Novel Designs of Multivalent Anti-CD20 Humanized Antibodies as Improved Lymphoma Therapeutics

Edmund A. Rossi,1 David M. Goldenberg,3 Thomas M. Cardillo,2 Rhona Stein,3 Yang Wang,3 and Chien-Hsing Chang2

1IBC Pharmaceuticals, Inc.; 2Immunomedics, Inc., Morris Plains, New Jersey; and 3Garden State Cancer Center, Center for Molecular Medicine and Immunology, Belleville, New Jersey

Abstract

Multivalent antibodies, either monospecific or bispecific, may improve the efficacy of current therapeutic interventions involving a single monoclonal antibody (mAb). We have applied the Dock-and-Lock (DNL) method, a new platform technology for the site-specific and covalent assembly of modular components into stably tethered complexes of defined composition, to prepare a hexavalent, anti-CD20 antibody, designated Hex-hA20, which comprises six Fabs with one Fc. We show that Hex-hA20 retains the binding activity of all six Fabs, associates with CD20 in lipid rafts, affects antibody-dependent cell-mediated cytotoxicity, but not complement-dependent cytotoxicity, and inhibits proliferation of Daudi, Raji, and Ramos cells in vitro at subnanomolar concentrations without the need for a cross-linking antibody. In addition, Hex-hA20 induces strong homotypical adhesion and is inefficient in stimulating calcium mobilization. Thus, Hex-hA20 exhibits biological properties attributable to both type I and type II anti-CD20 mAbs, as exemplified by rituximab and tositumomab, respectively. Although Hex-hA20 has a short serum half-life, it shows antitumor efficacy in tumor-bearing mice comparable with veltuzumab at equivalent doses. The versatile DNL method was also applied to generate two other multivalent anti-CD20 antibodies without the Fc region, Tri-hA20 and Tetra-hA20, comprising three and four Fabs of veltuzumab, respectively. Similar to Hex-hA20, these were purified to near homogeneity and shown to have potent antiproliferative activity in vitro, thus indicating the need for clustering three or more CD20 molecules on the cell surface to induce growth inhibition. [Cancer Res 2008;68(20):8384–92]

Introduction

With the approval of rituximab, a chimeric anti-CD20 monoclonal antibody (mAb), in 1997 for the treatment of relapsed or refractory low-grade or follicular non–Hodgkin lymphoma (NHL; ref. 1), a new era in mAb-based therapy of cancer was introduced whereas a profound management change in the treatment of CD20-positive lymphomas occurred. It not only presented a novel modality to treat B-cell malignancies but also provided an impetus to treat autoimmune diseases where B-cell dysregulation is implicated (2). Although only about half of patients with relapsed NHL respond to rituximab, its importance lies in its representing a category of therapeutics, which has a strikingly different toxicity profile than the typical cytotoxic drugs available. Its relative paucity of long-term toxicity and the short-term adverse event profile, being mostly associated with infusion reactions, show no overlap with chemotherapy, thus providing a rational combination of immunotherapy with chemotherapy without enhanced toxicity.

At present, next-generation anti-CD20 mAbs include human and humanized forms, with some claiming enhanced potency by antibody reengineering (1, 3–5). However, it is still unclear what improvements are required to show better efficacy than rituximab. Predicting on the basis of laboratory results has been difficult, because despite 10 years of clinical use of rituximab and an expansive preclinical literature on the use of anti-CD20 mAbs in lymphoma models in vitro and in vivo, how rituximab or other anti-CD20 mAbs kill lymphoma cells is still being debated (5) among the three mechanisms proposed: complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytoxicity (ADCC), and apoptosis or growth inhibition induced by direct signaling.

Multimeric forms of IgG made by genetic engineering (6–10) or chemical conjugation (11, 12) may become more effective antibodies than their monomeric counterparts as a result of increased valency, as well as enhanced effector functions. For example, a mAb for CD19 or CD20 that has little or no signaling activity was shown to induce growth inhibition or apoptosis when each is converted into an IgG-IgG homodimer (11, 12). Similar observations of attaining signaling activity or exhibiting higher cytotoxicity were reported for recombinantly produced fusion proteins comprising three identical anti-HER2 (13) single-chain Fvs (scFv), four identical anti-CD22 (14, 15) scFvs, or four identical anti-CD20 (16) scFvs. In addition, a dextran-rituximab polymer was found to inhibit the growth of Raji xenografts in mice, whereas the homodimer or monomer of rituximab was ineffective (17). Therefore, we propose that making multivalent or multimeric mAbs of a defined composition and proved homogeneity should be promising for developing more potent anti-CD20 agents, which may also help elucidate the most critical mechanism of action in vivo.

