a fluorescence microscope. (B) ICC of chimeric 1F5 under an inverted microscope. (C) The binding affinity of chimeric 1F5 in comparison with murine 1F5; using whole-cell ELISA. The data are the mean±SD of three experiments.

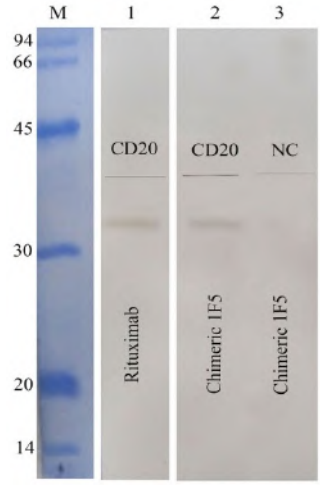

Figure 6. Binding specificity of chimeric 1F5 detected by non-reducing western blot. Lane 1, rituximab recognized CD20; Lane 2, chimeric 1F5 mAbs also detected CD20 about 33 kDa; Lane 3, negative control Jurkat cells; Lane M, protein standard markers

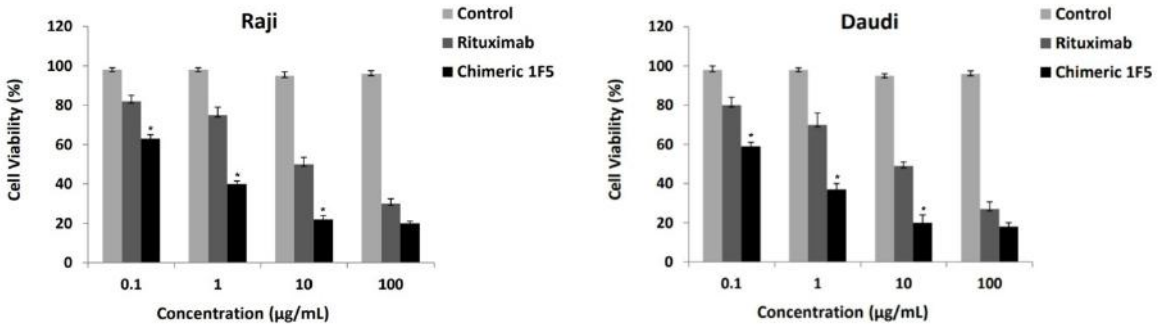

Figure 7. The effects of chimeric 1F5 mAbs in comparison with rituximab on the Raji cells proliferation. Controls were cells incubated in a medium without antibodies. The data are the mean±SD. *p<0.001 compared with rituximab determined by one-way analysis of variance (ANOVA).

