Properties and Structure-Function Relationships of Veltuzumab (hA20), a Humanized Anti-CD20 Monoclonal Antibody

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Abstract

Veltuzumab is a humanized anti-CD20 monoclonal antibody with complementarity-determining regions (CDRs) identical to rituximab, except for one residue at the 101st position (Kabat numbering) in CDR3 of the variable heavy chain (V_H), having aspartic acid (Asp) instead of asparagine (Asn), with framework regions of epratuzumab, a humanized anti-CD22 antibody. When compared to rituximab, veltuzumab has significantly reduced off-rates in 3 human lymphoma cell lines tested, as well as increased complement-dependent cytotoxicity in 1/3 cell lines, but no other in-vitro differences. Mutation studies confirmed that the differentiation of the off-rate between veltuzumab and rituximab is related to the single amino acid change in CDR3-V_H. Studies of i.p. and s.c. doses in mouse models of human lymphoma and in normal cynomolgus monkeys disclosed that low doses of veltuzumab control tumor growth or deplete circulating or sessile B cells. Low and high-dose veltuzumab were significantly more effective in vivo than rituximab in 3 lymphoma models. These findings are consistent with activity in patients with non-Hodgkin lymphoma given low i.v. or s.c. doses of veltuzumab. Thus, changing Asn_{101} to Asp_{101} in CDR3-V_H of rituximab is responsible for veltuzumab’s lower off-rate and apparent improved potency in preclinical models that could translate into advantages in patients.
Introduction

Advances in medical treatments during the last ten years have witnessed the introduction of 9 antibodies for the therapy of diverse cancers.\(^1\) Most of these new biological therapeutics are combined with conventional cytotoxic drugs, indicating that the antibodies require additional measures to improve their efficacy.\(^1\) This is best exemplified with rituximab, the first-generation chimeric anti-CD20 monoclonal antibody (MAb) that was approved initially as a monotherapy for the treatment of NHL.\(^2\) Based on this success, efforts are underway to introduce improved anti-CD20 antibodies.\(^3-8\)

Most of these new MAbs are intended to reduce the murine components while enhancing FcγR or complement-mediated functions.\(^8-10\) One of the first second-generation MAbs developed to mitigate the infusion-related reactions experienced with rituximab is the hA20 MAb,\(^3\) now termed veltuzumab, which has a shorter infusion time while indicating a higher complete response rate than has been reported for rituximab.\(^11,12\) Veltuzumab was constructed recombinantly on the framework regions (FRs) of epratuzumab, the humanized anti-CD22 MAb or hLL2,\(^13\) but has identical variable kappa light-chain (\(\kappa\)) complementarity-determining regions (CDRs), identical CDR1-\(\text{V}_{\text{H}}\) and CDR2-\(\text{V}_{\text{H}}\), but a different CDR3-\(\text{V}_{\text{H}}\), compared to rituximab. Our initial characterization of veltuzumab\(^3\) did not address whether these changes would result in different functions and therapeutic properties from those of rituximab. However, we postulated that, similar to the experience with epratuzumab,\(^14\) it would be well tolerated during more rapid infusions than rituximab, which has been confirmed in monkeys, as shown herein, and in patients.\(^11,12\) We now report that veltuzumab has unique characteristics in terms of significantly improved complement-dependent cytotoxicity (CDC) in 1 of 3 cell lines, slower
off-rates in all three lymphoma cell lines tested, and significantly improved therapeutic results \textit{in vivo} in 3 different lymphoma models, compared to rituximab, and potent anti-B-cell activity in cynomolgus monkeys, thus corroborating the activity observed in patients at very low doses. Surprisingly, we have determined that these differences between veltuzumab and rituximab, at least with regard to off-rates, are related to a single amino acid change in CDR3-\(V_H\).

\textbf{Methods}

\textbf{Antibodies}

The development of veltuzumab has been described previously,\textsuperscript{3} using the same human IgG donor FRs of epratuzumab.\textsuperscript{13} Specifically, FR1, FR2, and FR3 of EU and FR4 of NEWM were selected for grafting the CDRs of \(V_H\), and the FRs of REI were selected for grafting the CDRs of \(V_k\). Key murine residues were retained in the FRs to maintain the binding specificity and affinity of veltuzumab for CD20 similar to those of the parental murine antibody (A20). The amino acid sequences of \(V_H\) and \(V_k\) are shown for A20, C2B8, and veltuzumab in Figures S1A and S1B.

The chimeric form of veltuzumab, cA20, was generated by grafting the \(V_H\) and \(V_k\) domains of A20 to the human constant regions; thus, cA20 differs from veltuzumab in the variable FRs but has identical CDRs to veltuzumab. A mutant of veltuzumab, designated D101N, was engineered with a single amino acid change of Asp\textsubscript{101} to Asn\textsubscript{101} in CDR3-\(V_H\); thus, D101N has the same CDRs as rituximab but identical FRs to veltuzumab. The construction of the expression vector for D101N is provided in Supplementary Methods.

Veltuzumab, cA20, D101N, 1F5, labetuzumab (hMN-14, humanized anti-CEACAM5 MAb), and WR2, a rat anti-veltuzumab-idiotype MAb, were provided by Immunomedics. Rituximab and tositumomab (murine anti-B1) MAbs were obtained from commercial supplies.
Table 1 compares the CDR3-\(V_H\) of veltuzumab, cA20, D101N, rituximab, and 1F5, all of which have identical CDR1-\(V_H\) and CDR2-\(V_H\) sequences.

