B-Cell Depletion In Vitro and In Vivo with an Afucosylated Anti-CD19 Antibody

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ABSTRACT

The pan B-cell surface antigen CD19 is an attractive target for therapeutic monoclonal antibody (mAb) approaches. We have generated a new afucosylated anti-human (hu)CD19 mAb, MEDI-551, with increased affinity to human FcγRIIA and mouse FcγRIV and enhanced antibody-dependent cellular cytotoxicity (ADCC). During in vitro ADCC assays with B-cell lines, MEDI-551 is effective at much lower mAb concentrations than the fucosylated parental mAb anti-CD19-2. Furthermore, the afucosylated CD19 mAb MEDI-551 depleted B cells from normal donor peripheral blood mononuclear cell samples in an autologous ADCC assay, as well as blood and tissue B cells in huCD19/CD20 double transgenic (Tg) mice at lower concentrations than that of the positive control mAb rituximab. In huCD19/CD20 Tg mice, both macrophage-mediated phagocytosis and complement-dependent cytotoxicity contribute to depletion with rituximab; MEDI-551 did not require complement for maximal B-cell depletion. Furthermore, extended B-cell depletion from the blood and spleen was achieved with MEDI-551, which is probably explained by bone marrow B-cell depletion in huCD19/CD20 Tg mice relative to the control mAb rituximab. In summary, MEDI-551 has potent B-cell-depleting activity in vitro and in vivo and may be a promising new approach for the treatment of B-cell malignancies and autoimmune diseases.

Introduction

B lymphocytes are an important component of the immune system and are the source of humoral immunity. However, B cells can also be pathogenic and the origin of disease. Deregulated B-cell function has been implicated in several autoimmune diseases, including systemic sclerosis, systemic lupus erythematosus, and rheumatoid arthritis (Edwards and Cambridge, 2006; Gabrielli et al., 2009). B cells contribute to pathological immune responses through the secretion of cytokines, costimulation of T cells, antigen presentation, and the production of autoantibodies (Browning, 2006; Walker and Fritzler, 2007; Yanaba et al., 2008). Furthermore, the majority of human leukemias and lymphomas are of B-cell origin (Kuppers, 2005). Despite recent advances in the clinic, B-cell malignancies, such as acute lymphoblastic leukemia, chronic lymphocytic leukemia, follicular lymphoma, and diffuse large B-cell lymphoma (DLBCL), still present a significant challenge, and curative treatment is not possible in most cases (Coffier et al., 2005a; Coffier et al., 2005b).

B-cell depletion by targeting B-cell-restricted cell surface antigens with monoclonal antibodies (mAbs) has gained significant attention in recent years. In particular, the CD20 mAb rituximab, which is already approved for the treatment of non-Hodgkin’s lymphoma and DLBCL, has shown promising results in the treatment of B-cell malignancies. Although the combination of rituximab with chemotherapy has led to significant improvements in patient survival in follicular lymphoma and other malignancies, many patients relapse after treatment (Maloney, 2005). In addition, low CD20 expression has been associated with poor response to ritux-
imab in DLBCL, and rituximab treatment can lead to down-regulation of CD20 or the selection of CD20-deficient tumor clones (Davis et al., 1999; Kennedy et al., 2004; Johnson et al., 2009). Rituximab has also shown activity in autoimmune diseases, in particular in RA, but reductions in the levels of circulating autoantibodies, which may play an important role in systemic lupus erythematosus and other indications, are not consistent (Levesque and St Clair, 2008; Tedder, 2009).

CD19 is a B-cell-specific surface antigen that is expressed by early pre-B cells from the time of heavy chain rearrangement until the molecule is eventually down-regulated during terminal differentiation into plasma cells (Nadler et al., 1983). CD19 belongs to the immunoglobulin domain-containing superfamily of transmembrane receptors. As a component of the B-cell receptor complex, CD19 regulates the threshold for B-cell activation (Engel et al., 1995). Relatively small changes in CD19 surface expression can lead to loss of tolerance and autoantibody production (Sato et al., 2004).