Veltuzumab or hA20 is a complementarity-determined region–grafted, anti-CD20 humanized mAb that exhibits CDC and ADCC in vitro, as well as inhibits cell proliferation and induces apoptosis upon cross-linking with a second antibody (18). Initial clinical studies have shown good safety and efficacy results in NHL patients and confirmed that low doses of veltuzumab are effective and that all doses can be given in shorter infusion times than the approved dose of rituximab (19).

The Dock-and-Lock (DNL) method exploits the dimerization and docking domain (DDD) and the anchoring domain (AD) that are...
involved in the natural binding between cyclic AMP–dependent protein kinase and A-kinase anchoring proteins (20, 21) as a pair of linkers for specifically docking a DDD-containing module into an AD-containing module, with the resulting binary complex further stabilized covalently by locking with disulfide bonds (22, 23). One distinct advantage of the DNL method, as noted previously (23), is to provide a modular approach for generating multivalent antibodies of monospecificity or bispecificity via site-specific conjugation of two types of recombinantly produced fusion proteins that are derived from the same or different antibodies. Here, we describe a veltuzumab-based hexavalent antibody (Hex-hA20) made by DNL to consist of a bivalent IgG linked to four monovalent Fabs, and the evaluation of its antitumor activity against CD20-expressing human lymphoma cells in vitro and in vivo. The versatile DNL method was also applied to generate two other multivalent anti-CD20 antibodies without the Fc region: Tri-hA20, a trivalent construct composed of three hA20 Fabs, and Tetra-hA20, a tetravalent construct composed of four hA20 Fabs. All three multivalent anti-CD20 antibodies were purified to near homogeneity and shown to retain the binding activity of each constituent Fab. In addition, testing of Tri-hA20 allowed us to establish that clustering three CD20 molecules on the cell surface via antibody binding is sufficient to induce growth inhibition. Schematic diagrams for Hex-IgG, Tri-Fab, Tetra-Fab, and the modules used for building them are shown in Fig. 1.

Materials and Methods

Antibodies, reagents and culture media. Veltuzumab, epratuzumab (hL2, humanized anti-CD22), labetuzumab (hMN-14, humanized anti-CEACAM5), and WR2, a rat anti-idiotype mAb to veltuzumab, were provided by Immunomedics. Rituximab and tositumomab (murine anti-B1) were obtained from commercial supplies. The purity of rituximab or tositumomab was determined by size-exclusion high-performance liquid chromatography (SE-HPLC) to be >98%. Secondary antibodies were obtained from Jackson Immunoresearch. Mouse anti-human IgM was obtained from SouthernBiotech. Antimouse Gr-1 ascites was kindly supplied by Dr. Myron Czuczman (Roswell Park Cancer Institute). The anti-mouse interleukin 2 (IL-2) receptor mAb, TM1-1, was purchased from BD Biosciences. All restriction endonucleases and other enzymes were purchased from New England Biolabs. Oligonucleotides were synthesized by Sigma Genosys. PCR reactions were performed using AmpliTaq polymerase (Applied Biosystems) and a Perkin-Elmer GeneAmp PCR system 9600. Heat-inactivated fetal bovine serum (FBS) was purchased from Hyclone. All other cell culture media and supplements were from Invitrogen Life Technologies.

Cell lines. Daudi, Raji, and Ramos were purchased from the American Type Culture Collection. Sp/ESF, a variant of Sp2/0-Ag14 engineered to grow in serum-free medium, was purchased from the American Type Culture Collection. Sp/ESF, a variant of Sp2/0-Ag14 engineered to grow in serum-free medium, was purchased from the American Type Culture Collection. Sp/ESF, a variant of Sp2/0-Ag14 engineered to grow in serum-free medium, was purchased from the American Type Culture Collection.

