Novel humanized anti-CD20 monoclonal antibodies with unique germline VH and VL gene recruitment and potent effector functions

MICHIO NISHIDA[1], KEISUKE TESHIGAWARA[1], OTSURA NIWA[1], SADAKAZU USUDA[2], TETSUO NAKAMURA[2], PETER RALPH[3], ROLAND NEWMAN[4] and EDUARDO A. PADLAN[5]

[1]Late Effects Studies, Radiation Biology Center, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501; [2]BioMedics Japan, Inc., 1-1-10 Koraku, Bunkyo, Tokyo 112-0004, Japan; [3]Marin Biologic Laboratories Inc., 3152 Paradise Drive Tiburon, CA 94920; [4]RANA Consulting, 8919 Montrose Way, San Diego, CA 92122, USA; [5]Marine Science Institute, College of Science, University of the Philippines, Quezon City 1101, Philippines

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Abstract. The anti-CD20 chimeric monoclonal antibody (mAb) rituximab is the most widely used therapeutic antibody for B-cell malignancies. However, ~50% of non-Hodgkin's lymphoma (B-NHL) patients respond to treatment with this antibody. Novel humanized antibodies target membrane CD20 with enhanced effector properties should improve treatment for a broader patient population with relapsed and refractory disease. A novel chimerized form of the murine anti-CD20 1K1791 exerts more potent antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) activities and induces cell death by a non-caspase dependent process. Humanized mAbs derived from 1K1791 were designed using four different humanization techniques and characterized. In contrast to rituximab or 2F2 (human anti-CD20 mAb), several of these exhibited superior ADCC, CDC, inhibition of cell growth and cell death. There was a wide range of functional differences among the humanized forms of 1K1791 despite a modest replacement of amino acid residues in the CDRs. To determine whether the superior activities exhibited by parental murine mAb 1K1791 were due to differences in VH and VL rearrangement, we analyzed its germline and compared it to other anti-CD20 mAbs. A remarkable conservation of VH and Vk (VL kappa) gene usage was observed in the murine anti-CD20 mAbs. 18/23 used the same germline gene J*558.42* and 4/23 used closely related genes of the 'J*558*' group. Thus, 22/23 belonged to VH1 family. One exception was the mAb 1K1791, which was derived from the *VH9.12* germline gene. 1K1791 was also unique in its use of a Vk19/28 family gene whereas most other mAbs (21/23) used Vk4/5 family genes. A formal relationship between the particular germline gene recruitment and antibody functionality has not been established, however, the present findings identified humanized mAbs with functional activities that were superior to rituximab and 2F2. These *in vitro* results support future *in vivo* animal testing and subsequent clinical trials.

Correspondence to: Michio Nishida, Late Effects Studies, Radiation Biology Center, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan
E-mail: minishida@aol.com

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Introduction

Different anti-CD20 mAbs have been shown to have diverse functional properties and epitope specificities and mediate varied effects on cell death either by direct trans-membrane signaling or other cytotoxic mechanisms (1-3). Further, CD20 mAbs have been shown to sensitize tumor cells to both chemotherapy and immunotherapy (4,5). The exact function of the CD20 molecule is unknown. It has no known ligand and is purported to act as an ion channel to facilitate re-entry of intracellular calcium following BCR-induced efflux (6).

CD20 is a member of the membrane-spanning 4A gene family (MS4A). The size of the molecule is 33-35 kD but with only about 43 amino acids exposed on the extra-cellular surface. Deletion mutant studies by Polyak *et al* (7) demonstrated that the sequence AxP at position 170-172 is important in determining the secondary structure of the extracellular loops to allow antibody binding. However, it is not clear whether the sequence itself represents a contact point for CD20 antibody binding or is only responsible for maintaining a particular structural configuration. Most functional anti-CD20 mAbs do not react with synthetic linear epitopes or denatured antigen, suggesting that even though the extracellular portion is small there must be sufficient secondary structure to establish conformational epitopes. All of the monoclonal antibodies described to date that

recognize the extracellular loops, not surprisingly, partially or completely cross-block each other's binding (8-10). The binding specificities of several different antibodies were dissected by Polyak *et al* (7) who defined seven different patterns of binding, although many shared similar or overlapping epitopes with those already defined by other groups (11). Further, they showed that the mAb 1F5, which activates resting B cells, differed from the other mAbs in its inability to precipitate CD20 from detergent lysates. They concluded that the functional reactivity of 1F5 was dependent on the integrity of the CD20 oligomeric complex on the cell surface.

Although most CD20 mAbs exhibit CDC (complement-dependent cytotoxicity), and ADCC (antibody-dependent cellular cytotoxicity) *in vitro* and many exhibit apoptotic activity, the *in vivo* mechanisms of cell killing are far from clear. The activities of rituximab and ofatumumab are thought to be predominantly through CDC and ADCC (12,13); although a number of other anti-CD20 antibodies appear to operate in the absence of effector functions.

The overall response rate (complete response plus partial response) of B-NHL patients treated with rituximab ~50%. It is not clear why the remaining patients, though their tumors express CD20, do not sufficiently respond to treatment. In a recent study, we have reported on the generation of several murine anti-CD20 mAbs and demonstrated that many exhibited higher functional activities compared to 2B8 (the murine parental mAb for rituximab) or rituximab (14). We speculated such novel anti-CD20 mAbs with enhanced effector functions and different biological activities might potentiate the clinical response. One murine mAb in particular, 1K1791, showed significant direct inhibition of growth in several malignant B-cell lines. We assumed, therefore, that the generation of chimeric and humanized mAbs forms of 1K1791 would maintain or exceed the superior activities observed with murine 1K1791.

The present study was designed to test the above hypotheses and the following were investigated: i) characteristics of chimeric anti-CD20 mAbs and humanized mAbs derived from 1K1791; and ii) examination of the relationship between V gene usage of anti-CD20 antibodies and their functional activities *in vitro*, such as direct inhibition of cell growth and apoptosis, in comparison with 2B8. Eight murine anti-CD20 mAbs were selected and chimerized for comparison with rituximab for CDC and ADCC activities. One of these antibodies was selected for humanization based on the outcome of functional experiments with both original murine and the chimeric versions. The murine antibody parent was then subjected to a number of different humanization techniques.

Materials and methods

Cells and antibodies. The $CD20^+$ cell lines, Raji (Burkitt's lymphoma) and CCRF-SB (acute lymphoblastic leukemia) were obtained from JCRB (Riken Bioresource Center, Tsukuba, Japan). The SU-DHL4 (Diffuse large cell type B-NHL) and RC-K8 (Histiocytic lymphoma) were obtained from DSMZ GmbH (Braunschweig, Germany). CHO DG44 was from Invitrogen Japan (Tokyo, Japan) (15). The hybridoma producing murine anti-CD20 mAbs, with the prefix '1K', were described in our previous report (14). Rituximab (chimeric anti-CD20 mAb) and 2B8 were obtained from Zenyaku Kogyo (Tokyo, Japan) and infliximab was from Tanabe Pharmaceutical (Osaka, Japan). 2F2, human anti-CD20 mAb also known as ofatumumab, was generated through reverse engineering of the 2F2 DNA sequences described in the US patent publication and transfecting them into CHO cells (16).

Gene family analysis. Amino acid sequences of hybridoma producing anti-CD20 mAbs, as well as the published anti-CD20 mAb sequences, were assigned to germline variable genes. This was done by comparison to 97 Vk and 284 VH individual germline genes and their families from GenBank (National Center for Biotechnology Information, Bethesda, MD) and three other publications (17-19).

Chimeric antibody development. The murine anti-CD20 mAbs were chimerized as murine/human IgG1/kappa versions (designated with the prefix 'c1K' (c1K mAbs) and the Vk and VH DNA sequences inserted into pNOW-ab, a modified version of pNOW (20), for antibody expression in CHO DG44 cells.

Humanized antibody development. The humanized sequences were designed using four different humanization techniques. These were a) by 'veneering', whereby the exposed framework residues were replaced by amino acids found at corresponding positions in human antibody sequences (21); b) 'grafting of abbreviated CDRs', whereby only the parts of the CDRs, which contain antigen-contacting residues in antibody:antigen complexes of known three-dimensional structure, were grafted onto the most homologous human variable domains (22); c) 'SDR-transfer', whereby only the residues in direct contact with antigens, were transferred into the most homologous human domains (22); and d) grafting the whole CDRs onto a composite framework built from the most homologous segments from different human variable domains (23). In b), c) and d), human germline sequences were used. The sequences humanized using a), b), c) and d) were labeled 'ven', 'abb', 'sdr' and 'fra', respectively.