In hematopoietic malignancies of B-cell origin, expression of CD19 is maintained after malignant transformation of B cells, and the majority of B-cell-derived leukemias and lymphomas consistently express CD19 (D’Arena et al., 2000). It is important to note that malignant B cells that have lost CD20 expression during the course of rituximab therapy maintain expression of CD19 (Johnson et al., 2009). Thus, CD19 is an attractive target for antibody-mediated B-cell depletion therapy in multiple hematologic malignancies. Due to the expression of CD19 on plasmablasts, which are CD20 negative (Levesque and St. Clair, 2008), a depletion CD19 mAb may also have a more significant impact than CD20-targeted therapies on the levels of pathogenic autoantibodies in autoimmune diseases (Tedder, 2009).

Here, we report the generation of a new anti-human-CD19 mAb in development for the therapeutic depletion of B cells. The humanized and affinity-optimized MEDI-551 mAb has binding characteristics that are favorable for an ADCC-dependent mechanism. MEDI-551 was generated by removal of the fucosyl carbohydrate modification from the Fc portion of the humanized CD19 mAb anti-CD19-2, which resulted in a selective increase in the affinity for human FcγRIIA and mouse FcγRIV. The anti-CD19-2 mAb was developed by humanization and affinity optimization of the HB12b mAb, which has potent B-cell-depleting activity in transgenic (Tg) mice expressing human CD19 (huCD19) (Kansas and Tedder, 1991, Yazawa et al., 2005). During in vitro ADCC assays, MEDI-551 depleted primary human B cells at lower mAb concentrations than the CD20 mAb rituximab. Furthermore, at low mAb doses, MEDI-551 is more effective than rituximab in depleting blood and tissue B cells in huCD19/CD20 double Tg mice, despite the absence of complement-dependent cytoxicity (CDC), which contributes to in vivo depletion with rituximab. In the Tg mouse model, macrophages are important in vivo B-cell depletion with the afucosylated human IgG1, consistent with the increased binding to mouse FcγRIIb in a homogenous system. In summary, MEDI-551 is a new anti-CD19 mAb optimized for enhanced ADCC effector function and may be found to be a useful new agent for the treatment of B-cell malignancies and B-cell-dependent autoimmune disease.

Materials and Methods

Cells and Reagents. The Burkitt’s lymphoma cell lines Raji, Daudi, and Ramos and the human monocye line THP-1 were obtained from the American Type Culture Collection (Manassas, VA). The KC1333 NK cell line expressing human CD16 was obtained from BioWa Potellgent Technology (Princeton, NJ). All cell lines were maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum. Blood samples (peripheral blood mononuclear cells [PBMCs] and serum) were obtained from healthy donors after obtaining informed consent. PBMCs were isolated by Ficoll density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instructions, and B cells were purified by negative selection using the Naive B Cell Isolation Kit II according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). The mAbs used were anti-CD22 clone S-HCL-1 (Immunocytometry Systems, San Jose, CA) and rituximab (Biogen Idec, Inc., Cambridge, MA) (Anderson et al., 1997). The human IgG1 mAb R347 (MedImmune, LLC, Gaithersburg, MD) was used as isotype control. Mouse specific mAbs were used were B220 (CD45R; Invitrogen, Carlsbad, CA) and 1D3 (CD19; BD Biosciences Pharmingen, San Diego, CA).

Antibody Engineering. To generate a CD19 mAb with enhanced ADCC effector function, the mouse IgG1 mAb HB12b (Kansas and Tedder, 1991), which recognizes human CD19, was humanized and affinity-optimized, resulting in mAb anti-CD19-2. To generate a homogenously afucosylated antibody, the humanized IgG1 mAb anti-CD19-2 was expressed in a fucosyltransferase-deficient producer Chinese hamster ovary cell line (BioWa Potellgent Technology) to generate MEDI-551.

Cell Proliferation Assay. To test the effect of immobilized (cross-linked) mAb on B-cell function, 100 ng of anti-CD19-2 mAb or isotype control mAb in phosphate-buffered saline (PBS) was used to coat triplicate wells of a flat-bottomed high-binding 96-well plate (Costar 3361; Corning Life Sciences, Lowell, MA) overnight at room temperature. Next, the wells were washed and the cells were seeded at a density of 10,000 cells/well. Cells were assayed on day 3 using the CellTiter-Glo luminescent cell viability assay according to the manufacturer’s instructions (Promega; Madison, WI).