**Cell lines**

The murine hybridoma 1F5 and the human Burkitt lymphoma lines, Daudi, Raji, and Ramos, were purchased from ATCC. The non-Burkitt lymphoma cell lines were: SU-DHL-6 from Dr. Alan Epstein (University of Southern California, Los Angeles, CA), and WSU-FSCCL from Dr. Mitchell Smith (Fox Chase Cancer Center, Philadelphia, PA). The cells were grown as suspension cultures in DMEM (Life Technologies, Inc. Gaithersburg, MD), supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 \(\mu\)g/ml), and L-glutamine (2 mM).

**Scatchard analyses**

The maximum number of binding sites per cell and the apparent dissociation constants were determined by nonlinear regression analysis of the saturation binding data obtained with the radioiodinated samples and Raji cells, using Prism software (GraphPad Software Inc., San Diego, CA).

**In-vitro cytotoxicity**

The colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, first described by Mosmann,\(^{15}\) was used to evaluate the *in-vitro* cytotoxicity by quantifying viable cells after treatments with anti-CD20 MAbs (5 \(\mu\)g/mL final concentration) for 4 days at 37°C in the absence or presence of goat-anti-human (GAH) IgG (20 \(\mu\)g/mL final concentration).

**Ex-vivo depletion of B- and T-cells compared also to Daudi and Raji lymphoma cells**

The effects of veltuzumab and rituximab on peripheral blood lymphocytes from healthy volunteers were evaluated *ex-vivo* using flow cytometry. Blood specimens were collected under
a protocol approved by the New England Institutional Review Board (Wellesley, MA) and informed consent was obtained in accordance with the Declaration of Helsinki. Heparinized whole blood (150 μL) was incubated with veltuzumab or rituximab for 2 days at 37°C and 5% CO₂ in a final volume of 250 μL. In some experiments, 5 x 10⁴ Daudi or Raji cells were included in the mixture. FITC-labeled anti-CD3, anti-CD19, anti-kappa light chain, or mouse IgG₁ (isotype control), all purchased from BD Biosciences (San Jose, CA), were added to appropriate tubes, and incubation continued for an additional 30 min. Following lysing of erythrocytes, cells were analyzed using a FACSCalibur (BD Biosciences) with Cell Quest software. Both Daudi and Raji cells separate from lymphocytes on forward scatter vs. side scatter flow cytometry dot plots, and are gated with the monocyte population. Daudi cells express high surface kappa light chain and are identified as kappa-positive cells in the monocytes gate. Raji cells are identified as CD19+ cells in the monocyte gate. The normal B-cells are identified as CD19+ cells in the lymphocyte gate. In these experiments, Student’s t-test was used to evaluate statistical significance (P≤.05).

Measuring antibody off-rates by flow cytometry

Veltuzumab, rituximab, cA20, D101N, 1F5, and tositumomab were labeled with phycoerythrin (PE) using an appropriate Zenon R-Phycoerythrin IgG labeling kit (Invitrogen, Molecular Probes, Z-25455) following the manufacturer’s protocol. Cells (Daudi, Raji, or Ramos) in 0.5 mL of CM (phenol red-free RPMI 1640 media supplemented with 10% FBS) at 1 x 10⁶ cells/mL were incubated with 5 μg of each PE-labeled MAb at room temperature for 30 min, pelleted at 400 x g, washed twice with CM, resuspended in 1.5 mL of CM, and split into two 0.75-mL aliquots. To prevent rebinding, N-ethyl-maleimide (NEM)-blocked veltuzumab-Fab’ was added to each replicate (1 mg/mL final concentration) and the mean fluorescence intensity (MFI) was
immediately measured to determine the maximal binding (T=0) using a Guava PCA and Guava Express software (Guava Technologies, Inc., Hayward, CA). Subsequent measurements were taken at 30-min intervals. The percent maximal binding, which is the quotient of the MFI at T=X divided by that at T=0, was plotted against time, and the results analyzed by Prism software to yield the half-life or off-rates.

**CDC assays**

Daudi, Raji or Ramos cells (1 x 10^6/mL) were seeded (50 μL per well) in black 96-well plates (Nunc) and incubated for 3 h at 37°C and 5% CO2 with each test MAb (0.001 to 10 μg/mL) in the presence of human complement (Quidel Corp., San Diego, CA) at 1/20 final dilution. The indicator dye, AlamarBlue (BioSource, Camarillo, CA), was added and the incubation continued overnight. Viable cells were then quantified by measuring the fluorescence intensity with excitation at 530 nM and emission at 590 nM using a BioTek Synergy™ HT Multi-Detection Microplate Reader and KC4 Signature Software (BioTek Instruments, Inc., Winooski, VT). The dose-response curves generated from the mean of 6 replicate determinations were analyzed using Prism software to obtain EC50 values. In the case of Daudi cells, to account for day-to-day variations in the assay, as well as to increase the precision of the EC50 estimates, the experiments used a multi-factorial design, where the assay for each antibody was performed in triplicate each day, and repeated on three different days for a total of 9 assays per antibody. The samples included 3 different lots of veltuzumab and one of rituximab. Statistical analysis of the EC50 data was based on a 2-way analysis of variance (ANOVA) model with day and antibody type as factors. Dunnett’s multiple comparison procedure was utilized to perform the 3 comparisons of all 4 constructs at an overall experimental error rate of 0.05.