The conversion of amino acids to nucleotides was done with reference to the consensus DNA sequences of human immunoglobulin Vk and VH genes in GenBank. The nucleotide sequences of humanized Vk and VH were synthesized by Hokkaido System Science (Sapporo, Japan) inserted into pNOW-ab and transfected into CHO DG44 cells with assistance from the Bacteriology Division at the Tottori University (Yonago, Japan). The humanized anti-CD20 mAbs derived from 1K1791 were named by the abbreviated names of the humanization technique used for both light and heavy chains, i.e. light chain/heavy chain: abb/abb, abb/fra, abb/sdr, abb/ven, fra/abb, fra/fra, fra/sdr, fra/ven, sdr/abb, sdr/fra, sdr/sdr, sdr/ven, ven/abb, ven/fra, ven/sdr and ven/ven, respectively. Amino acid replacements are shown in Table I.

Inhibition of cell growth by murine mAbs or humanized mAbs. A total of 19 murine and 16 humanized anti-CD20 mAbs originating from 1K1791 were used in this study to

Table I. Amino acid replacement during humanization.

	A. Heavy chain variable domain						
	FR1	CDR1	FR2	CDR2	FR3	CDR3	J
1K1791	QIQLVQSGPELKKPGETVKISCKASGYTFT	NFGVN	WVKQAPGKGLKWMG	WINTYTGEPSYADDFKG	RFAFSLEASANTAYLQINNLKNDDMSTYFCTR	RTNYYGTSYYYAMDY	WGQGTSVTVSS
abb	--------S----------V----------	-----	--R-------E---	----------QG-T-	--V------V----------TA------	----------	----------
fra	--------S----------V----------	-----	--K-------K---	----------DD-K-	--A------A----------MA------	----------	----------
sdr	--------S----------V----------	-----	--R-------K---	----------QG-T-	--A------V----------TA------	----------	----------
ven	--------P----------I----------	-----	--K-------K---	----------DD-K-	--A------V----------TS------	----------	----------
	B. Light chain variable domain						
	FR1	CDR1	FR2	CDR2	FR3	CDR3	J
1K1791	STVMTQTPKFLLVSAGDRVTITC	KASQSVSNDVA	WYQQKPGQSPKVLIY	FASNRYT	GVPDRFTGSGYGTDFTFTINTVQAEDLAVYFC	QQDYSSPLT	FGAGTKLELK
abb	---------DS-AV-L-E-A--N-	-S----V------	----------	-------S	----------L----L----------	---------	--------I-
fra	--------SF-SA-V-D-V--T-	-A----V------	----------	-------T	----------L----L----------	---------	--------I-
sdr	--------DS-AV-L-E-A--N-	-S----N------	----------	-------S	----------L----L----------	---------	--------L-
ven	--------DS-AV-L-E-V--N-	-A----V------	----------	-------T	----------F----V----------	---------	--------L-

The humanized antibodies were various combination of the 'abb', 'fra', 'sdr' and 'ven' independently humanized light and heavy chain variable domains. The table demonstrates the amino acid replacements from the parental murine mAb 1K1791. The replacement at CDRs was minimal so that the biological properties of humanized anti-CD20 mAbs were not predominantly dependent on the CDRs but rather influenced by the framework modification. The modification of light chain J-regions was a conservative change of leucine to isoleucine. Sequences of CDRs of the parental murine mAb are underlined.

define their inhibitory effects on the growth of Raji or SU-DHL4 cells in the absence of lytic components. The studies were done in a concentration and time-dependent manner using either 0.3 μg/ml or 1.0 μg/ml after 24, 48 and 83 h of continuous culture for the murine mAbs, and 0.5 μg/ml after 24 and 81 h for the humanized mAbs, respectively. The concentration used for humanized mAbs was determined in reference to the results of murine mAb studies suggesting 0.3-1.0 μg/ml was appropriate to examine. Chromogenic reagent (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan) was added and incubated for an additional 4 h. The plates were then read in a micro-plate reader (Hitachi High-Technologies, Tokyo, Japan) and the absorbance at 492 nm was recorded. The cell growth in the presence of each antibody was compared to that without antibody and the inhibition determined. The murine mAbs were compared to 2B8 and humanized mAbs compared to rituximab and 2F2.

CDC assay of chimeric mAbs or humanized mAbs. The ability of chimeric mAbs to promote CDC against RC-K8 cells or humanized mAbs against RC-K8 and SU-DHL4 cells was examined using the methods of Gazzano-Santoro *et al* (24) and Idusogie *et al* (25). The chimeric mAbs were compared with rituximab and humanized mAbs compared with rituximab and 2F2. Infliximab and human complement (Quidel, San Diego, CA) solution without antibody were used as negative controls and Triton X-100 was used to give a maximum value of cell lysis. Each of the test antibodies was diluted with RHB buffer; RPMI-1640, 0.1% bovine serum albumin (BSA) (Sigma, St. Louis, MO), 20 mM HEPES at pH 7.2, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin resulting in 0.01-10.0 μg/ml of chimeric antibodies with RC-K8 cells. A range of 0.08-10.0 μg/ml was applied to the humanized antibodies with either RC-K8 or SU-DHL4 cells. The cells were washed in RHB buffer and re-suspended at $1x10^6$ cells/ml density and 50 μl of the cell suspension was loaded into 96-well TC black plate (Nunc A/S, Roskilde, Denmark). Each antibody solution (50 μl) was added with 50 μl of human serum complement and cell suspension and incubated thereafter at 37˚C for 2 h. Alamar blue (BioSource, Camarillo, CA) was added and the culturing continued for another 18 h. Heat-inactivated human serum complement was used as a negative control. The plate was quenched at room temperature for 10 min. Fluorescence was read using a 96-well fluorometer with excitation at 530 nm and emission at 590 nm. The results are expressed in relative fluorescence units (RFU) which are proportional to the number of viable cells. The activity of various sample antibodies was examined by plotting the CDC activity by percent against the log of antibody concentration, present before the addition of Alamar blue, using 4-parameter curve fitting program (Kaleidagraph).

ADCC assay of chimeric mAb or humanized mAbs. The ADCC of chimeric mAbs or humanized mAbs against RC-K8 and SU-DHL4 cells was assayed according to a standard protocol (26). Peripheral blood mononuclear cells (PBMC) were isolated from blood of three normal donors (A, B and C) by Ficoll-Hypaque separation (GE Healthcare/Amersham Biosciences, Piscataway, NJ) as effector cells and washed twice with Hanks Balanced Salt Solution (Invitrogen) to remove platelets, and re-suspended in RPMI media with 10% heat-inactivated fetal bovine serum (HIBS) ($5x10^6$ cells/ml) and antibiotics. An E:T ratio (effector cells to target cells) of 50:1 was used. The chimeric mAbs were compared with

rituximab and the humanized antibodies compared with rituximab and 2F2. Infliximab was used as a negative control for both studies. Target cells were labeled with 1 mCi of ^{51}Cr (1 mCi/ml Na_2CrO_4/6x10^6 cells, GE Amersham) for 1 h and incubated at 37˚C for 2 h with PBMC in the presence of antibody. The cells were washed twice, incubated at 37˚C for 15 min and then washed twice again. The cells were counted and re-suspended in RPMI media with 15% HIBS at 1x10^5 cells/ml. ^{51}Cr labeled target cells (100 μl) were added to each well (1x10^4 cells/ml) with 100 μl of sample antibody solution (30 μg/ml). The final concentration of sample antibody was 10 μg/ml. The supernatant was removed for quantification of ^{51}Cr release. Controls with target cells and PBMC but no antibody determined spontaneous ^{51}Cr release for individual assays. Triton (5%) was used as a measure of complete cell lysis. Four replicates were performed for each sample.

Affinity measurement and competitive binding assay by ^{125}I-labeled antibody. The assay of equilibrium dissociation constant (Kd), total amount of antibody bound to the target cells (B_{max}) and EC_{50} were performed according to standard protocols. The ^{125}I-labeled antibody was prepared using sodium [^{125}I]-iodide and Iodogen (GE Healthcare/Amersham Biosciences), purified by size exclusion chromatography and diluted in PBS with 0.25% BSA. Raji cells were incubated in RPMI-1640 with 10% fetal bovine serum (FBS) plus antibiotics in the presence of a range of antibody concentration under equilibrium conditions. Detection of ^{125}I-labeled antibody bound to cells after thorough washing was performed on a gamma counter. All data analyses were performed using non-linear regression curve fitting provided by GraphPad Prism software (version 3.02) (27). Competition of rituximab binding to target cells by the humanized anti-CD20 mAbs or cold rituximab was also measured.