BLAcore Affinity Measurements. The affinity ($K_d$) for the binding of human and murine FcγRs to IgG mAbs was measured on a BLAcore 3000 instrument (BLAcore AB, Uppsala, Sweden). In brief, the fucosylated and afucosylated forms of the IgG1-humanized CD19 mAb were immobilized onto separate flow cells on a CM5 sensor chip. Stock solutions of FcγRs (Oganesyan et al., 2008) were serially diluted using instrument buffer (HBS-EP buffer containing 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% P20 detergent). FcγRs were injected over both the IgG and reference cell surfaces at a flow rate of 5 μl/min. Binding data were collected for 50 min, followed by a 60-s pulse of 5 mM HCl between injections to remove bound FcR from the IgG surfaces. After all binding data were collected, individual data sets were averaged for binding (response at equilibrium) at each concentration, and then fit to a 1:1 binding isotherm (response at equilibrium concentration). From this, the $K_d$ values were derived. Such calculations were performed with BLAevaluation software, version 4.1 (BLAcore AB).

ADCC Assays. ADCC assays were performed with a set effector-target cell ratio of 2.5:1. In brief, Daudi target cells were washed with PBS, resuspended in RPMI 1640 phenol-free media at a cell density of 0.4 × 10^6/ml. KC1333 NK cells were washed once in PBS and resuspended in RPMI 1640 phenol-free media at a cell density 1 × 10^6/ml. Assays were performed in U-bottomed 96-well plates. Experimental wells were set up by combining 50 μl of the appropriate mAb dilution, 50 μl of target cell suspension, and 50 μl of effector cell suspension. Reactions were set up in triplicates. After setup, plates were spun at 120g for 3 min to pellet the cells. Plates were incubated at 37°C, 5% CO2 for 4 h. Target cell lysis was measured by detecting the release of lactate dehydrogenase (LDH) from the cytoplasm of lysed cells using the CytoTox 96 nonradioactive cytotoxicity assay.
*Hill, NJ). In total, 2 × 10^6 cells/ml in culture media (RPMI 1640 medium, 10% heat inactivated fetal bovine serum, and 2 mM l-glutamine), supplemented with 200 ng/ml recombinant human interleukin-2 (PeproTech, Rocky Hill, NJ). In total, 2 × 10^6 PBMCs were added to each well in U96-well round-bottomed microwell plates (Nalge Nunc International, Rochester, NY). Serial dilutions of MEDI-551 or rituximab were added in 10 µl to wells in triplicate and incubated for 20 h at 37°C with 5% CO2. Cells were then washed once in stain buffer (PBS containing 0.5% bovine serum albumin) and resuspended in 100 µl of a cocktail of fluorescently labeled antibodies in stain buffer containing anti-CD19 phycoerythrin-Cy7, anti-CD20 Pacific Blue, anti-CD22 allophycocyanin, or anti-CD22 phycocerythrin, and anti-FcR1α fluorescein isothiocyanate. The cells were stained for 30 min on ice, washed once in stain buffer, and then resuspended in a final volume of 100 µl of stain buffer containing 5 µl of the viability solution, 7-aminocoumarin D. As a counting standard, 20 µl/well CountBright absolute counting beads was added to determine cell concentration of cell subsets. Samples were acquired on an LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ) equipped with a high-throughput capability for running 96-well microwell plates. The fluorescence-activated cell sorting (FACS) data were analyzed with FlowJo software, version 7.2.2 for PC (TreeStar, Inc., Ashland, OR). The gating strategy used to quantify B-cell depletion was as follows. Initially, dead 7-aminocoumarin D-positive cells were excluded, and then live lymphocytes were gated using their forward and light scatter characteristics. Basophils, eosinophils, and mast cells within the lymphocyte gate were excluded on the basis of their expression of FcR1α. With rituximab, it was evident that the antibody treatment reduced CD19 expression. Consequently, for this antibody treatment, the total number of surviving B cells was defined by a single gate of all CD22+ cells. For MEDI-551 treatment, the number of surviving B cells in the CD20−CD22+ gate was determined. The IgG1 afucosylated antibody R347-aFuc was used as a nondepleting treatment control and was used to define these gates. The number of absolute counting beads in each sample was quantified in a forward scatter gate. The numbers of surviving B cells in the total CD22+ or CD20−CD22+ gates were converted to cell concentrations using the standard counting beads according to the manufacturer’s instructions.