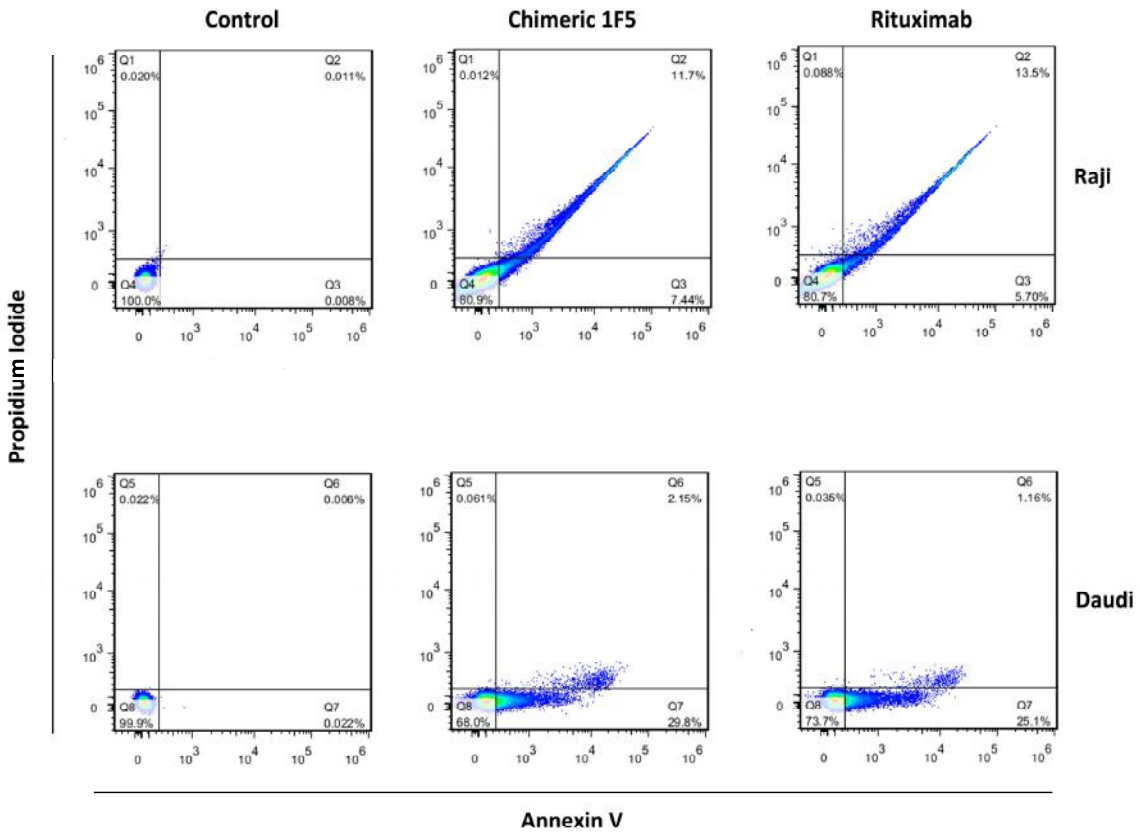

Figure 8. Flowcytometry analysis of complement-mediated cell death. Complement-dependent cytotoxicity (CDC) activity was determined by Annexin V/PI staining and flow cytometry analysis. Raji or Daudi cells were treated with 1 µg/mL of each antibody (1F5 chimeric mAbs or rituximab) and 20% normal human serum (NHS) for 2 hours at 37 ºC. Negative controls included background (cells plus Ab) and complement cytotoxicity (cells plus complement).