Cytotoxicity by apoptosis or necrosis. Apoptosis and necrosis induced by humanized mAbs were measured by flow cytometry using the Annexin V/FITC and propidium iodine (PI) staining methods (Annexin V/FITC apoptosis detection kit, BD Biosciences, Franklin Lakes, NJ). This method allows the examination of live cells (Annexin$^-$/PI$^-$), early stage of apoptosis (Annexin$^+$/PI$^-$), late-stage of apoptosis (Annexin$^+$/PI$^+$) and necrotic cells (Annexin$^-$/PI$^+$). Eleven humanized and three control antibodies-rituximab and 2F2 as positive controls and murine anti-CD3 mAb as a negative control (BioLegend, San Diego, CA) were tested against RC-K8 and Raji cells. Goat anti-human IgG (Jackson Immunoresearch Laboratories, West Grove, PA) was used as a cross-linking antibody. The amount of each antibody used in this study was 10 μg/ml. RC-K8 cells were maintained in RPMI-1640 media with 15% FBS plus antibiotics and Raji cells were in RPMI-1640 with 10% FBS plus antibiotics. Cells (5x10^5 cells/ml) were sub-cultured for one or two days prior to seeding for the assay, to assure the cells were in logarithmic growth at the start of the assay and then incubated in duplicate with 10 μg/ml test or control antibodies, in the presence or absence of 10 μg/ml cross-linking antibody. Following incubation for 24 h, cells were pelleted and washed in PBS, re-suspended in FACS staining buffer, and stained with Annexin V/FITC and PI, according to the manufacturer's specification. Flow cytometry was performed with a FACS Calibur 2 and analyzed by CellQuest software (BD Biosciences).

Analysis of caspase activation. Three humanized anti-CD20 mAbs, sdr/abb, sdr/fra and abb/abb, that demonstrated greater relative apoptosis values compared to other humanized antibodies, were used in the activation study of caspase-3, 7 and 9 (Promega, Madison, WI) using Raji cells as the target. Rituximab was used as a control. Antibodies were incubated in the presence or absence of a cross-linking antibody, goat anti-mouse IgG for murine antibodies or goat anti-human IgG for humanized antibodies (Pierce Biotechnology, Rockford, IL). Anti-CD3 mAb and infliximab were used as negative controls. Samples were incubated for 24 h and caspase activity was measured using a commercial detection assay with luminescence readout. Raji cells were incubated in RPMI-1640 containing 10% HIBS and penicillin/streptomycin at 37˚C and sub-cultured every 2-3 days. Raji cells were plated at 100 μl/well in a 96-well flat bottom TC plate. Test and control antibodies (5-10 μg/ml) were added in the presence or absence of cross-linking antibody (5-10 μg/ml). Ionomycin (1 μM; EMD Chemicals, San Diego, CA) treated cells served as an additional positive control. A pan-caspase inhibitor, zVAD-fmk (Promega), was added to some wells (10-20 μM), to demonstrate specificity. Cells were incubated for up to 48 h. An equal volume of caspase-3, -7 or -9 substrate was added and the plates were agitated on a tabletop shaker for 30 sec at 300 rpm and further incubated at room temperature from 45 min to 2 h (based on the Promega technical bulletin of Caspase-Glo™ 3/7 Assay System: TB323, Caspase-Glo™ 9 Assay System). The luminescence was read after 1 and 2 h with a Wallac Victor2 plate reader (Perkin-Elmer, Waltham, MA).

Results

Potentiation of effector functions of chimeric and humanized anti-CD20 mAbs in comparison with rituximab and 2F2; *Complement-dependent cytotoxicity* (CDC). Our recent findings demonstrated that the novel murine anti-CD20 mAb 1K1791 exhibited unique cytotoxic effects in comparison with several other generated mAbs and rituximab (14). Consequently, we generated chimeric mAbs from several murine anti-CD20 mAbs and examined their effector properties. Analysis of CDC against the RC-K8 cells revealed that the chimeric mAb c1K1791 was the most cytotoxic with ~75% of cell death at a concentration of 10 μg/ml whereas the other chimeric mAbs induced cytotoxicity ranging from 10-50% when used at the same concentration. Rituximab showed 29% cytotoxicity. At a lower concentration of 1.0 μg/ml, chimeric 1K1791 (c1K1791) induced 50% cell death whereas the other mAbs showed 5-40% and rituximab gave 18% (Fig. 1A).

The humanized mAbs derived from 1K1791 were tested for CDC against both RC-K8 and SU-DHL4 cells. All of the humanized antibodies except for fra/ven induced up to 80% of cell death against RC-K8 cells (Fig. 1B) and close to 100% against SU-DHL4 cells (Fig. 1C) at 10 μg/ml concentration. By comparison, the humanized antibodies, excluding fra/ven, were more cytotoxic against RC-K8 cells

than rituximab (5-6-fold) and 2F2 (>120%) at 10 μg/ml concentration. More significant differences of cytotoxicity of the humanized antibodies against RC-K8 cells were seen at a lower concentration. However, there was only a slight difference among antibodies with SU-DHL4 cells and this cell line was more sensitive than RC-K8.

The above findings demonstrate that humanized and chimeric antibodies from 1K1791 were more cytotoxic than rituximab and 2F2 against RC-K8 cells. The cytotoxicity of humanized antibodies was slightly higher than rituximab or 2F2 with SU-DHL4 cells. The findings also established that humanization retained the CDC activity of the chimeric mAb in most cases.

Antibody-dependent cellular cytotoxicity (ADCC). The chimeric and humanized anti-CD20 mAbs were tested for ADCC activity using human PBMCs derived from three normal donors as effectors and both radiolabeled RC-K8 and SU-DHL4 cells as targets. The ADCC activity of the chimeric mAbs against RC-K8 and SU-DHL4 cells varied from donor to donor although the level of cytotoxic activity was similar for all the antibodies tested. Rituximab was effective in inducing ADCC though slightly less than c1K mAbs (Fig. 2A and B). Similar findings were observed with the humanized mAbs though there were some differences among the antibodies tested, especially against RC-K8 (Fig. 2C and D). These findings from three different normal donors demonstrate that the chimeric and humanized mAbs exerted significant ADCC activities against two cell lines and that these were higher than those induced by rituximab or 2F2.

Inhibitory effect for tumor cell proliferation. The humanized mAbs were tested for their inhibitory effect on RC-K8 and SU-DHL4 tumor cell growth. The tumor cells were incubated for 81 h (Raji) and 57 h (SU-DHL4) in the presence or absence of 0.5 μg/ml antibody. Cell growth was determined as described in Materials and methods. For RC-K8 humanized mAbs inhibited 20-40% of cell growth whereas rituximab or 2F2 showed only 15% inhibition (Fig. 3A). For SU-DHL4 the humanized mAbs inhibited a range of 30-50% whereas rituximab inhibited 50% and 2F2 inhibited only 25% (Fig. 3B). Of note, the humanized sdr/abb, sdr/fra and sdr/sdr mAbs exhibited the more pronounced inhibitory effects against Raji and SU-DHL4 cells among the tested antibodies.

Induction of cytotoxic effects. The humanized mAbs were tested for cytotoxic activity against RC-K8 and Raji cells in