**Antibody-dependent cellular cytotoxicity (ADCC) assays**
Daudi cells were incubated with each test article in triplicate at 5 μg/mL for 30 min at 37°C and 5% CO₂. Freshly isolated peripheral blood mononuclear cells (PBMCs) obtained from healthy volunteers were then added at a predetermined optimal effector-to-target ratio of 50:1. Following a 4-h incubation, cell lysis was assessed by CytoTox-One (Promega, Madison, WI).

**Tolerability and toxicokinetics in cynomolgus monkeys**

An exploratory single- and repeated-dose study of i.v. and s.c. injections of veltuzumab was conducted in cynomolgus monkeys (*Macaca fascicularis*) at SNBL USA, Ltd. (Everett, WA). Sixteen male and 16 female monkeys weighing 2.5 to 6.6 kg (3-7 years old) were given i.v. or s.c. doses of 0, 6.7, 33.5, and 67 mg/kg (which correspond to 80, 375, and 800 mg/m² doses, respectively, in humans), either once or three times (2 weeks apart). The monkeys were examined regularly, with blood samples taken for MAb titers and PK, blood chemistry, coagulation, and hematology testing, as well as urinalysis, and then post-mortem evaluation of lymphoid tissue status in spleen, mandibular and mesenteric lymph nodes.

**Serum pharmacokinetics (PK) in mice after i.p. or s.c. administration**

Twelve 9-week-old naïve female Swiss-Webster mice (Taconic Farms, Germantown, NY) were administered veltuzumab (150 μg in 200 μL) either i.p. or s.c. Serum samples were taken by retro-orbital bleeding at 0.5, 1, 4, 6, 24, 48, 120, 168 and 336 h and stored frozen until analysis for veltuzumab, which involved capturing it with WR2-coated microtiter plates and quantifying the bound veltuzumab with a peroxidase-conjugated goat anti-human (GAH) polyclonal antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Non-compartmental analysis was performed on both the i.p. and s.c. data, representing the best-fit model.

**Evaluation of in vivo efficacy in mouse models**
C.B.17 homozygous severe combined immune deficient (SCID) mice of approximately 20 g (7 weeks old when received from Taconic, Germantown, NY) were used for studies with Daudi and WSU-FSCCL lymphomas. For the Daudi model, mice were inoculated i.v. on Day 0 with 1.5x10^7 cells, weighed, and randomly assigned to treatment and control groups. On Day 1 or 5, mice received a single dose of veltuzumab s.c. or i.p., and those in the control groups received either saline (200 μL) or labetuzumab (60 μg). In addition to this study, a minimal effective dose experiment was performed in the same model; groups of 14 mice received a single dose of veltuzumab (0.5, 0.25, 0.1, or 0.05 μg) i.p with saline given to controls. In the WSU-FSCCL follicular lymphoma model, each mouse (15/group) was inoculated with 2.5 x 10^6 cells i.v. and 5 days later received a single dose of veltuzumab (0.035, 0.35, 3.5, or 35 μg) i.p.

Comparative studies of veltuzumab and rituximab also were conducted in the Daudi and WSU-FSCCL lymphoma models. Likewise, experiments were conducted at Roswell Park Cancer Institute (RPCI) comparing velltuzumab to rituximab under identical conditions in RPCI-bred SCID mice grafted with 1 x 10^5 Raji lymphoma cells via tail vein injection, and treated with either MAb (10 mg/kg or 200 μg, total N=15/group) administered i.v. 5, 10, 15, and 20 days post grafting.

Animals were monitored daily and sacrificed humanely when hind-limb paralysis developed, when they became moribund, or if they lost more than 20% of initial body weight. Statistical differences in survival between treatment groups were analyzed using Kaplan-Meier plots (log-rank analysis of P values, which were considered significant at <.05) provided by Prism software.
**Effects of depleting natural killer (NK) cells and neutrophils on therapy**

Depletion of NK cells and neutrophils was conducted as described.\textsuperscript{16} Briefly, each mouse received 100 μL of anti-mouse Gr-1 ascites i.p. and 100 μg anti-mouse IL-2 receptor antibody (TMβ-1, BD PharMingen, Inc., San Jose, CA) one day before inoculating 1 x 10^6 Raji cells, followed by two more i.p. injections of anti-mouse Gr-1 ascites on Days 6 and 13. Depletion was confirmed by FACS analysis of blood samples taken from 1 depleted and 1 non-depleted mouse on Days 3 and 13. Veltuzumab (200 μg) or saline was administered i.v. on Days 3, 5, 7, and 11.

All animal studies were approved by the respective Institutional Animal Care and Use Committees of the Center for Molecular Medicine and Immunology and Roswell Park Cancer Institute, and performed in accordance with the AAALAC, USDA, and DHHS regulations.