Generation of Hex-hA20. The pHIL2 vector has been used to mediate the expression of recombinant IgGs in mammalian hosts (24). A plasmid

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shuttle vector was produced to facilitate the conversion of any IgG-pdHL2 vector into a C_{115T}-AD2-IgG-pdHL2 vector. The expression vector encoding C_{115T}-DDD2-Fab-hA20 was generated from the C_{115T}-AD2-IgG-hA20-pdHL2 by excising the coding sequence for the C_{115T}-Hinge-I_{C2}-C_{115T} domains with SacI and EagI and replacing it with a 507-bp sequence encoding C_{115T}-DDD2, which was excised from the C-DDD2-hMv-14-pdHL2 expression vector (22) with the same enzymes. The expression vector C_{115T}-AD2-IgG-hA20-pdHL2 or C_{115T}-DDD2-Fab-hA20-pdHL2, each 30 μg, was linearized by digestion with SacI and transfected into Sp/ESF (2.8 × 10^6 cells) by electroporation (450 V, 25 μF). The pdHL2 vector contains the gene for dihydrofolate reductase, thus allowing clonal selection, as well as gene amplification, with methotrexate (MTX). After transfection, the cells were plated in 96-well plates and selected in media containing 0.2 μM MTX. Clones were screened for C_{115T}-AD2-IgG-hA20 or C_{115T}-DDD2-Fab-hA20 productivity by a sandwich ELISA using 96-well microtiter plates coated with WR2 to capture the fusion protein, which was detected with horseradish peroxidase-conjugated goat anti-human IgG F(ab')2. Wells giving the highest signal were expanded and ultimately used for production.

C_{115T}-AD2-IgG-hA20 and C_{115T}-DDD2-Fab-hA20 were produced in roller bottles, purified by Protein A (MabSelect, Amersham-Biosciences) and Protein L (Cibind L, Fluka), respectively, and stored in PBS. To generate Hex-hA20, a mixture of C_{115T}-DDD2-Fab-hA20 (134 mg) and C_{115T}-AD2-IgG-hA20 (100 mg) was treated with 1 mmol/L reduced glutathione at room temperature for 16 h, followed by 2 mmol/L oxidized glutathione for 24 h, from which Hex-hA20 was purified by Protein A. DNL-20/14 was made similarly by reacting C_{115T}-AD2-IgG-hA20 with C_{115T}-DDD2-Fab-hMv-14 (22), excising the coding sequence for the CH1-Hinge-CH2-CH3 domains with SacI and placing it into SacI-digested DNL-20/14 shuttle vector (Deans, 2008).

The maximum number of binding sites per cell and the apparent affinity constants were determined by Scatchard analysis. The results were scored semiquantitatively according to Polyak and Deans (25).

**Results**

Hexavalent antibodies made by DNL. Hex-hA20 was readily obtained by mixing C_{115T}-DDD2-Fab-hA20 and C_{115T}-AD2-IgG-hA20 under redox conditions followed by purification with Protein A. Both C_{115T}-DDD2-Fab-hA20 and C_{115T}-AD2-IgG-hA20 were produced with good yields as fusion proteins in myeloma cells, with subsequent purification from culture supernatants by Protein A, and Protein A, respectively.

The purity of Hex-hA20 was shown by reducing SDS-PAGE, which showed only three bands corresponding to the monomeric form of C_{115T}-DDD2-Fab-hA20 covalently to C_{115T}-AD2-Fab-hA20, which was produced as described for Hex-hA20 (22).

**Molecular size.** SE-HPLC was performed on a Beckman System Gold Model 116 with a Bio-Sil SEC 250 column (Bio-Rad) and 0.04 mol/L PBS (pH 6.8) and 1 mmol/L EDTA as the mobile phase.

**SDS-PAGE.** Reducing and nonreducing SDS-PAGE analyses were performed using 4% to 20% gradient Tris-glycine gels (Cambrex Bio Science).

**Mass spectrometry.** Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry was performed in a sinapinic acid matrix by Scripps Center for Mass Spectrometry.