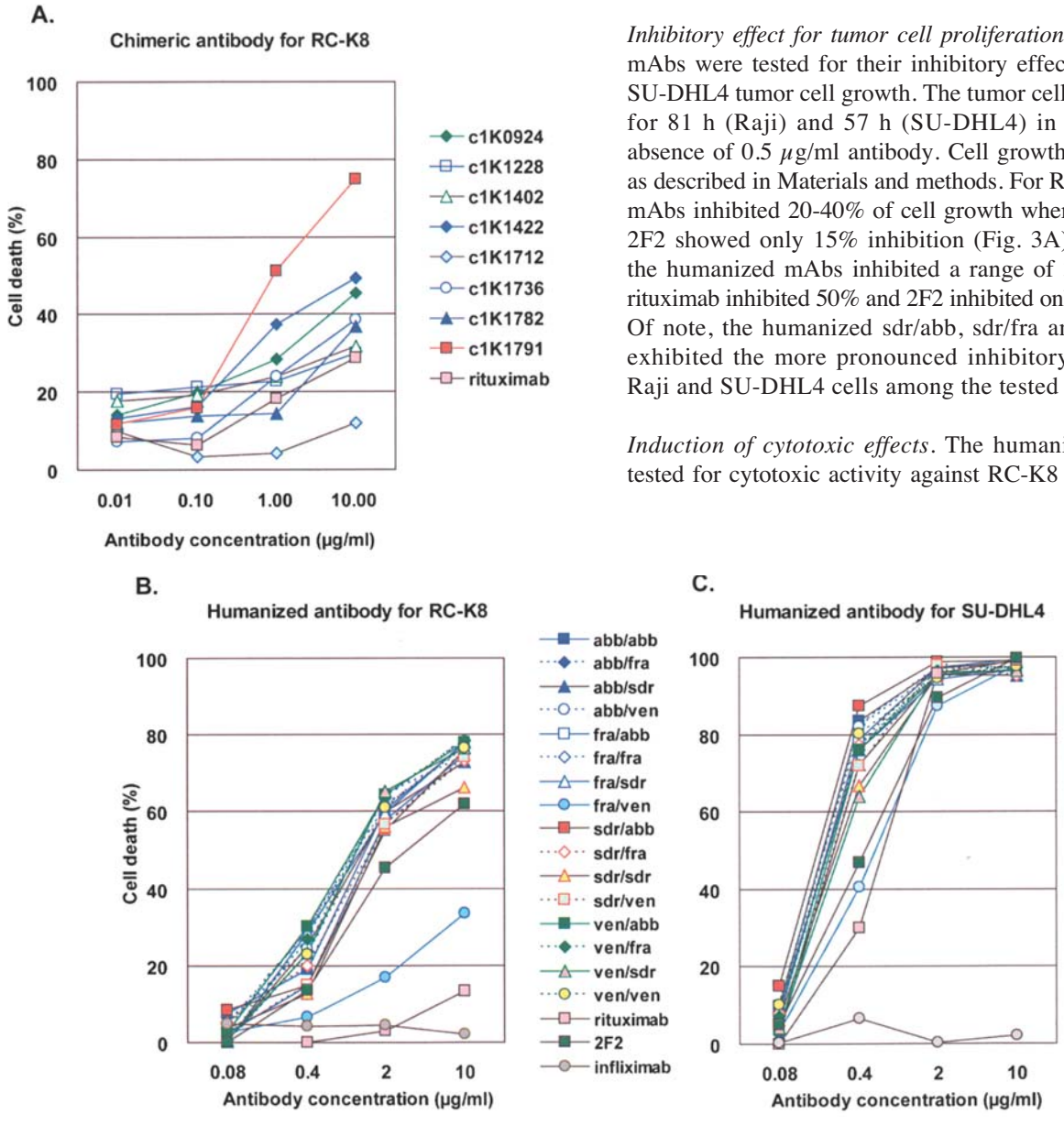


Figure 1. CDC induction by chimeric mAbs or humanized mAbs. CDC induction against RC-K8 cells by chimeric mAbs (A). CDC by humanized mAbs against RC-K8 or SU-DHL4 cells (B and C). Rituximab and infliximab were used as positive and negative controls respectively for the chimeric mAb study. Rituximab and 2F2 were used as positive controls and infliximab as a negative control for humanized mAb studies. Tests were done in concentration-dependent manner for chimeric mAb study ranging from 0.01 to 10.00 μg/ml and for humanized mAb study ranging from 0.08 to 10.0 μg/ml based on the results of minimum concentration of antibody effectively induced CDC. The results of cell death (lysis) are shown graphically from 0 to 100% by adjusting 0% for (target cells + human serum complement) and 100% for (target cells + Triton X-100). Samples were run in 3 replicates (n=3) for both chimeric and humanized mAbs.

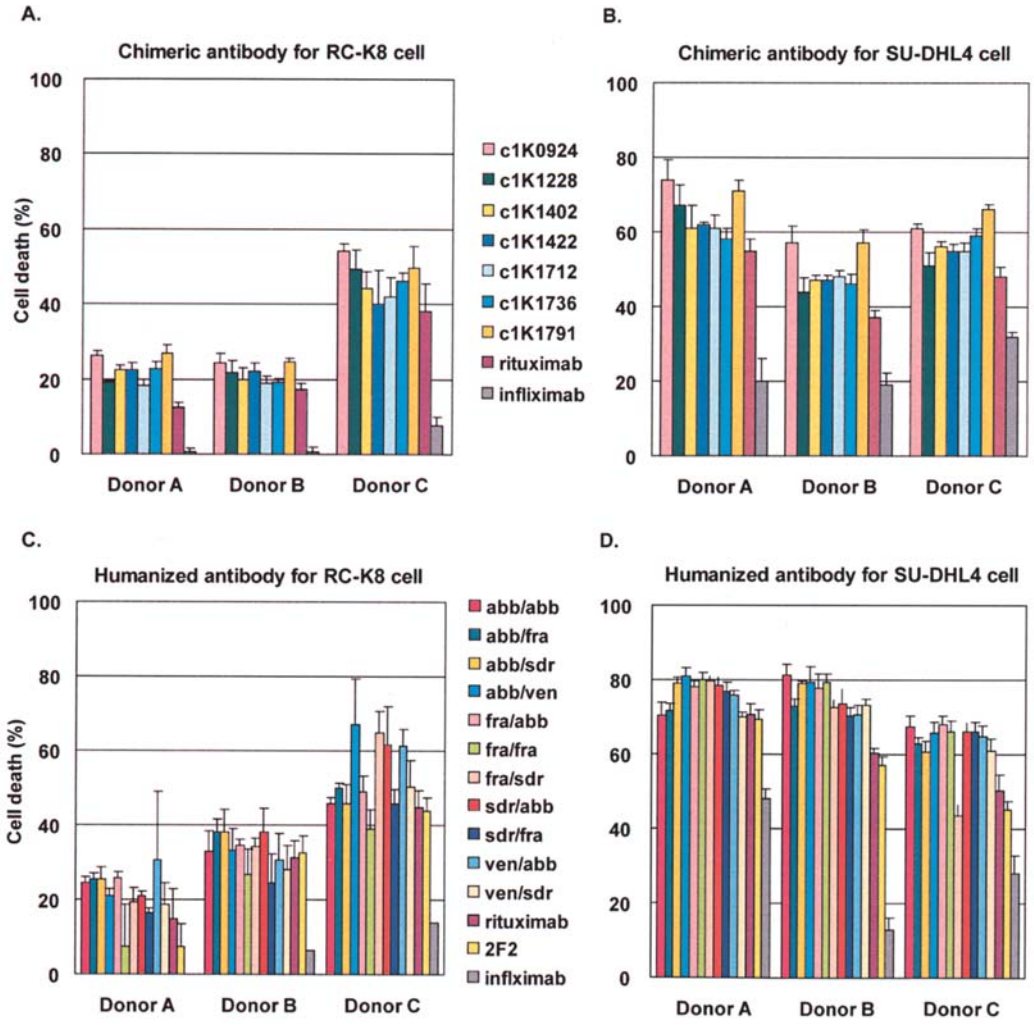


Figure 2. ADCC induction by chimeric or humanized mAbs. ADCC induction against RC-K8 or SU-DHL4 cells by chimeric mAbs (A and B), or by humanized mAbs (C and D) was measured using PBMCs from three donors (donors A, B and C). For studies on SU-DHL4 by humanized mAbs, donors B and C were substituted for other donors because of their unavailability at the requested time. Rituximab and infliximab were used as positive and negative controls respectively for the study of chimeric mAbs. Rituximab and 2F2 were used as positive controls and infliximab as a negative control for humanized mAbs. The results of cell death (lysis) are shown graphically from 0 to 100% by adjusting 0% for (target cells + PBMCs) and 100% for (target cells + Triton X). Samples were run in 5 replicates (n=5) for chimeric mAbs and in 4 replicates (n=4) for humanized mAbs.

the presence or absence of a secondary cross-linking antibody. Total cell death, apoptosis and necrosis were assessed as described in Materials and methods. Moderate cytotoxic effects were observed against RC-K8 cells with sdr/abb and sdr/fra and more significant cytotoxicity against Raji cells (Fig. 4A and C). By comparison, neither rituximab nor 2F2 showed any effect. In the presence of cross-linking antibody, there were modest but significant increases in cytotoxicity with most of the mAbs, including rituximab and 2F2 (Fig. 4B and D). These findings demonstrate that some humanized antibodies can induce a higher level of apoptosis in the presence or absence of a secondary cross-linking antibody relative to rituximab or 2F2.