The B-cell depletion (percentage of cytotoxicity) was then calculated according to the following formula: for rituximab, 1 - [rituximab CD22+ (cells/milliliter)/(R347-aFuc CD19+ CD22+ (cells/milliliter)) ÷ 100; and for MEDI-551, and 1 - [MEDI-551 CD20+ CD22+ (cells/milliliter)/(R347-aFuc CD20+ CD22+ (cells/milliliter)) ÷ 100. The half-maximal effective concentration (EC50) of B-cell depletion was calculated using a four-variable curve-fit equation in Prism, version 5.01 software (GraphPad Software Inc., San Diego, CA). The results are shown in Table 2 and include the 95% confidence interval as well as the R² goodness-of-fit value.

**Complement-Dependent Cytotoxicity Assay.** The CDC assay was performed using the same LDH readout assay as the ADCC assay. In brief, human donor serum was collected and divided into two separate aliquots. One aliquot was treated at 56°C for 30 min to inactivate complement followed by centrifugation for 10 min at 3000g, whereas the other aliquot remained untreated. Media containing phenol-free RPMI 1640 medium and 10% of either heat-inactivated or nonheat-inactivated serum were prepared. Target cells were washed with PBS, resuspended in either RPMI 1640 medium containing heat-inactivated or nonheat-inactivated serum, at a cell density of 0.4 × 10^5/ml. Experimental wells were set up by combining 50 µl of the appropriate antibody dilution (in either heat-inactivated or nonheat-inactivated media), 50 µl of target cell suspension (in either heat-inactivated or nonheat-inactivated media), and 50 µl of media (either heat-inactivated or nonheat-inactivated). Reactions were set up in triplicates, and plates were incubated at 37°C, 5% CO2 for 4 h. Forty-five minutes before the end of incubation, 15 µl of manufacturer-provided lysis buffer was added to the target cell maximal release control well and detergent background wells. After incubation, the plate was centrifuged at 120g for 3 min. Then, 50 µl of the supernatant from each well was transferred to a new flat-bottomed 96-well plate. Next, 50 µl of reconstituted substrate mix (assembled from manufacturer-provided components) was added, and the plate was incubated at room temperature for 10 to 20 min, protected from light. Finally, 50 µl of manufacturer-provided stop buffer was added, and absorbance at 490 or 492 nm was measured in a plate reader. Percentage of cytotoxicity was calculated as described above for ADCC.

**B-Cell Depletion in huCD19/CD20 Transgenic Mice.** All mouse experiments were carried out in a pathogen-free environment at the MedImmune, LCC, animal facility in accordance with Institutional Animal Care and Use Committee-approved protocols. In vivo depletion of B cells by CD19 and CD20 mAbs was evaluated in huCD19/CD20 double Tg mice, which were generated by crossing huCD19 Tg with huCD20 Tg animals (Zhou et al., 1994; Ahuja et al., 2007). Tg mice (10–15 weeks old; five to eight animals/group) were randomized and treated with MEDI-551 or rituximab by tail vein injection. PBS-treated animals were used as controls (three to five animals/group). Blood, spleen, and bone marrow samples were collected for further analysis using flow cytometry. B-cell numbers were determined in each sample by staining with allophycocyanin-Cy5.5-conjugated B220 (CD45R) and PerCP-Cy5.5-conjugated mCD19 antibodies. Single-cell bone marrow and spleen cell suspensions were loaded at 1.5 million cells/well for flow cytometry analysis.

**Mechanism of In Vivo B-Cell Depletion.** Tg mice were treated with MEDI-551 or rituximab in the absence of complement or of one of the potential effector cell populations.

To study the contribution of complement for B-cell depletion, hCD19 Tg mice (three mice/group; 8–12 weeks old) were treated with 30 µg/mouse cobra venom factor (CoVF, Sigma-Aldrich) or PBS every other day for 5 days (Minard-Colin et al., 2008). Twenty-four hours after the first intraperitoneal dose of CoVF, mice were treated with either MEDI-551 or rituximab (10 mg/kg i.v.) or PBS. Serum C3 levels were detected before mAb injection and at the study endpoint by enzyme-linked immunosorbent assay (Immunochemistry Consultants Laboratory, Newberg, OR; data not shown).