**Results**

**Cell binding analyses**

The number of binding sites per Raji cell and the apparent dissociation constants of veltuzumab were determined by direct cell surface saturation binding and Scatchard analyses, and compared with those of rituximab. The results (Figure S2) confirm that both parameters are similar for veltuzumab and rituximab (2.0 x 10^5 – 4.2 x 10^5 vs. 1.8 x 10^5 – 3.6 x 10^5 sites/cell, \( P = .8563 \); 6.23 – 12.02 vs. 6.70 -8.63 nM; \( P = .6837 \)). These values are comparable to those reported previously by us \textsuperscript{3} and others, \textsuperscript{17} as well as in the prescribing information for rituximab.\textsuperscript{18}

**Off-rates**

The dissociation of veltuzumab, rituximab, and cA20 from Daudi (Figure 1A), Ramos (Figure 1B), and Raji (Figure 1C) were compared in the presence of excess veltuzumab-Fab’-NEM at
37°C. Additional measurements were performed to compare the dissociation of veltuzumab, rituximab, and D101N from Raji (Figure 1D) under similar conditions. For each cell line tested, the half-life of veltuzumab on average was 2.7-fold (±0.3) longer than that of rituximab ($P<.0001$), but indistinguishable from that of cA20 ($P>.2$). In contrast, the D101N mutant dissociated from Raji cells with an off-rate two- and six-fold faster than rituximab and veltuzumab, respectively. These results suggest that the change of Asn$_{101}$ to Asp$_{101}$ is responsible for the slower dissociation of veltuzumab. We also compared the dissociation of veltuzumab, rituximab, D101N, 1F5, and tositumomab from Raji cells (Figure 1E) in the absence of the competing veltuzumab-Fab’-NEM, and found that veltuzumab has the longest half-life (281 min), followed by 1F5 (195 min), rituximab (94 min), tositumomab (50 min), and D101N (42 min).

In vivo anti-proliferative activity

The ability of veltuzumab and rituximab to inhibit proliferation was examined on four lymphoma cell lines, SU-DHL-6, Daudi, Raji, and WSU-FSCCL, which differ in their expression levels of CD20, using the MTT cell viability assay. While the sensitivity to both MAbs correlated with CD20 expression (SU-DHL-6 > Raji > Daudi > WSU-FSCCL), no significant differences in potency were observed between veltuzumab and rituximab within a cell line (Figure S3). Although crosslinking with GAH increased the efficacy of veltuzumab and rituximab on Raji and Daudi cells (both with intermediate levels of CD20 expression and sensitivity to killing by anti-CD20 MAbs), the addition of GAH did not further enhance the inhibition of proliferation of the highly sensitive SU-DHL-6 or the less sensitive WSU-FSCCL under the conditions tested.

B- and T-cell, and lymphoma depletion studies ex vivo
The effects of veltuzumab and rituximab on human peripheral blood lymphocytes of healthy volunteers were assessed *ex vivo* using flow cytometry. Aliquots of whole blood were incubated with the MAbs for two days, followed by FACS analysis of B cells (CD19+) and T cells (CD3+). Controls included no antibody and an isotype-matched, irrelevant antibody (labetuzumab). Incubation of whole blood with veltuzumab at 5 and 1 μg/ml led to a statistically significant (*P* < 0.05) decrease of 26 – 80% and 11 – 61%, respectively, in the number of B cells (Figure 2A), but not T cells (data not shown). Similar results were obtained using rituximab (not shown). Because whole blood was used in the incubation mixtures, the decreases observed in the cell counts could be due to CDC, ADCC, as well as direct apoptotic signaling.

Figure 2B compares the depletion of human B cells vs. Raji lymphoma cells by veltuzumab and rituximab, showing that both MAbs have virtually identical effects, including depleting normal B cells proportionately less than Raji lymphoma cells. Similar results were obtained in a comparison with Daudi lymphoma cells (data not shown).

**CDC**

With Daudi as the target cells for CDC, we observed consistently a lower value of EC$_{50}$ for veltuzumab (Table 2) when compared to rituximab. Further measurements addressing any effect of day-to-day variation as well as any differences in EC$_{50}$ patterns amongst the antibodies across different days indicated that the mean difference in EC$_{50}$ observed between rituximab and each of the three lots of veltuzumab was consistently statistically significant (*P* < 0.0001). However, no differences between veltuzumab and rituximab were observed with CDC results in the other two cell lines, Raji and Ramos.

**ADCC**
With PBMCs as effector cells for ADCC, veltuzumab and rituximab produced similar extents of cell lysis (40-45%; $P = .12$), which were significantly higher ($P < .0001$) than labetuzumab (9.9%; data not shown).

**PK studies in mice**

While serum concentrations and clearance of veltuzumab were very similar between those animals injected i.p. and s.c. (Figure S4), several of their respective PK-parameters were significantly different (Tables S1 and S2). In terms of maximum serum concentrations ($C_{max}$) and the time to $C_{max}$ ($T_{max}$), there were no significant differences between the injection routes. This was also true for comparisons between clearance ($Cl$) and area under the curve (AUC) values. However, notable differences were observed in the terminal half-life ($T_{1/2}$) and mean residence time (MRT), with the i.p. route yielding significantly higher values for each ($P = .0316$ and $P = .0357$, respectively).