Apoptosis induction by humanized mAb is caspase-independent. We previously reported (14) that the apoptosis induced by some murine anti-CD20 mAbs was caspase-independent and consistent with the reports by other groups (28-30); including the observation that rituximab-mediated death in Raji cells lacked certain characteristic apoptotic features and appeared to be independent of the usual caspase cascade activity. Addition of rituximab (10 μg/ml) and cross-linking antibody (10 μg/ml) did not result in increased luminescence when compared to untreated cells, suggesting that rituximab does not activate caspase-3, 7 and 9 under these conditions (data not shown). Both substrates gave a titratable luminescence signal within the range of 1000-5000 cells. Independence of caspase-3 and 7 activation was demonstrated using murine 1K mAbs or humanized mAbs such as sdr/abb, sdr/fra and abb/abb wherein incubation of these antibodies with Raji cells did not result in an increase in luminescence (Fig. 5). Ionomycin, a positive control for induction of apoptosis, induced significant activation (80%) of caspase-3/7 in Raji cells in the same assay, suggesting that anti-CD20 induced apoptosis or necrosis was caspase-independent.

Affinity measurement and competitive binding assay by ^{125}I-labeled antibody. It is usually assumed that an antibody being bivalent binds to two independent non-interacting sites and

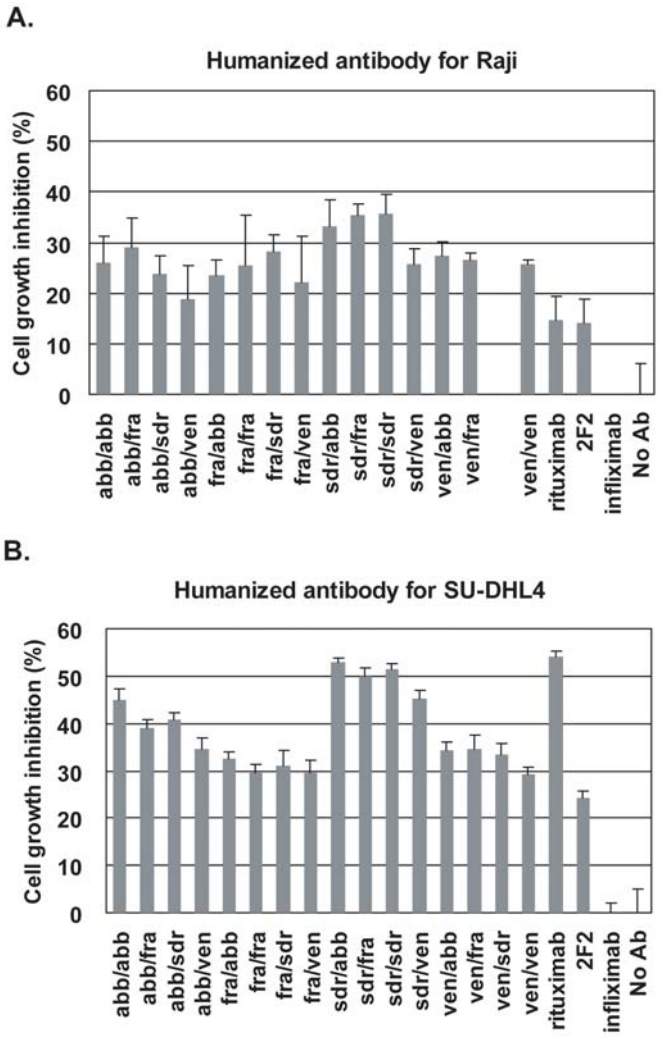


Figure 3. Direct inhibition of cell growth of Raji or SU-DHL4 cells by humanized mAbs. The growth of Raji and SU-DHL4 cells was measured in the presence of 0.5 μg/ml of each humanized mAb without effector components. The percentages of inhibitory effect in the figure are shown minus the percentage of the culture without antibody. The effect of each mAb against Raji cells was determined after 81 h culture and against SU-DHL4 cells after 57 h culture, respectively. Rituximab and 2F2 were used as positive controls, and infliximab or the culture without antibody as negative controls. Ven/sdr was not available for the study of Raji cells. Samples were run in 4 replicates (n=4) for humanized mAbs including control mAbs.

that binding to each site follows the law of mass action. In this study, we used GraphPad Prism software to individually analyze whether the binding of each antibody best fitted single-site or two-site binding kinetics through determination of Kd, B_{max} and EC_{50} (Table II). Most humanized antibodies had binding characteristics that fitted a two-site binding equation. The Kd values for the humanized mAbs ranged from 0.92 to 2.54 nM while repeated measurements of Kd for rituximab gave an average value of 1.38 nM. B_{max} values for the humanized mAbs were grouped into three, one ranged from 1.7-2.9x10^5 cpm which was most common, a larger group of 7.5-8.6x10^5 cpm and a middle group of 3.4-3.7x10^5 cpm, although any correlation to physical or functional properties was not identified. The EC_{50} for each humanized anti-CD20 in competition with radiolabeled [^{125}I]-rituximab was measured using Raji cells in suspension. The humanized antibodies most competitive with rituximab were fra/abb, sdr/abb and fra/sdr and were equivalent or stronger than rituximab binding itself. Other humanized mAbs although weaker, still showed significant competition.

Relationship between biological properties of anti-CD20 mAbs and their germline gene origin. There was no clear relationship between the variable region germline gene of origin for the various anti-CD20 mAbs (Table III) and their biological properties described in our previous study (14). Nevertheless, the murine antibody 1K1791 did exhibit significant inhibition of cell growth and apoptosis even without a secondary cross-linking antibody and the chimerized version of 1K1791 also demonstrated significant CDC and ADCC activity (Figs. 1 and 2). It is noteworthy because 1K1791 was the only monoclonal antibody selected that was not derived from the VH1 gene family. The heavy-chain CDR3 region of 1K1791 was 15 amino acids long, which was the longest of any of the mAbs examined in this study. Rock *et al* examined 1620 murine VH regions and showed an average CDR3 length of 8-9 residues in the mouse (31).

Relationship between physical properties and biological properties of humanized mAbs. The considerably larger B_{max} of ven/abb, abb/sdr and abb/ven, almost four times larger than rituximab and other common humanized mAbs, did not show any correlation with any of their biological properties (Table II, Figs. 1-4). The Kd values also did not have any impact on the biological properties within <3-fold range. Sdr/abb and sdr/fra which demonstrated significant biological effects compared to rituximab, showed similar Kds (1.32, 1.33 nM, respectively whereas 1.38 nM for rituximab) but >2-fold difference of B_{max} (1.7x10^5, 4.0x10^5, respectively whereas 2.1x10^5 for rituximab). These results suggest the strongest binding is not a sufficient enough factor alone to induce the most potent cytotoxicity, inhibition of cell proliferation or apoptosis.

Discussion

The present study examined a number of anti-CD20 mAbs that were developed by us as well as some pre-existing anti-CD20 mAbs whose properties and sequences have been published. We identified a chimeric antibody, c1K1791, which showed significantly enhanced functional activities compared to other antibodies including rituximab. The original murine antibody 1K1791 was the top choice for humanization using four different methods to derive 16 humanized variants. Most of these humanized mAbs showed superior functional activity in comparison with rituximab and 2F2.

In a recent study, we reported the unique activity of the murine 1K1791 anti-CD20 mAb over other generated mAbs, including 2B8 (the murine parent of rituximab). We pursued the development of chimeric and humanized antibodies from 1K1791, therefore, with the objective of validating their unique features and their potential clinical applications. The present findings demonstrate that the chimeric mAb c1K1791 showed significant functional activities and humanization of 1K1791 resulted in the generation of several mAbs that