To deplete NK cells and neutrophils, hCD19 Tg mice were treated with intraperitoneal injections of 0.25 mg/mouse anti-NK1.1 (clone PK136) or anti-Gr1 (clone RB6-8C5) (Biolegend, San Diego, CA), respectively (Gong et al., 2005). Anti-NK1.1 treatment occurred every other day for 5 days (days −1, 1, and 3). Anti-Gr1 was given as a single injection (day −1). Treatment with MEDI-551 or rituximab (10 mg/kg mAb) followed 24 h after the first injection (day 0). The depletion of NK cells and neutrophils was verified with FACS analysis before treatment with MEDI-551 or rituximab with FACS cytometry to determine the duration of B-cell depletion. NK cells were detected as CD49b+CD335+ in the blood and CD49b+Ly49+ in the spleen and...
bone marrow. Neutrophils were detected as CD11b+SSC<sup>high</sup> (data not shown).

To assess the role of macrophages, hCD19 Tg mice were treated with injections of liposome-encapsulated clodronate (Encapcula, Nashville, TN; five to seven animals/group). PBS-loaded liposomes were used as control (three to four animals/group). Loaded liposomes were intraperitoneally injected at 0.2 ml/mouse every other day for 5 days before treatment with 10 mg/kg MEDI-551 or rituximab. An additional 0.2 ml/mouse was injected 2 days after mAb treatment. The depletion of macrophages was verified by FACS and immunofluorescence histology. The number of macrophages in spleen was determined by FACS staining of F4/80<sup>+</sup> cells before the treatment with MEDI-551 and again at the endpoint. The spleens of mice were also harvested for histology. Slides of spleen sections, prepared from optimal cutting temperature blocks, were stained for B cells (Alexa Fluor 555 or Pacific Blue-conjugated B220), T cells (Alexa Fluor 647-conjugated CD90), red pulp macrophages (Alexa Fluor 488-conjugated F4/80), marginal zone metaphilic macrophages (biotinylated MOMA-1 followed by Pacific Blue-conjugated streptavidin), and/or marginal zone macrophages (Alexa Fluor 488-conjugated ERTR9) (data not shown).

Statistical Analysis. The data were analyzed and graphed using Prism software (GraphPad Software Inc.). Mean values and S.D. were calculated for experiments as indicated in the figure legends. Statistics were performed using a two-tailed, two-sample t test in Prism and are determined from triplicate wells of each donor tested. Significant differences are noted by p value: *, p < 0.05; **, p < 0.01; and ***, p < 0.005.

Results

Removal of Fucose Enhances the ADCC Effector Function of the Anti-CD19-2 mAb. ADCC is an important mechanism by which therapeutic mAbs mediate target cell depletion (Glennie et al., 2007). Engagement of effector cells requires the interaction of the mAb Fc with Fcγ receptors on the surface of NK cells, monocytes/macrophages, or neutrophils. It has been demonstrated that the removal of fucose from the carbohydrate modification of the mAb Fc preferentially increases the affinity to FcγRIIIA, resulting in increased ADCC effector function (Shields et al., 2002; Nimmerjahn and Ravetch, 2008).

To generate a CD19 mAb with enhanced effector function, the humanized and affinity-optimized CD19 mAb anti-CD19-2 was expressed in a fucosyltransferase-deficient Chinese hamster ovary cell line, which generates homogenously afucosylated mAbs (BioWa Potelligent Technology). The afucosylated form of anti-CD19-2 is termed MEDI-551. We first tested the effect of fucosylation on the binding of the humanized IgG1 anti-CD19 mAb to various human Fcγ receptors by measuring the equilibrium binding constant (K<sub>d</sub>) on a BIAcore instrument. The removal of fucose had no significant effect on the binding to human FcγRI and FcγRIIa (Table 1). However, the afucosylated mAb has approximately 9-fold increased affinity to the activating FcγRIIIA. Likewise, removal of fucose resulted in a 10-fold increase in binding affinity to mouse FcγRI, the mouse ortholog of human FcγRIIIA. The binding to mouse FcγRIIB and FcγRIII was unaffected by the removal of fucose. These results are consistent with previously published data and further demonstrate a selective increase in the binding of afucosylated IgG1 mAbs to human FcγRIIIA and mouse FcγRIV (Shields et al., 2002; Nimmerjahn and Ravetch, 2008).