PK results of veltuzumab in normal mice are summarized in the Supplementary Results online. After injecting 150 μg of veltuzumab into these 30-g mice, we achieved a $C_{max}$ of 30 μg/mL, or 5.3-fold less than what could be expected maximally. These findings suggested that we could inject 50 ng (0.05 μg or 0.0025 mg/kg) into ~20-g tumor-bearing SCID mice for our lowest therapy dose, which under ideal conditions would provide a maximum serum concentration of 87.7 ng/mL, which could yield an estimated $C_{max}$ concentration of approximately 16.5 ng/mL veltuzumab in the serum (87.7 ng/mL divided by 5.3).

**Tolerability and PK studies in cynomolgus monkeys**

Veltuzumab administered i.v. or s.c. as single or multiple doses was well tolerated, with no clinical or persistent laboratory test abnormalities noted other than B-cell depletion in the circulation and lymphatic organs. Post-mortem changes in the animals receiving all doses
included follicular lymphoid depletion of the spleen, mandibular, and mesenteric lymph nodes at all doses (data not shown). Transient decreases in white blood cells, neutrophils, lymphocytes, and basophils were noted, but only a rapid reduction in the number of peripheral blood B cells was observed (results not shown). These effects occurred within 2 days of dosing by either route and were present at doses of 6.7 mg/kg or higher. The animals recovered at either 28 days when treated once, or at 56 days when given 3 doses. PK analyses (data not shown) indicated that the half-life was estimated to be 5 to 8 days after i.v. injection or 6-13 days following s.c. administration, and the $T_{\text{max}}$ for both routes ranged from 2 to 5 days. $C_{\text{max}}$ following i.v. injection was linear and showed no accumulation, and the $\text{AUC}_{0-27 \text{ days}}$ was greater for i.v. administration than for the s.c. route. This is likely related to the longer period required for the MAb to enter the blood via the s.c. route with a similar rate of clearance. The mean volume of distribution was greater after s.c. administration than after i.v. infusion at all dose levels (not shown). These results indicate that at the lowest single dose of 6.7 mg/kg (equivalent to 80 mg/m² in humans), rapid depletion of peripheral and splenic B cells occurs for veltuzumab given either by i.v. or s.c. routes at this low dose.

**Intraperitoneal vs. s.c. therapy of Burkitt lymphoma xenografts**

Mice bearing disseminated disease were treated with single i.p. or s.c. injections of veltuzumab. All three doses (5, 20, and 60 μg, or 0.25, 1.0, and 3.0 mg/kg, respectively), regardless of whether administered i.p. or s.c., significantly increased survival of mice in comparison to the saline and labetuzumab control groups ($P=.0001$). Comparisons between equal doses administered i.p. and s.c. did not yield significant differences (Figure 3A). While the control mice succumbed to disease (hind-limb paralysis) on day 28, the mean survival times (MSTs) of the two 60-μg groups were 101.9 ± 26.8 and 114.6 ± 21.8 days for i.p. and s.c., respectively, with
4/8 and 6/8 mice still alive when the study ended on Day-126. Similar results were obtained for the animals given 20 μg, with the MSTs of 116.4 ± 14.0 (i.p.) and 108.4 ± 26.2 (s.c.) days, and 5/8 mice alive at the end of the study in both groups. Only at the lowest dose (5 μg, or 0.25 mg/kg) was a >50% mortality rate observed (3/8 and 1/8 mice were still alive at the end of the study in each i.p./s.c. group), but these mice still had a >3.2-fold increase in the MSTs (91.1 ± 30.9 and 91.6 ± 22.5 days for i.p. and s.c., respectively) compared to controls.

**Minimum effective dose of veltuzumab in Daudi lymphoma xenografts**

Since a single 5-μg dose of veltuzumab proved to be potent in the Daudi disseminated Burkitt lymphoma model, still lower doses (0.5, 0.25, 0.1, and 0.05 μg) were examined. Remarkably, all four doses improved survival significantly \( P < 0.0001 \) when compared to saline control mice (Figure 3B). For example, mice receiving a single dose of 0.5 μg (0.025 mg/kg) had a 3-fold improvement in the MST compared to controls (69.5 ± 23.9 vs. 21.4 ± 1.1 days). Even the lowest tested dose of 0.05 μg (50 ng, or 0.0025 mg/kg) increased the MST (50 ± 8 days) by more than 2-fold over the controls.

**Minimum effective dose of veltuzumab in follicular cell lymphoma xenografts**

In disseminated follicular cell lymphoma, WSU-FSCCL, xenografts, mice were administered a single dose of veltuzumab (35, 3.5, 0.35, and 0.035 μg) i.p., 5 days after tumor inoculation. All four doses improved survival of the mice significantly \( P < 0.0001 \) when compared to the saline controls (Figure 3C). The MST of mice administered the 35-μg dose (44.3 ± 4.9 days) was not significantly different from that of the 3.5-μg group (39.5 ± 4.6 days), but was significantly \( P < 0.021 \) longer than that of the 0.35- and 0.035-μg (35 ng or 0.002 mg/kg) groups (40.5 ± 1.6 days and 33.3 ± 2.1 days, respectively).