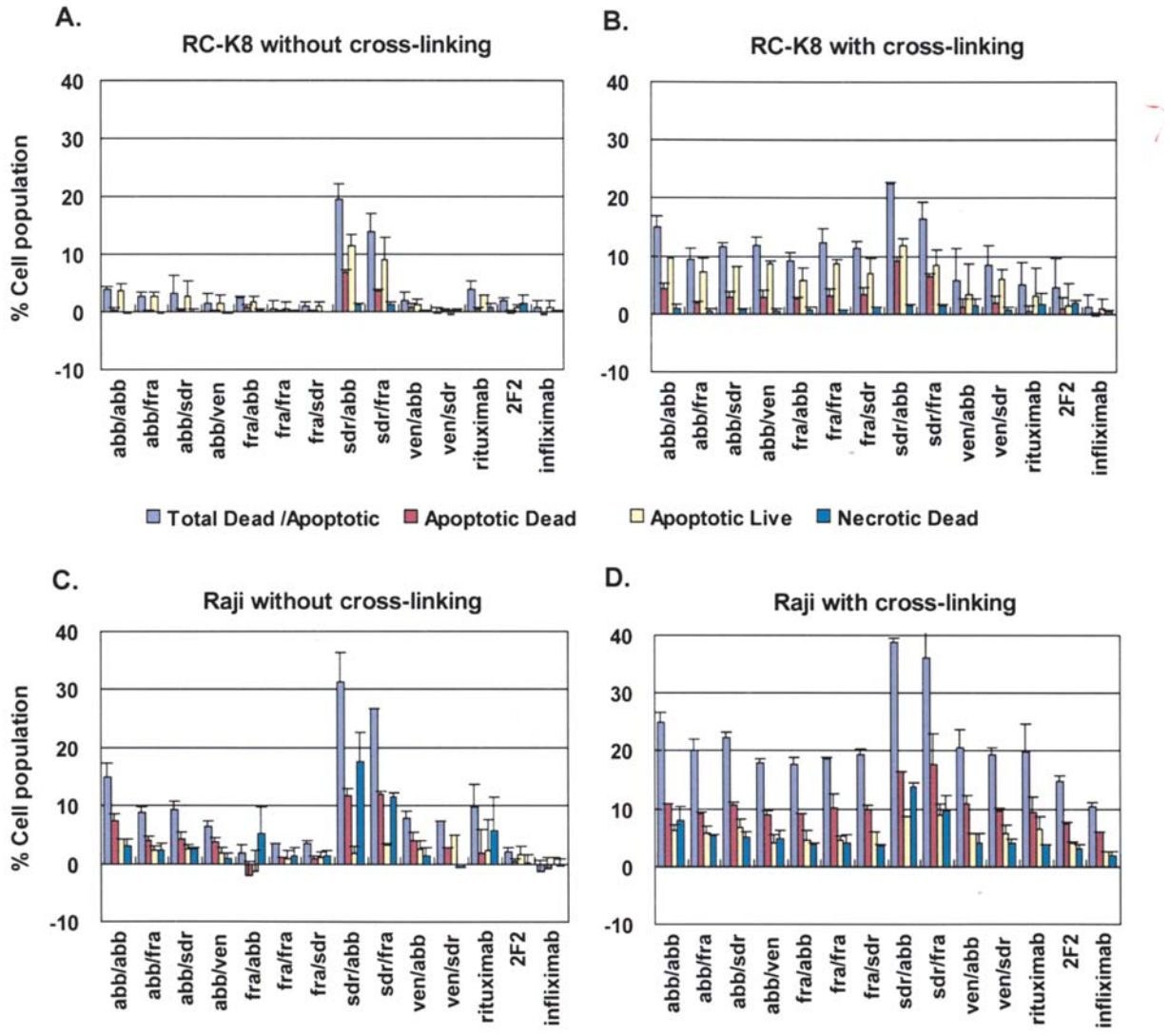
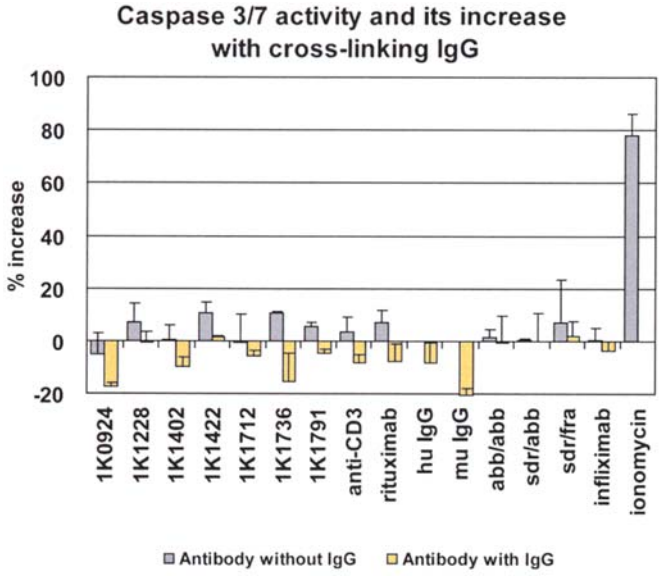


Figure 4. Analysis of cell death mediated by humanized mAbs. RC-K8 and Raji cells were treated with humanized mAbs for 24 h, without or with cross-linking antibody. This cell culture with antibody treatment was performed in duplicate. Apoptotic dead, apoptotic live and necrotic dead were measured by flow cytometry with Annexin V/FITC and PI staining. Rituximab and 2F2 were used for the comparison and infliximab as the negative control. Cells were incubated in duplicate with test or control antibodies (10 μg/ml), in the presence or absence of cross-linking antibody (10 μg/ml). The results of the flow cytometric analysis of RC-K8 cells either with or without cross-linking IgG (A and B) and that of Raji cells either with or without cross-linking IgG (C and D) were demonstrated.



also retained such potent functional activities. These newly developed humanized antibodies were generally superior to rituximab and 2F2 in many *in vitro* activities such as CDC, ADCC, inhibition of cell growth and cell death including apoptosis. There were two humanized antibodies, sdr/abb and sdr/fra, that exerted more significant inhibition of cell growth and induction of cell death and these became candidates for potential follow-up of their anti-tumor effects in animals and in clinical trials.

Figure 5. Failure to activate caspase-3/7 by humanized or murine anti-CD20 mAbs. Increase in caspase-3/7 activities with anti-CD20 mAbs, murine 1K mAbs, humanized mAbs or rituximab were measured in Raji cells in the presence or absence of cross-linking antibody. Murine IgG (mu IgG), human IgG (hu IgG), anti-CD3 mAb and infliximab were used as negative controls. Ionomycin was used as a positive control for the induction of caspase-dependent apoptosis also in Raji cells. All values are shown minus the value using cells with caspase-3/7 substrate alone. Ionomycin was tested without antibody.

Table II. Binding affinities of humanized mAbs and competition against ^{125}I-labeled rituximab.

	Binding affinity			Total amount bound		Binding competition with rituximab	
	Kd (nM)	SD	R^2	B_{max}	SD	EC_{50} (nM)	R^2
abb/abb	1.624	0.006	0.992	$2.8x10^5$	$2.0x10^4$	51	0.971
abb/fra	1.936	0.011	0.993	$2.3x10^5$	$2.4x10^4$	71	0.983
abb/sdr	1.439	0.016	0.998	$7.5x10^5$	$0.7x10^4$	75	0.972
abb/ven	1.360	0.005	0.993	$7.7x10^5$	$3.4x10^4$	51	0.874
fra/abb	0.927	0.001	0.996	$2.1x10^5$	$0.7x10^4$	14	0.918
fra/fra	1.871	0.000	0.995	$3.7x10^5$	$2.5x10^4$	68	0.949
fra/sdr	1.016	0.001	0.996	$2.4x10^5$	$0.84x10^4$	37	0.919
sdr/abb	1.321	0.001	0.991	$1.7x10^5$	$0.7x10^4$	35	0.988
sdr/fra	1.334	0.003	0.993	$3.4x10^5$	$5.6x10^4$	94	0.973
ven/abb	2.537	n/a	0.991	$8.6x10^5$	n/a	54	0.934
ven/sdr	1.456	0.007	0.999	$2.9x10^5$	$1.9x10^4$	66	0.804
rituximab	1.382	0.013	0.996	$2.1x10^5$	$0.9x10^4$	38	0.982

Kd and B_{max} were determined in suspended Raji cells by non-linear regression analysis. The EC_{50} of each humanized mAb in competition with radiolabeled [^{125}I]-rituximab was analyzed using non-linear regression curve fitting using a single site parabolic curve fit. Raji cells were used as the target cell. R^2, Correlation coefficient. n/a, not available.

Table III. Germline VL and VH gene recruitment.

		VL		VH		
	Isotype	Best germline fit	Family	Best germline fit	Family	CDR3 length
B1	IgG1, k	*am4*	Vk4/5	*J558.9.99*	VH1	13
2H7	IgG1,k	*am4*	Vk4/5	*J558.9.99*	VH1	13
1F5	IgG1,k	*am4*	Vk4/5	*J558.42*	VH1	13
2B8	IgG1, k	*am4*	Vk4/5	*J558.42*	VH1	12
1k0911	IgG2b, k	*am4*	Vk4/5	*J558.42*	VH1	10
1K0924	IgG2b, k	*am4*	Vk4/5	*J558.42*	VH1	10
1K1712	IgG2a, k	*am4*	Vk4/5	*J558.42*	VH1	13
1k1728	IgG2a, k	*am4*	Vk4/5	*J558.42*	VH1	13
1k1409	IgG1, k	*am4*	Vk4/5	*J558.42*	VH1	12
1k1773	IgG2b, k	*am4*	Vk4/5	*J558.42*	VH1	13
1K1402	IgG1, k	*am4*	Vk4/5	*J558.42*	VH1	12
1k1405	IgG1, k	*am4*	Vk4/5	*J558.42*	VH1	11
1k1428	IgG1, k	*am4*	Vk4/5	*J558.42*	VH1	12
1k1436A	IgG1, k	*am4*	Vk4/5	*J558.42*	VH1	12
1K1736	IgG2b, k	*am4 (23-48)*	Vk4/5 (Vk23)	*J558.42*	VH1	13
1k1264	IgG1, k	*al4*	Vk4/5	*J558.42*	VH1	10
1k1301B	IgG1, k	*al4*	Vk4/5	*J558.42*	VH1	12
1k1316	IgG1, k	*al4*	Vk4/5	*J558.42*	VH1	12
1K1228	IgG1, k	*kf4*	Vk4/5	*J558.42*	VH1	10
1k1257	IgG1, k	*kf4*	Vk4/5	*J558.42*	VH1	10
1K1422	IgG1, k	*af4*	Vk4/5	*J558.55.149*	VH1	14
1K1782	IgG1, k	*23-48*	Vk23	*J558.45*	VH1	7
1K1791	IgG1, k	*19-32*	Vk19/28	*VH9.12*	VH9	15

The best germline VL and VH fit was identified for each murine anti-CD20. The CDR3 length shown is for the heavy chain variable region. Vk of 1K1736 did not fit well to a specific germline Vk gene but had an *am4*- like 5' sequence and a *23-48*-like 3' sequence. CDR3 length of 1K1791 was the longest whereas that of 1K1782 was the shortest.