**Competition ELISA.** Microtiter plates were coated overnight with veltuzumab at 5 μg/mL and blocked with PBS containing 2% bovine serum albumin (BSA) for 1 h. Hex-hA20 and veltuzumab, serially diluted in PBS to 504 cells inlane 3 and 5% CO_2, became moribund, were humanely sacrificed. Additionally, if mice lost >20% of initial body weight, they were sacrificed. Survival curves were analyzed using Kaplan-Meier plots (log-rank analysis) and Prism software. All animal studies were performed after approval of the local animal care and use committee.
Remarkably dissociates veltuzumab that Hex-hA20 dissociates monomeric CH3-AD2-IgG-hA20, veltuzumab, heavy chain-AD2, heavy chain, Fd-DDD2, and light chain are indicated as Hex, (IgG), H, AD2, H, d-DDD, and K, respectively.

Figure 2. SDS-PAGE analysis of protein A-purified samples under reducing (lanes 1–4) and nonreducing (lanes 5–8) conditions. Lanes 1 and 5, Hex-hA20 (2 μg); lanes 2 and 6, Hex-hA20 (0.5 μg); lanes 3 and 7, CH3-AD2-IgG-hA20; lanes 4 and 8, veltuzumab. Left, positions at prestained M, standards; right, arrows, positions of Hex-hA20, dimeric CH3-AD2-IgG-hA20, monomeric CH3-AD2-IgG-hA20, veltuzumab, heavy chain-AD2, heavy chain, Fd-DDD2, and light chain are indicated as Hex, (IgG), H, AD2, H, d-DDD, and K, respectively.

Reducing SDS-PAGE. These results are corroborated by the HPLC data shown in Supplementary Fig. S1A-D.

Binding analysis. As shown by competition ELISA in Fig. 3A, Hex-hA20 has a 3-fold higher avidity than veltuzumab, suggesting that at least three or more of the six Fab components are capable of binding simultaneously to the WR2 antibody epitope. The binding of Hex-hA20 to CD20 on live cells was compared with that of veltuzumab by flow cytometry. Figure 3B shows that Hex-hA20 resulted in 40% to 50% greater fluorescence intensity than veltuzumab when probed by PE-anti-Fab. In contrast, the signal observed for Hex-hA20 with PE-anti-Fc was lower than that of veltuzumab. These results are consistent with Hex-hA20 having four more Fabs than veltuzumab, but only one Fc like veltuzumab. Additional evidence for the higher valency and avidity of Hex-hA20 is provided by Scatchard analysis of binding to Raji cells (Fig. 3C), which shows that the apparent association constant of Hex-hA20 

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3.9 × 10^8 (mol/L)^{-1} is ~4-fold higher (P < 0.0001) than that of veltuzumab ~1 × 10^8 (mol/L)^{-1}, whereas an F test determined that the saturation binding curves for rituximab and veltuzumab were similar (P = 0.1859). More importantly, the number of receptors per cell calculated from the maximum number of binding sites (Bmax) obtained for Hex-hA20 was found to be ~1/4 of that obtained with veltuzumab (~100,000 versus ~437,500), suggesting that all six Fabs of Hex-hA20 are capable of binding to CD20 on the cell surface, because the same number of CD20 molecules would require thrice as many veltuzumab to occupy them. The data obtained from the off-rate measurements (Fig. 3D) also indicate that Hex-hA20 dissociates ~3-fold slower than veltuzumab, which remarkably dissociates ~2-fold to 3-fold slower than rituximab.

Antiproliferative activity. Based on the MTS assay (Fig. 4A), Hex-hA20 strongly inhibited proliferation in three Burkitt lymphoma cell lines, Raji, Ramos, and Daudi, with an EC50 of 0.064, 0.15, and 0.15 nmol/L, respectively. In contrast, veltuzumab in the absence of a cross-linking antibody showed detectable potency in all three cell lines only at >10 nmol/L. As expected, cross-linking of veltuzumab with goat anti-human Fc resulted in significant inhibition of proliferation. For Hex-hA20, cross-linking did not increase its potency further in Daudi. Additional experiments compared the effects of Hex-hA20, Tri-hA20, and Tetra-hA20 on cell proliferation over 5 days by viable cell counting. Representative results are shown in Fig. 4B for Raji and Ramos, which show the ability of Tri-hA20, Tetra-hA20, and Hex-hA20 to prevent 50% or greater cell proliferation without cross-linking at concentrations as low as 0.5 nmol/L. Similar results were obtained for Daudi (data not shown). The cell counting assay also showed that Hex-hA20 (EC50 = 0.17 nmol/L) was considerably more potent than anti-B1 (EC50 = 4.65 nmol/L) when evaluated with Ramos for 3 days.