**Comparative therapeutic effects of veltuzumab and rituximab**
Studies in Daudi, WSU-FSCCL, and Raji tumor models showed statistically significant improved survival of veltuzumab over rituximab. The MST of 0.05 and 0.1 μg single doses (0.0025 and 0.005 mg/kg, respectively) given 1 day after grafting were 28 and 35 vs. 24 and 28 days for veltuzumab and rituximab, respectively ($P=0.0011$) in the Daudi Burkitt lymphoma, and also at the single low dose of 0.035 μg (0.0021 mg/kg) also given 1 day post transplantation of the WSU-FSCCL model ($P=0.005$) (data not shown). Figure 4A shows the results of treating SCID mice with Raji lymphoma cells after administering veltuzumab or rituximab, indicating a statistically significant survival advantage for veltuzumab ($P=0.002$), where median survival of veltuzumab was not reached but rituximab-treated animals showed a median survival of 48 days after receiving 3 doses of 10 mg/kg (200 μg) on days 5, 10, 15, and 20 post tumor transplantation.

**Effect of depleting NK cells and neutrophils on anti-lymphoma activity**

We also examined the role of effector cells on veltuzumab’s inhibition of Raji tumor growth in vivo (Figure 4B). In those animals depleted of NK cells and neutrophils, there was no difference between saline control and treated mice, both having the same MST (16.4 ± 1.3 days). In the non-depleted group, veltuzumab-treated mice had a significantly improved MST over the saline controls (38.6 ± 7.3 vs. 18.4 ± 0.9 days; $P<0.0035$).

**Discussion**

Due to our observations that rituximab combined with epratuzumab showed increased complete responses in NHL patients, with no increased side-effects over those resulting from monotherapy with rituximab,\textsuperscript{14,19-21} our original purpose was to develop a humanized anti-CD20 MAb that could be combined with epratuzumab, but would be more tolerable for rapid infusions due to
having the FRs of epratuzumab. The first characterization of veltuzumab reported similarities to rituximab in terms of epitope binding, affinity, ADCC, CDC, and cell growth inhibition in vitro.\textsuperscript{3} Treating patients with NHL demonstrated a favorable tolerability and infusion profile, but also a high rate of complete responses (CR/CRu). The Phase I/II trial in 82 NHL patients is now being summarized for publication, but it has been reported already that the complete response rates for all doses tested (27% for all follicular lymphoma patients for doses between 80 and 750 mg/m\textsuperscript{2} once-weekly x 4 weeks)\textsuperscript{11,12} exceed those reported for repeated use of rituximab at its conventional dose in comparable patients.\textsuperscript{22} Ongoing studies also showed that single absolute doses of 80 mg of veltuzumab induced rapid B-cell depletion in patients with NHL or immune thrombocytopenic purpura, including reversal of thrombocytopenia in the latter patients (data on file, Immunomedics, Inc.). These findings prompted us to re-evaluate the functional properties of veltuzumab, also in comparison to rituximab, which is the subject of this article.

Studies in cynomolgus monkeys have now confirmed the effects of various i.v. and s.c. doses, showing that a single dose as low as the equivalent of 80 mg/m\textsuperscript{2} in humans, given by either route, is sufficiently potent to induce peripheral blood and lymphatic organ B-cell depletion. In addition, enhanced survival and even cures were demonstrated in mice bearing CD20+ lymphoma xenografts after a single i.p. or s.c. dose as low as 0.035 or 0.050 μg (or ~0.002 mg/kg). In these mouse studies, a dose-response was observed, but no significant difference between the i.v. or s.c. routes was noted.

In studies involving 3 lymphoma models in SCID mice, comparisons of low and high, single or multiple, doses showed a significantly increased survival time after veltuzumab compared to rituximab treatment, as conducted in two different laboratories. However, this was not consistent with ex-vivo cell studies showing equal potency of veltuzumab and rituximab in
killing human B cells and either Daudi or Raji lymphoma cells, but it is interesting that both antibodies were more effective against the tumor cells than the normal human B cells, suggesting that there may be a higher density of CD20 on these lymphoma cells.

Although different anti-CD20 MAbs have shown some variations in functional properties and epitope specificities, mediating different CDC and cell-killing effects, virtually all recognize the large, extracellular loop and partially or completely crossblock each other, except ofatumumab, which is reported to bind to a novel epitope of CD20. Veltuzumab crossblocks binding by rituximab, suggesting either the same epitope is recognized by both MAbs or binding to an adjacent epitope could result in steric hindrance.

In this study, the binding and dissociation parameters of veltuzumab and rituximab were compared both by Scatchard analyses and off-rate measurements. Scatchard analyses confirmed that veltuzumab and rituximab have similar affinity for cell-surface CD20 and number of binding sites per cell. Interestingly, statistical differences between veltuzumab or cA20 vs. rituximab or D101N were found in a slower off-rate (i.e., longer cell-surface retention) in all 3 human lymphoma cell lines tested, and a higher CDC-mediated cell killing in Daudi lymphoma cells (but not Raji and Ramos cells) by veltuzumab, compared to rituximab or D101N. Whether measured in the presence or absence of a competitive binder, veltuzumab and cA20, both containing Asp101 instead of Asn101 in CDR3-VH, had significantly (P<.0001) slower off rates (~2.5-fold) than rituximab or D101N. The demonstration of CDC activity for veltuzumab in Daudi being significantly more than rituximab or D101N is also intriguing, since the Fc portion of veltuzumab is derived from that of epratuzumab, which fails to show CDC functions. This suggests that rapid internalization of epratuzumab may prevent it from residing on the cell surface long enough to form membrane attack complexes needed for CDC. Our results also
suggest that the off-rate difference between veltuzumab and rituximab is not related to the enhanced CDC observed in Daudi cells, as first postulated for ofatumumab,\textsuperscript{4} since this difference was not observed for CDC in two other cell lines (Raji and Ramos) that also showed a significantly slower off-rate with veltuzumab compared to rituximab. Although these findings with veltuzumab involve evidently a different targeted epitope of CD20 than ofatumumab,\textsuperscript{28} we are not convinced that such off-rate changes are due to the position of the epitope, as postulated by these authors. Nevertheless, we agree with Teeling \textit{et al.}\textsuperscript{4} that such off-rate changes, as also described herein, may explain an antibody functioning at lower concentrations than other MAbs, such as we have found with veltuzumab, but whether CDC plays a role remains speculative.