The augmented functional activities observed with 1K1791 and its chimeric and humanized derivatives prompted us to examine whether these activities are associated with VH and Vk germline gene sequences. In this study, 16/19 anti-CD20 antibodies that we developed were derived from the same VH germline gene, namely '*J558.42*' and two closely related genes of the '*J558*' group which belong to the VH1 family. The exception was the mAb 1K1791 which used '*VH9.12*' belonging to the VH9 family. Examination of the light chain sequences showed that 17/19 anti-CD20 antibodies were from the Vk4/5 family and 21/23 if the 4 published anti-CD20 mAb sequences were included. 11/19 mAbs used the '*am4*' gene. 1K1791 was the only antibody to use the '*19-32*' gene which belonged to the Vk19/28 family. It is remarkable that so many antibodies independently generated against cell surface CD20 selected the same or closely related heavy and light variable region germline sequences.

Further examination of the published sequences for B1 and 2H7 showed minimal deviation from the germline Vk gene '*am4*' and the heavy chain region showed 100% identity to the germline VH gene '*J588.9.99*'. Little or no affinity maturation had occurred in these two antibodies. This germline gene combination, apparently, seems to have a natural affinity for the CD20 molecule without the need to undergo significant affinity maturation. 2B8 showed minor modifications from the germline Vk in the framework region and CDRs and only a single amino acid change in FR2 of the germline VH gene '*J558.42*'. 1F5 also showed several changes in the Vk gene but only a single amino acid change in FR1 of the germline VH gene '*J558.42*'.

It is possible that certain germline variable region genes have a 'pre-determined' affinity for certain antigens. There are precedents in the literature for this, for example, most murine antibodies against phosphatidylcholine have been shown to use the same VH11/Vk9 germline genes (32,33). Autoantibodies in cold agglutinin disease stemming from lymphoproliferative disorders have been shown to be highly correlated with the use of the human VH4-34 gene (34). The lack of extensive mutation due to affinity maturation and the predominant use of the same germline gene seen in the antibodies we describe may well be due to the paucity of strong immunogenic T cell epitopes on CD20 and/or reflect the highly conserved nature of the molecule. Alternatively, the gene families used by 1K1791 may recognize certain epitopes that either impart an enhanced ability to cross-link the CD20 molecule or promote its localization to lipid rafts. Cragg *et al* (35) have shown this to be a prerequisite for intracellular signaling by bringing together other cell surface molecules such as BCR, src, CD52. However, Chan *et al* (28) have stated that cross-linking of CD20 alone is insufficient to promote localization to lipid rafts and quote B1 as an example of an antibody unable to promote localization of CD20 into this compartment, even though B1 is a potent inducer of apoptosis.

Another intriguing observation is that many of the immunoglobulin molecules isolated from CLL cells share common germline sequences. Johnson *et al* (36) observed that 63% of CLL patients with functional VH gene rearrangements expressed VH1-69. The D and J region usage was restricted and, in addition, the average length of the heavy chain CDR3 was longer than that for normal B cells. These exhibit very similar characteristics to those seen by us in the murine antibodies raised against CD20. CLL cells are believed to arise from a minority population of B cells, the so called $CD5^+$ subset, the equivalent cells in the mouse being the Ly-1B subset. It may be possible that monoclonal antibodies against human CD20 arise from a relatively restricted set of cells, such as the Ly1-b subset. Alternatively, CD20 may be highly specific in selecting B cells with a pre-determined VH gene and Vk gene usage, and longer than average CDR3 regions, that have a high natural affinity for it without the need for extensive affinity maturation.

Teeling *et al* (10) reported that some of the anti-CD20 antibodies they tested recognized a region in the small loop of CD20 whereas other CD20 mAbs bound to the large loop of CD20 (3,37). There could also be such differential recognition of the small or large loops of CD20 among our 1K mAbs. However, examination of many of the biological properties of the 1K mAbs, c1K mAbs and humanized mAbs derived from 1K1791, as well as 2F2 and rituximab, showed that emphasis on the recognition of either small or large loops may not be predictive. Elucidating the mechanism of apoptosis induction may likely prove more important than the localization of the epitopes. Cardarelli *et al* (38), later confirmed by Cragg and Glennie (37), demonstrated that B1 and its $F(ab')_2$ could induce apoptosis of B cells directly, even in the absence of secondary cross-linking by anti-IgG. The apoptosis induced by 1K1791 and its humanized mAbs was found to be caspase-independent consistent with the findings of Chan *et al* (28) for other anti-CD20 mAbs.

All humanized mAbs except fra/ven induced significant and similar CDC in both RC-K8 and SU-DHL4 cells which was far stronger than rituximab or 2F2. It was notable that RC-K8 cells were extremely resistant to rituximab. Similarly, ADCC against SU-DHL4 was similar among the humanized mAbs with different donors of PBMCs and all of these mAbs demonstrated stronger ADCC activity than rituximab or 2F2. However, the level of ADCC activity achieved using RC-K8 as target cells varied among different donors and also among humanized mAbs.

The relationship between antibody structure, binding affinity and epitope recognition is a very complex one. In the present study, the best antibody that we have developed originated from a different gene family compared to all others tested or from those previously published in the scientific literature. However, with only one example of the heavy chain VH9 or the light chain Vk19/28 family it is premature to draw conclusions on the significance of these gene families and their associated functions. Furthermore, different methods of humanization affected and even enhanced the activity of IK1791. This finding is supported by evidence from the comparison of sequences of framework region and CDRs (Table III), and their functional activities represented in Figs. 1-4. Even a very small number of amino acid replacements (made predominantly in the framework regions) gave a diversified response in apoptosis induction although the affinity, CDC and ADCC remained relatively unchanged. One could speculate that future selection of mAbs based on gene family rather than antigen affinity may yield antibodies with different functional activities.

Our present study resulted in the selection of a specific humanized version sdr/abb of 1K1791 that exhibited enhanced functional activities, compared to other antibodies that were developed, but also compared to other published and approved anti-CD20 mAbs. Future studies will be aimed at validating selected humanized antibodies in preclinical animal models that showed strongest *in vitro* activities. In addition, we will select those mAbs that can be developed for clinical trials to examine whether a humanized anti-CD20 antibody with different or enhanced functional activities will respond to refractory or recurrent B-cell malignancies. Such novel mAbs will also improve the response rates among a broader population of B-NHL patients.