Apoptosis and the roles of caspases and calcium. Raji cells were treated with the three multivalent anti-CD20 constructs at 0.5 or 5 nmol/L and analyzed with the Guava Nexin assay after 24 hours (Fig. 5A, left), Treatment with Tri-hA20, Tetra-hA20, or Hex-hA20 resulted in more cells in early apoptosis (12–16%) compared with the bivalent veltuzumab (6–9%) and the untreated control (3%). Comparable results were obtained with Daudi and Ramos cells (data not shown).

The extent of apoptosis was also assessed for Raji cells treated with 5 nmol/L Hex-hA20 or veltuzumab over a 24-hour period using the Guava MultiCaspase assay (Fig. 5A, right). The results at 24 hours were 17% and 6% for Hex-hA20 and veltuzumab, respectively, which agree with those determined by the Guava Nexin assay.

The effect of Z-VAD-FMK (a broad-spectrum caspase inhibitor) on apoptosis induced by Hex-hA20 was examined in Ramos, and the results (Supplementary Fig. S2) indicate that Z-VAD-FMK at 100 μmol/L completely prevented the apoptosis induced by anti-human IgM, but not that induced by Hex-hA20, suggesting that both caspase-dependent and caspase-independent pathways occur for Hex-hA20, as reported for rituximab with cross-linking (27). Although CD20 clustering is presumably achievable either

indirectly by cross-linking the antigen-bound veltuzumab with a second antibody or directly via multivalent engagement of Hex-hA20, the former, but not the latter, leads to a rapid rise in intracellular calcium levels (Supplementary Fig. S3).

**Effector functions.** CDC activity was evaluated in vitro using human complement and Daudi cells (Fig. 5B, left). Veltuzumab exhibits potent CDC activity. Surprisingly, Hex-hA20 fails to induce CDC in Daudi cells. Because CH3-AD2-IgG-hA20 induces CDC with similar potency as veltuzumab, modification of the carboxyl termini of veltuzumab by the addition of the small AD2 peptide does not affect CDC, suggesting that the addition of the four Fab-DD2 groups apparently prevents complement fixation, despite the ability of Hex-hA20 to bind C1q (data not shown). Hex-hA20 and veltuzumab have comparable ADCC (Fig. 5B, right). Thus, the Fc of Hex-hA20 induces ADCC.

**Homotypical adhesion.** Hex-hA20, Tetra-hA20, and Tri-hA20 induced homotypical adhesion of Daudi cells, resulting in >50% of cells having medium-size to large-size aggregates, whereas under the same conditions, the extent of cell aggregation observed for veltuzumab was similar to that of the untreated control (Supplementary Fig. S4).

**Membrane localization of CD20/Hex-hA20.** Veltuzumab translocates CD20 into lipid rafts, as shown previously (28) by Western blot analysis of the cell lysates in fractions isolated from sucrose density gradient centrifugation. The distribution of CD20 at the cell surface upon binding to veltuzumab or Hex-hA20 was examined further with immunofluorescence microscopy using cholera toxin subunit B-Alexa Fluor 488 as the reporter for ganglioside GM-1, a common lipid raft marker (29). As shown in Supplementary Fig. S5A-D, incubation of Daudi cells with veltuzumab or Hex-hA20 led to the formation of membrane patches or caps with punctuate spots, which were superbly matched by the images obtained with cholera toxin subunit B, indicating the localization of CD20 in lipid rafts. Although the fluorescent patterns observed were similar, veltuzumab seemed to form larger and fewer patches than Hex-hA20.

**Serum stability.** Hex-hA20 was found to have the same stability in serum as veltuzumab, maintaining 86% binding activity after 11 days (Supplementary Fig. S6). These results are similar to those of the bispecific Tri-Fab complexes reported previously (22).