Table 1

<table>
<thead>
<tr>
<th>Fcγ Receptor</th>
<th>Fucosylated Anti-CD19 IgG1 K&lt;sub&gt;d&lt;/sub&gt;</th>
<th>Afucosylated Anti-CD19 IgG1 K&lt;sub&gt;d&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Human</td>
<td>Mouse</td>
</tr>
<tr>
<td>I</td>
<td>N.T.</td>
<td>20</td>
</tr>
<tr>
<td>IIa</td>
<td>N.A.</td>
<td>3030</td>
</tr>
<tr>
<td>IIb</td>
<td>1230</td>
<td>14,500</td>
</tr>
<tr>
<td>IIIA (V158)</td>
<td>N.A.</td>
<td>656</td>
</tr>
<tr>
<td>III</td>
<td>3440</td>
<td>N.A.</td>
</tr>
<tr>
<td>IV</td>
<td>306</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

N.A., not applicable; N.T., not tested.

Although the relative contribution of different effector cell populations in various tissues is still not fully established for human subjects, NK cells probably play an important role. In ADCC assays with human PBMCs as the source of the effector cells, almost all activity is provided by the NK cells (Desjarlais et al., 2007). Therefore, we tested the effector function of MEDI-551 and the fucosylated version the mAb (anti-CD19-2) in assays with NK cells as the effector cell population. The increased affinity of MEDI-551 for FcγRIIIA resulted in a substantial enhancement of its ADCC activity in vitro (Fig. 1A). This increase in ADCC effector function was reflected in a lower EC<sub>50</sub> value for cytotoxicity (3.1 ng/ml for MEDI-551 versus 57.6 ng/ml for the fucosylated mAb) as well as an increase in overall cytotoxicity (50% for MEDI-551 versus 17% for mAb anti-CD19-2). In repeated assays with Daudi cells as targets, MEDI-551 was also more effective at low mAb concentrations than the rituximab CD20 mAb (EC<sub>50</sub> = 41.0 ng/ml), which was included as a positive control (Fig. 1A).

To further characterize the anti-CD19 mAb, we tested mAb anti-CD19-2 for additional Fc-dependent and independent effects on B cells. In addition to ADCC, CDC is an Fc-dependent mechanism by which mAbs against cell surface antigens can mediate target cell killing and is well characterized for rituximab (Glennie et al., 2007). When tested in vitro with Daudi cells and human serum as a source of complement, the anti-CD19-2 mAb was not active in a CDC assay, whereas the rituximab CD20 mAb showed significant cytotoxicity (Fig. 1B).

Antibodies against cell surface antigens can also affect proliferation and/or viability of the target cell. Previous reports demonstrated that some, but not all, mAbs against CD19 can inhibit proliferation of B leukemia or lymphoma cells (Ghetie et al., 1990). Therefore we tested the effect of anti-CD19-2 mAb on cell proliferation of Raji, Daudi, and Ramos B lymphoma cell lines, using a luminescent viability assay. As a control, we also included the monocytic cell line THP-1. As shown in Fig. 1C, incubation with anti-CD19-2 mAb resulted in a significant decrease in the number of viable B cells compared with cells treated with the isotype control mAb. Proliferation of THP-1 cells, which do not express CD19, was not affected by anti-CD19-2 mAb. When added in solution, anti-CD19-2 mAb also inhibited the proliferation of primary human B cells stimulated with anti-IgM and CpG-B oligonucleotide (data not shown).

Depletion of Primary Human B Cells Ex Vivo with MEDI-551. Having demonstrated potent activity in an ADCC assay with a B-cell line and NK cells, we asked...
whether DI-551 was also effective in depleting primary human B cell in an autologous assay in which donor PBMCs are the source of both the target and effector cell populations. Figure 2 shows the results from three representative assays (of six individual PBMC samples) in which the activities of MEDI-551 and rituximab were compared. The calculated EC50 values for B-cell depletion with MEDI-551 mAb and rituximab for all PBMC samples are summarized in Table 2.

In all assays, MEDI-551 demonstrated potent ADCC activity against primary human B cells. The EC50 values determined ranged from 3 to 29 pM for MEDI-551 and from 18 to 431 pM for rituximab. The results demonstrate that MEDI-551 is active against primary human B cells in autologous ADCC assays.