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References

1. Golay J, Zaffaroni L, Vaccari T, Lazzari M, Borleri GM, Bernasconi S, Tedesco F, Rambaldi A and Introna M: Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab *in vitro*: CD55 and CD59 regulate complement-mediated cell lysis. Blood 95: 3900-3908, 2000.
2. Semac I, Palomba C, Kulangara K, Klages N, van Echten-Deckert G, Borisch B and Hoessli DC: Anti-CD20 therapeutic antibody rituximab modifies the functional organization of rafts/microdomains of B lymphoma cells. Cancer Res 15: 534-540, 2003.
3. Deans JP, Haidong LI and Polyak MJ: CD20-mediated apoptosis signaling through lipid rafts. Immunology 107: 176-182, 2002.
4. Jazirehi AR and Bonavida B: Cellular and molecular signal transduction pathways modulated by rituximab (rituxan, anti-CD20 mAb) in non-Hodgkin's lymphoma: implications in chemosensitization and therapeutic intervention. Oncogene 24: 2121-2143, 2005.
5. Jazirehi AR, Huerta-Yepez S, Cheng G and Bonavida B: Rituximab (chimeric anti-CD20 monoclonal antibody) inhibits the constitutive nuclear factor-{kappa}B signaling pathway in non-Hodgkin's lymphoma B-cell lines: role in sensitization to chemotherapeutic drug-induced apoptosis. Cancer Res 65: 264-276, 2005.
6. Deans JP, Schieven GL, Shu GL, Valentine MA, Gilliland LA, Aruffo A, Clark EA and Ledbetter JA: Association of tyrosine and serine kinases with the B cell surface antigen CD20. Induction via CD20 of tyrosine phosphorylation and activation of phospholipase C-gamma 1 and PLC phospholipase C-gamma 2. J Immunol 151: 4494-4504, 1993.
7. Polyak MJ and Deans JP: Alanine-170 and proline-172 are critical determinants for extracellular CD20 epitopes; heterogeneity in the fine specificity of CD20 monoclonal antibodies is defined by additional requirements imposed by both amino acid sequence and quaternary structure. Blood 99: 3256-3262, 2002.
8. Polyak MJ, Tailor SH and Deans JP: Identification of a cytoplasmic region of CD20 required for its redistribution to a detergent-insoluble membrane compartment. J Immunol 161: 3242-3248, 1998.
9. Perosa F, Favoino E, Caragnano MA and Dammacco F: Generation of biologically active linear and cyclic peptides has revealed a unique fine specificity of rituximab and its possible cross-reactivity with acid sphingomyelinase-like phospho-diesterase 3b precursor. Blood 107: 1070-1077, 2006.
10. Teeling JL, Mackus WJ, Wiegman LJ, van den Brakel JH, Beers SA, French RR, van Meerten T, Ebeling S, Vink T, Slootstra JW, Parren PW, Glennie MJ and van de Winkel JG: The biological activity of human CD20 monoclonal antibodies is linked to unique epitopes on CD20. J Immunol 177: 362-371, 2006.
11. Glennie MJ, French RR, Cragg MS and Taylor RP: Mechanism of killing by anti-CD20 monoclonal antibodies. Mol Immunol 44: 3823-3837, 2007.
12. Golay J, Gramigna R, Facchinetti V, Capello D and Introna M: Acquired immunodeficiency syndrome-associated lymphomas are efficiently lysed through complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity by rituximab. Br J Haematol 119: 923-929, 2002.
13. Teeling JL, French RR, Cragg MS, van den Brakel J, Pluyter M, Huang H, Chan C, Parren PW, Hack CE, Dechant M, Valerius T, van de Winkel JG and Glennie MJ: Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. Blood 104: 1793-1800, 2004.
14. Nishida M, Usuda S, Okabe M, Miyakoda H, Komatsu M, Hanaoka H, Teshigawara K and Niwa O: Characterization of novel murine anti-CD20 monoclonal antibodies and their comparison to 2B8 and c2B8 (rituximab). Int J Oncol 31: 29-40, 2007.
15. Urlaub G, Mitchell PJ, Kas E, Chasin LA, Funanage VL, Myoda TT and Hamlin J: Effect of gamma rays at the dihydrofolate reductase locus: deletions and inversions. Somat Cell Mol Genet 12: 555-566, 1986.
16. Teeling J, Ruuls S, Glennie M, von de Winkel JDG, Parren P, Petersen J, Baadsgaard O and Huang H: Human monoclonal antibody against CD20. United State Patent Publication No. US 2004/0167319 A1, 2004.
17. Johnston CM, Wood AL, Bolland DJ and Corcoran AE: Complete sequence assembly and characterization of the C57BL/6 mouse Ig heavy chain V region. J Immunol 176: 4221-4234, 2006.
18. Thiebe R, Schäble KF, Bensch A, Brensing-Küppers J, Heim V, Kirschbaum T, Mitlöhner H, Ohnrich M, Pourrajabi S, Röschenahaler F, Schwendinger J, Wichelhaus D, Zocher I and Zachau HG: The variable genes and gene families of the mouse immunoglobulin kappa locus. Eur Immunol 29: 2072-2081, 1999.
19. Brekke KM and Garrard WT: Assembly and analysis of the mouse immunoglobulin kappa gene sequence. Immunogenetics 56: 490-505, 2004.
20. Ohtani K, Suzuki Y, Eda S, Kawai T, Kase T, Keshi H, Sakai Y, Yamamoto S, Sakamoto T and Wakamiya N: High-level and effective production of human mannan-binding lectin (MBL) in Chinese hamster ovary (CHO) cells. J Immunol Methods 222: 135-144, 1999.
21. Padlan EA: A possible procedure for reducing the immunogenicity of antibody variable domains while preserving their ligand-binding properties. Mol Immunol 28: 489-498, 1991.
22. Padlan EA, Abergel C and Tipper JP: Identification of specificity-determining residues in antibodies. FASEB J 9: 133-139, 1995.
23. Wu TT and Kabat EA: Possible use of similar framework region amino acid sequences between human and mouse immunoglobulin for humanizing mouse antibodies. Mol Immunol 29: 1141-1146, 1992.
24. Gazzano-Santoro H, Ralph P, Ryskamp TC, Chen AB and Mukku VR: A non-radioactive complement-dependent cytotoxicity assay for anti-CD20 monoclonal antibody. J Immunol Methods 202: 163-171, 1997.
25. Idusogie EE, Presta LG, Gazzano-Santoro H, Totpal K, Wong PY, Ultsch M, Meng G and Mulkerrin MG: Mapping of the C1q binding site on Rituxan, a chimeric antibody with a human IgG1 Fc. J Immunol 164: 4178-4184, 2000.
26. Rose AL, Smith BE and Maloney DG: Glucocorticoids and rituximab *in vitro*: synergistic direct antiproliferative and apoptotic effects. Blood 100: 1765-1773, 2002.
27. GraphPad Software, Inc: Fitting Models to Biological Data Using Linear and Nonlinear Regression, 2003.
28. Chan HT, Hughes D, French RR, Tutt AL, Walshe CA, Teeling JL, Glennie MJ and Cragg MS: CD20-induced lymphoma cell death is independent of both caspases and its redistribution into triton X-100 insoluble membrane rafts. Cancer Res 63: 5480-5489, 2003.
29. Stanglmaier M, Reis S and Hallek M: Rituximab and alemtuzumab induce a non-classic caspase-independent apoptotic pathway in B-lymphoid cell lines and in chronic lymphocytic leukemia cells. Ann Hematol 83: 634-645, 2004.
30. Daniels I, Abulayha AM, Thomson BJ and Haynes AP: Caspase-independent killing of Burkitt lymphoma cell lines by rituximab. Apoptosis 11: 1013-1023, 2006.

31. Rock EP, Sibbald PR, Davis MM and Chien YH: CDR3 length in antigen-specific immune receptors. J Exp Med 179: 323-328, 1994.
32. Seidl KJ, MacKenzie JD, Wang D, Kantor AB, Kabat EA, Herzenberg LA and Herzenberg LA: Frequent occurrence of identical heavy and light chain Ig rearrangement. Int Immunol 9: 689-702, 1997.
33. Arnold LW, Spencer DH, Clarke SH and Haughton G: Mechanisms that limit the diversity of antibody: three sequentially acting mechanisms that favor the spontaneous production of germline encoded anti-phophatidyl choline. Int Immunol 5: 1365-1373, 1993.
34. Li Y, Spellerberg MB, Stevenson FK, Capra JD and Potter KN: The I binding specificity of human VH 4-34 (VH 4-21) encoded antibodies is determined by both VH framework region 1 and complementarity determining region 3. J Mol Biol 256: 577-589, 1996.
35. Cragg MS, Morgan SM, Chan HT, Morgan BP, Filatov AV, Johnson PW, French RR and Glennie MJ: Complement mediated lysis by anti-CD20 mAb correlates with segregation into lipid rafts. Blood 101: 1045-1052, 2003.
36. Johnson TA, Rassenti LZ and Kipps TJ: Ig VH1 genes expressed in B cell chronic lymphocytic leukemia exhibit distinctive molecular features. J Immunol 158: 235-246, 1997.
37. Cragg MS and Glennie MJ: Antibody specificity controls *in vivo* effector mechanisms of anti-CD20 reagents. Blood 103: 2738-2743, 2004.
38. Cardarelli PM, Quinn M, Buckman D, Fang Y, Colcher D, King DJ, Bebbington C and Yarranton G: Binding to CD20 by anti-B1 antibody or F(ab')2 is sufficient for induction of apoptosis in B-cell lines. Cancer Immunol Immunother 51: 15-24, 2002.