It is doubtful that these differences are related to veltuzumab having the FRs of epratuzumab. The $V_H$ and $V_K$ chains of cA20 differ from those of rituximab in six positions, but except for the 101-residue in CDR3-$V_H$, the remaining five residues (two in FR4-$V_H$ and three in FR1-$V_K$) are unlikely to be responsible for the differential off-rates. Du \textit{et al.} reported a weaker interaction of the CDRs of another anti-CD20 MAb, c2H7, compared to rituximab, suggesting that the amino acid residues of 2H7 at the equivalent positions in CDR3-$V_H$ have more bulky side chains, resulting in a wider pocket to accommodate the CD20 peptide.\textsuperscript{29} The fact that cA20 and veltuzumab have virtually the same affinity and off-rate, whereas cA20 has more similar FRs to rituximab than to veltuzumab, emphasizes the more critical role of CDRs than FRs in interacting with CD20. Thus, the significant difference observed in the off-rate between veltuzumab/cA20 and rituximab/D101N is apparently due to the single amino acid difference in CDR3-$V_H$, and not to the more extensive differences in the FRs between veltuzumab and rituximab. Accordingly, we believe this is the first single amino-acid change in a CDR that is shown to cause a functional effect, possibly resulting in a more potent antibody.
As discussed already, these in-vitro off-rate and CDC differences are comparable to the findings with another anti-CD20 MAb, ofatumumab, which was reported to bind to a different epitope than rituximab, and claimed to be therapeutically more active than rituximab in-vitro. However, this is not consistent with the relatively high doses chosen for clinical studies, also requiring long infusion times like rituximab, or the lowest dose of 0.5 mg/kg (10 μg/mouse) shown to elicit growth inhibition in lymphoma xenografts.

Still another recently developed human anti-CD20 MAb, G101, which has properties of a Type-II anti-CD20 MAb, has been shown to be more potent in-vitro and in animal models than rituximab, when mice were given repeated doses of 10-30 mg/kg. This translates to each dose of repeated applications being between 200 and 600 μg in a 20-g mouse, which are at least 5,000- to 15,000-fold higher than the single doses of 0.05 to 0.35 μg (0.002 mg/kg) of veltuzumab showing high anti-growth activity in the lymphoma xenografts tested. Depletion of murine NK cells and neutrophils prevented these effects of veltuzumab, emphasizing the role of ADCC in vivo, as shown previously for rituximab.

In conclusion, in-vitro, mouse, monkey, and other human studies indicate that (i) veltuzumab is active at a fraction of the conventional clinical dose of rituximab or of the minimal therapeutic doses of two other second-generation anti-CD20 MAbs in preclinical models, (ii) survival studies in 3 different lymphoma models showed a significantly higher potency of veltuzumab over rituximab, (iii) the two distinguishing differences in activity vs. rituximab in-vitro involve CDC and off-rate functions, and (iv) the lower off-rate appears to be related to a single amino acid mutation at the Kabat-101 residue in the CDR3-VH. This is the first description, to our knowledge, of a single amino acid difference in an antibody’s CDR affecting
its function, but whether this functional change in veltuzumab depends specifically on Asp\textsubscript{101} or can be reproduced with other amino acid substitutions is of interest to investigate.

**Acknowledgments**

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**Authorship**

Contribution: D.M.G., E.A.R., H.J.H., R.S., and C.-H. C. designed research, analyzed data, and wrote the paper; T.M.C., M.S.C., and F.J.H. performed research, collected and analyzed data, and revised the paper.

Conflict-of-interest disclosure: All of the authors, except R.S., M.S.C., and F.J.H., have employment, stock, and/or stock options with Immunomedics, Inc., which owns and has patented veltuzumab.


The online version of the article contains a data supplement.
References


Table 1. Comparison of CDR3-V<sub>H</sub>.

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<tr>
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<tr>
<td>D101N</td>
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<td>•</td>
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</tr>
<tr>
<td>1F5</td>
<td>•</td>
<td>H</td>
<td>•</td>
<td>G</td>
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1 Residue marked as • is identical to that of veltuzumab in the same position.
Table 2. Comparison of Veltuzumab versus Rituximab: Summary of CDC Results (EC$_{50}$) in the Daudi cell line.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>No. of Experiments (N)</th>
<th>EC$_{50}$ (µg/mL) Mean ±Std. Dev.</th>
<th>Mean Difference (Vmab – Rmab)</th>
<th>95% Confidence Interval [1]</th>
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<td>Rituximab</td>
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<td>0.1485 ± 0.0200</td>
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<td>Veltuzumab (Lot 1)</td>
<td>9</td>
<td>0.0990 ± 0.0232</td>
<td>-0.0495</td>
<td>(-0.0611, -0.0378)</td>
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<td>Veltuzumab (Lot 2)</td>
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<td>0.0843 ± 0.0215</td>
<td>-0.0642</td>
<td>(-0.0758, -0.0525)</td>
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<tr>
<td>Veltuzumab (Lot 3)</td>
<td>9</td>
<td>0.0904 ± 0.0239</td>
<td>-0.0581</td>
<td>(-0.0697, -0.0464)</td>
</tr>
</tbody>
</table>