**Pharmacokinetic analysis.** Using radio-iodinated preparations, it was found that Hex-hA20 cleared ~4.5 times faster than...
that received Hex-hA20 or veltuzumab had significantly improved (MST = 17 days for all three groups). In contrast, nondepleted mice saline control and mice treated with veltuzumab or Hex-hA20 NK cells and neutrophils, there was no difference between the growth using the Raji model (Fig. 6).

Although no significant difference in survival was observed with the saline control (66.5 and 42 versus 21 days; \( P < 0.0001 \)), the \( C_{\text{max}} \) was only half as high for Hex-hA20 compared with veltuzumab (12.9 nmol/L versus 25.7 nmol/L). Pharmacokinetic variables determined for Hex-hA20 and veltuzumab are provided in Supplementary Table S1.

**In vivo efficacy.** In the multiple-dose study with the Daudi model, mice were treated with Hex-hA20 at 30 \( \mu \)g (q7dx2) and 6 \( \mu \)g (q7dx2). As shown in Fig. 6A, both treatments resulted in significantly improved median survival time (MST) when compared with the saline control (66.5 and 42 versus 21 days; \( P < 0.0001 \)). Althou...
antibodies made by DNL is the ability of each Fab constituent in the respective structure to engage its cognate antigen either in the form of immobilized antiidiotype antibody or on the target cell surface, as shown collectively for Hex-hA20 by competition ELISA, flow cytometry, and Scatchard analysis. Moreover, the fact that Tri-hA20, but not veltuzumab, can potently inhibit the proliferation of CD20-positive cells in vitro is consistent with the notion that all three Fabs in Tri-hA20 are capable of simultaneously binding to CD20, resulting in clustering of CD20 and the onset of signal transduction, which leads us to conclude that a minimum valency of 3 is required for an anti-CD20 antibody to effectively induce growth inhibition without cross-linking. A similar observation was reported by Miller and colleagues (30) that, at 2 to 3 nmol/L, trivalent and tetravalent anti-CD20 constructs made by linearly stitching two or more Fab groups of rituximab were able to induce maximal apoptosis (up to 25%) of the B-lymphoma cell line, WIL2-S, without requiring further cross-linking. More recently, tetravalent anti-CD20 antibodies made by genetically fusing an identical pair of scFvs in tandem to the hinge CH2-CH3 were also shown by Li and colleagues (16) to have significant antiproliferative and apoptotic activities in Daudi and Raji lymphoma cells in the absence of a second antibody, despite the lack of direct evidence that all of the four scFvs are engaged in antigen-binding.

Based on their efficacy in certain in vitro assays, anti-CD20 mAbs have been classified by Cragg and colleagues (35) as either type I, represented by rituximab, or type II, represented by tositumomab (36). Type I anti-CD20 mAbs are characterized by potent CDC, rapid calcium mobilization upon the addition of a second antibody (37, 38), weak induction of homotypical adhesion (39), enhanced association of CD20 with lipid rafts (40), and a general requirement of cross-linking for efficient antiproliferation or apoptosis (39). In contrast, type II anti-CD20 mAbs lack CDC, fail to bring CD20 into lipid rafts (3, 4), induce a high level of homotypical adhesion (39), and do not stimulate calcium mobilization upon further cross-linking (37). A further differentiation is that apoptotic cell death mediated by type I mAbs involves both caspase-dependent and caspase-independent pathways, but only caspase-independent pathways seem to be associated with type II mAbs (4). However, both type I and type II anti-CD20 mAbs are effective in ADCC and apparently bind to the same region of the extracellular loop of CD20 on the plasma membrane (41–43). Interestingly, at saturation, the number by which a type II mAb binds to each CD20-positive B cell only approaches half of that observed with a type I mAb (3, 4), suggesting that a type II mAb may act virtually as a tetravalent antibody by engaging two adjacent extracellular loops of CD20 with each antigen-binding site.