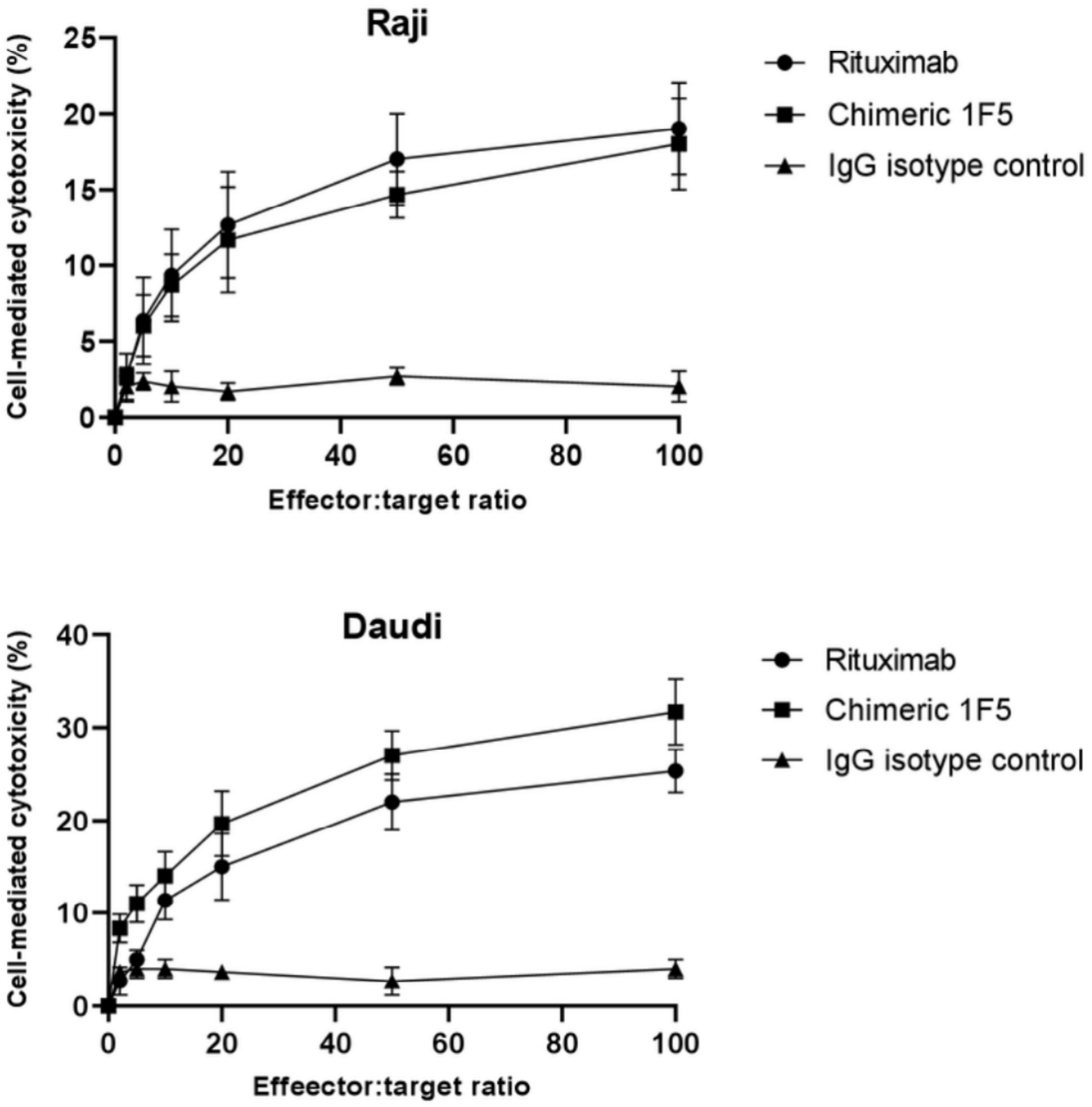

Figure 9. Antibody-dependent cellular cytotoxicity (ADCC) mediated by 1.0 µg/mL 1F5 chimeric mAbs or rituximab against Burkitt's lymphoma Raji and Daudi cell lines was evaluated; using lactate dehydrogenase (LDH) assay. E:T ratio was 2:1, 5:1, 10:1, 20:1, 50:1 and 100:1.The data are the mean±SD of three tests. Comparison between groups was analyzed by one-way ANOVA (* p<0.01 compared with IgG isotype control)

CDC and ADCC Mediated by 1F5 Chimeric mAbs is Comparable with Rituximab

CDC mediated against the Raji and Daudi cell lines by 1.0 µg/mL 1F5 chimeric mAbs was 19.14±3.51% and 32±2.7% (mean±SD), respectively, which is comparable with CDC activity mediated by 1.0 µg/mL rituximab (19.2±3.6% and 26.25±3%, respectively) (Figure 8).

ADCC was measured by determining the activity of cytosolic LDH is released by treating Raji and Daudi

cells. The test was performed in 3 independent experiments using fresh peripheral blood mononuclear cell fractions as effector cells. The cell-mediated cytotoxicity (%) reached the maximum level at E: T ratio of 100:1 in these two cell lines. At E:T ratio of 100:1, 50:1, and 20:1, the ADCC mediated against Raji and Daudi cell lines by 1.0 μg/mL 1F5 chimeric mAbs or rituximab showed significantly higher levels compared with that of control groups (p<0.01). Any significant cell lysis (less than 10%) was observed by the control IgG (Figure 9). The results indicated that regarding ADCC, a similar relative potency between chimeric 1F5 and rituximab was obtained.