[1] Based on 2-way ANOVA model adjusted for multiple comparisons using Dunnett’s method.
Figure Legends

**Figure 1. Comparison of off-rates from live cells.** Daudi (A), Ramos (B), and Raji (C-E) cells were stained with PE-labeled rituximab (▲), veltuzumab (■), cA20 (▼), D101N (●), 1F5 (○) or B1 (□). The labeled MAbs were incubated at 37°C with (A-D) or without (E) excess veltuzumab Fab’-NEM and the cells analyzed by flow cytometry over time. The off-rate was determined by non-linear regression (one-phase exponential decay) and *P*-values were generated by F-test using GraphPad Prism software.

**Figure 2. Ex vivo depletion of B-cells and lymphoma cells.** (A) The effect of veltuzumab on peripheral blood lymphocytes from healthy volunteers was evaluated *in vitro* using flow cytometry. Decrease in the percent of CD19+ cells present in the lymphocyte gate after a two-day incubation of heparinized whole blood of healthy volunteers with veltuzumab is shown. Each line represents a different blood donor. Error bars, SD. (B) The effects of veltuzumab and rituximab on peripheral blood B cells and Raji lymphoma cells are sown as the number of CD19+ events relative to untreated cell mixtures. B cells are derived as the CD19+ cells in the lymphocyte gate, while Raji cells are located in the monocytes gate. Error bars, SD.

**Figure 3. Evaluation of *in vivo* efficacy in mouse models.** (A) Survival of mice in a disseminated Burkitt lymphoma xenograft model was compared for veltuzumab treatment via intraperitoneal *versus* subcutaneous administration. SCID mice were administered 1.5 x 10^7 Daudi cells i.v. on day 0. Therapy with veltuzumab began on day 1 with mice receiving either a
single i.p. or single s.c. injection of veltuzumab at doses of 60, 20, or 5 μg. Control mice received an i.p. injection of either saline or 60 μg hMN-14 IgG (labetuzumab, anti-CEACAM5 isotype-matched antibody). (B) The minimal effective dose of veltuzumab was determined in a disseminated Burkitt lymphoma xenograft model. SCID mice were administered $1.5 \times 10^7$ Daudi cells i.v. on day 0. Therapy with veltuzumab began on day 1 with a single i.p. injection of veltuzumab. Doses administered were 0.5, 0.25, 0.1, or 0.05 μg veltuzumab. Control mice received a 200 μL i.p. of saline. (C) Survival of mice bearing disseminated follicular cell lymphoma was examined for treatment with decreasing doses of veltuzumab. SCID mice were administered $2.5 \times 10^6$ WSU-FSCCL cells i.v. on day 0. On day 5, mice received a single i.p. injection of veltuzumab at a dose of 35, 3.5, 0.35, or 0.035 μg. Control mice received only saline.

**Figure 4. In-vivo effects of veltuzumab compared to rituximab and after NK/neutrophil depletion in Raji lymphoma model.** (A) Comparison of therapeutic effects on survival of RPCI-SCID mice bearing Raji lymphoma cells treated with 10 mg/kg veltuzumab or rituximab (or untreated control) on days 5, 10, 15, and 20 post tumor inoculation i.v. (N=15 per group), indicating significantly improved survival ($P=.005$) of veltuzumab group compared to rituximab group. (B) The effect of depleting NK cells and neutrophils on anti-lymphoma activity in SCID mice, as described in the Methods section. Veltuzumab therapy consisted of 200 μg given on days 3, 5, 7, and 11 i.v.; control mice received 100 μL saline. Depletion of NK cells and neutrophils abrogated the anti-lymphoma activity of veltuzumab.
Fig 2

A

% Decrease

Log Concentration (µg/mL)

Donor A
Donor B
Donor C

B

% Gated Events Relative to Untreated

Concentration (nM)

Rituximab - Raji
Rituximab - B cells
Veltuzumab - Raji
Veltuzumab - B cells
Fig 4

A

Cumulative Survival

Time to limb paralysis (days)

Untreated
Rituximab
Veltuzumab

B

Percent Survival

Days

Therapy
Inject Raji

NK/Neutrophil Depleted Mice

Median Survival

- 200 μg Veltuzumab: 17 days
- 100 μL Saline: 17 days

Non-Depleted Mice

- 200 μg Veltuzumab: 41 days
- 100 μL Saline: 19 days

Veltuzumab vs. Rituximab P = 0.002

Treatment | Median (Days) | Std. Error | 95% Confidence Interval
--- | --- | --- | ---
Rituximab | 38 | 7.454 | 23.391 | 52.59
Veltuzumab | Not Reach (NR) | NR | NR
Properties and structure-function relationships of veltuzumab (hA20), a humanized anti-CD20 monoclonal antibody

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