![Figure 5. A. apoptosis measured by Guava Nexin (left) showing percentage of early apoptotic cells (Annexin V–PE positive/7-AAD negative) induced in Raji after 24-h incubation with veltuzumab, Tri-hA20, Tetra-hA20, or Hex-hA20 at 0.5 nmol/L (black columns) or 5 nmol/L (gray columns); apoptosis measured by Guava MultiCaspase (right) for which Raji cells were cultured in the presence of Hex-hA20 (5 nmol/L), veltuzumab (5 nmol/L), or anti-IgM (5 μg/mL) and analyzed at 3, 7, 16, and 24 h by flow cytometry after staining with Srt-VAD-FMK. Cells were plated at 2 × 10^5 cells/mL in fresh media and incubated at 37°C with each test article at the indicated concentrations for up to 24 h, and duplicate wells were processed for Guava analysis.

B. CDC (left) was measured in Daudi cells for Hex-hA20 (C), epratuzumab ( ), veltuzumab ( ), or C95–A2D2-lgG-hA20 ( ) in the presence of human complement. The percentage complement control (number of viable cells in the test sample compared with cells treated with complement only) was plotted versus the log of the nanomolar concentration. ADCC (right) was measured for Hex-hA20, veltuzumab, epratuzumab, or labetuzumab at 5 μg/mL using Daudi as the target cells and freshly isolated peripheral blood mononuclear cells from two donors as the effector cells. A 100% lysis reference was generated by the addition of detergent to wells containing target cells only. The bar graphs show percentage of lysis obtained for each of the two donors.](image-url)
We note that Hex-hA20 exhibits biological properties attributable to both type II (for example, negative for CDC and calcium mobilization; positive for antiproliferation, apoptosis, and homotypic adhesion) and type I (for example, positive for trafficking to lipid rafts). Thus, one effective approach to converting a type I anti-CD20 mAb to a type II can be achieved by making the type I mAb multivalent. Preliminary investigation of the signaling pathway indicates that Hex-hA20 induces caspase-dependent, as well as caspase-independent, apoptosis. Additional studies are in progress to identify the subcellular events associated with the binding of CD20 by Hex-hA20 or Tri-hA20, which may reveal unequivocally the molecular factors that account for the antiproliferative potency of a multivalent anti-CD20 antibody with defined composition.

When tested against human lymphoma xenografts in mice, the therapeutic activity of Hex-hA20 was comparable, but not better, than the parental veltuzumab, except when a higher mole-equivalent dose (such as 465 µg) was given. Because mouse complement was reported to be inactive in mediating CDC of the DHL-4 cell line in vitro by rituximab (26), the shorter half-life of Hex-hA20 rather than the lack of CDC may explain why the in vivo antitumor efficacy of Hex-hA20 was not superior to that of the bivalent veltuzumab, suggesting that more frequent or higher doses of Hex-hA20 may be indicated. It is also noted that cross-linking of veltuzumab may occur in vivo via binding to effector cells (44), thus mimicking the advantage of Hex-hA20 over veltuzumab in vitro.

The observation that neither veltuzumab nor Hex-hA20 is effective in mouse models depleted with NK cells and neutrophils emphasizes the importance of ADCC for in vivo killing of tumor cells. Whether a multivalent anti-CD20 construct can be made by DNL to retain CDC and show more potent antitumor effects in vivo.

Figure 6. Efficacy of Hex-hA20 in human lymphoma xenograft models. A, Daudi cells (1.5 × 10^7) were injected i.v. into SCID mice on day 0. On days 1 and 8, groups of mice (n = 9–10) were given either Hex-hA20 at two different doses (30 or 6 µg) or equimolar amounts of veltuzumab (12.4 or 2.4 µg). B, SCID mice were depleted of NK cells and neutrophils before the administration of Raji cells with antimouse Gr-1 ascites and TM±1 mAb specific for mouse IL-2 receptor, as described in Materials and Methods. On day 0, Raji cells (1 × 10^6) were injected i.v. into both depleted and nondepleted mice. Hex-hA20 (465 µg) or veltuzumab (200 µg) was given i.v. on days 3, 5, 7, and 11, whereas the control group received saline.
is worthy to determine. On the other hand, because CDC has been suggested to be a major factor in infusion-related toxicity for rituximab, it is tempting to speculate that HexaH20 may be a viable alternative where reduced side effects are desired.

Disclosure of Potential Conflicts of Interest

References
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