DISCUSSION

CD20 mAbs have proved as the most successful therapeutics in non-Hodgkin's lymphomas.[2-4,27] Nowadays, rituximab with chemotherapy is considered as the current treatment of NHL and CLL.[28] Furthermore, rituximab is applied for the treatment of several autoimmune diseases such as lupus and multiple sclerosis.[5,6] Currently, several novel CD20 mAbs have been produced and some of them have been approved by the FDA.[1,7,8] Despite the unparalleled success of anti-CD20-targeted immunotherapy, patients often relapse with the current treatments. Therefore, there is a need to produce CD20 mAbs with ameliorated biological properties. 1F5 as the first CD20 mAb utilized in lymphoma serotherapy is an IgG2a mouse antibody.[18] Despite the efficacy of murine 1F5 mAbs in lymphoma patients, the chimeric 1F5 antibodies with human effector functionality have so far not been approved and widely used in the treatment of lymphoma. In this paper, we report the construction of 1F5 chimeric mouse-human anti-CD20 antibody genes; using an efficient SOE-PCR technique, cloning, and expression of 1F5 chimeric mAbs in CHO-K1cells.

Complement has crucial importance in treatment with rituximab.[15,29,30] Rituximab and murine 1F5 (type I CD20 mAbs) can activate complement efficiently.[14,15] Interestingly, the generated 1F5 chimeric mAbs showed complement-dependent cytotoxicity comparable with rituximab.

Recruiting effector cells by mAbs via Fc: Fcγ R interaction known as ADCC is critical to the therapeutic effects of anti-CD20 mAbs.[31] Herein, we further assessed the ability of 1F5 chimeric mAbs to induce ADCC on Burkitt's lymphoma cell lines. Our results indicate that the generated 1F5 chimeric mAbs can induce remarkable ADCC effects, comparable with rituximab, on effector cells. So, our findings show that 1F5 chimeric mAbs is a potentially effective antibody that may further be used in the therapy of B-cell lymphomas.

The method of overlap extension PCR for splicing two fragments of DNA was described more than two decades ago.[32] In 2005, Jones and Barnard[20] used this technique for the chimerization of multiple antibody classes using J-region similarity. Powerful as it is, the technique is not used widely for the production of a specific mouse-human chimeric mAbs. Here we describe the utility of the SOE-PCR technique for the chimerization and conversion of 1F5 mAb from mouse IgG2a to human-mouse chimeric IgG1, with several improvements as described below. First, intermediate PCR products should be purified by 'bind-and-wash' methods, such as Qiaquick. Although acceptable for other PCR products, gel purification of therapeutic antibody genes may render them unusable due to exposure to UV light. If one has to gel-purify PCR products of therapeutic antibody chimeric genes or other genes, it is recommended that the gel is never exposed to direct or indirect UV light. We achieved this approach as follows. After agarose gel electrophoresis, one lane from gel the vertical side containing separated genes was cut out, and only this part of the gel was exposed to UV light. After the location of the target gene in the agarose gel, the marker gel piece was placed at its original position, and the target gene band was cut carefully with a scalpel blade. So the separated gene bands never are exposed to UV light, suggesting low levels of DNA damage by UV.

Second, if one has to produce chimeric antibody genes; using SOE-PCR, it is better to design highly specific J-region primers than previously reported ones[20] that allowed the isolation of all variable and constant regions. To allow generation of the chimeric heavy-chain sequence through SOE-PCR, V_H and C_H should ideally overlap by at least 15 bp. With the overall sequence similarity of 1F5 and IgG1 heavy-chain J-regions at 10-bp (ggggccaagg), new J_HF and J_HR primers were designed to increase the overlap sequences. The new primers were more specific for the amplification of the *1F5 V_H* gene, but the amplified V_H and C_H domains comprised the most overlapping sequences at theV_H3' end and the C_H5' end.

In conclusion, the generated 1F5 chimeric anti-CD20 mAbs offer potential reagents for *in vivo* study involving anti-CD20 immunotherapy. Here we describe the utility of an efficient SOE-PCR technique for the chimerization and conversion of 1F5 mAb from mouse IgG2a to human-mouse chimeric IgG1. Our results showed that 1F5 chimeric mAbs exhibit CDC and ADCC activity on Burkitt's lymphoma cell lines. Also 1F5 chimeric mAbs exhibit superior cell growth inhibitor activity, compared to rituximab. Our present data contribute to the further studies of *in vivo* biological functions and application of 1F5 chimeric antibody.

CONFLICT OF INTEREST

No conflict of interest is reported by the authors.

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