MEDI-551 Depletes Blood and Tissue B Cells In Vivo.

The HB12b and anti-CD19-2 mAbs are specific for human CD19 and do not bind rodent CD19 or CD19 from nonhuman primates (Yazawa et al., 2005; data not shown). In addition, rituximab does not cross-react with rodent CD19 (Gong et al., 2005). Therefore, we made use of a Tg mouse model to compare the in vivo B-cell depletion activity of the two mAbs. Double Tg animals were generated by crossing huCD19 Tg mice with huCD20 Tg mice. Both strains have been well...
characterized previously, express the transgene in a B-cell restricted manner, and have been used successfully to study B-cell depletion with CD19 and CD20 mAbs, respectively (Zhou et al., 1994; Yazawa et al., 2005; Ahuja et al., 2007). As shown in Fig. 3A, the B-cell-restricted expression of both transgenes is also maintained in the huCD19/CD20 double Tg animals. To further characterize the expression of huCD19 and huCD20 in this model, purified spleen B cells were incubated with serial dilutions of anti-CD19-2 mAb, rituximab, or the R347 isotype control mAb and mAb binding was determined by FACS. The results demonstrate that maximal surface binding to huCD19/CD20 Tg B cells is comparable for the anti-CD19-2 and rituximab mAbs. The anti-CD19 mAb, however, seems to have stronger binding at low mAb concentrations, suggesting a higher affinity than rituximab (Fig. 3B).

To compare in vivo B-cell depletion with MEDI-551 and rituximab, huCD19/CD20 Tg mice received a single dose of each mAb at 0.5, 2, or 10 mg/kg, and remaining blood and spleen B-cell numbers were determined 4 days later by flow cytometry. At the highest dose of 10 mg/kg, both mAbs were very effective in depleting B cells from blood and spleen (Fig. 4, A and B). At the lower doses, however, B-cell depletion with rituximab was incomplete, whereas depletion with MEDI-551 was as effective at 0.5 mg/kg as at 10 mg/kg mAb. For example, a single dose of 0.5 mg/kg rituximab reduced splenic B cells on average by 42%, whereas MEDI-551 mAb used at this dose eliminated 85% of B cells from treated mice (Fig. 4B).

Further analysis showed that B-cell depletion in blood and spleen was maintained for more that 2 wk after a single 10 mg/kg administration of MEDI-551 (Fig. 4, C and D). In mice treated with rituximab, significant recovery of blood and spleen B-cell numbers was observed by day 14, despite almost complete depletion of blood and spleen B cells during the first days after dosing. In clinical studies with rituximab, immature B cells are often the first B cells detectable in peripheral blood during B-cell recovery, suggesting repopulation from the bone marrow (BM) (Roll et al., 2008). To test whether differences in the depletion of BM B cells between CD19 and CD20 mAbs could account for the differences in the kinetics of B-cell recovery in the periphery and secondary lymphoid organs, we analyzed the extent of B-cell depletion in this compartment with the two mAbs. As shown in Fig. 4E, a single dose of MEDI-551 mAb resulted in a substantial reduction (on average by 91.4% by day 3) in BM B220+muCD19+ B cells, whereas rituximab depleted fewer than 50% of the cells. Thus, the shorter time to B-cell recovery in mice treated with rituximab is probably caused by the partial depletion of BM B cells.

**Macrophages Are Required for Efficient In Vivo B-Cell Depletion with MEDI-551.** Several cell types, including NK cells, neutrophils, and macrophages, can potentially contribute to antibody-mediated target cell depletion in vivo (Glennie et al., 2007; Nimmerjahn and Ravetch, 2008). Previous studies have emphasized the importance of monocytes/macrophages for B-cell depletion in mice with mouse IgG2a mAbs (Uchida et al., 2004; Gong et al., 2005; Yazawa et al., 2005). However, the mechanism by which an afucosylated human IgG1 mediates its effector function has not yet been determined in a Table 2

<table>
<thead>
<tr>
<th>Donor</th>
<th>MEDI-551 EC_{50} (95% CI)</th>
<th>R^2</th>
<th>Rituximab EC_{50} (95% CI)</th>
<th>R^2</th>
</tr>
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<tr>
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<td>0.016 (0.0096–0.0264)</td>
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<tr>
<td>310</td>
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<td>374</td>
<td>0.029 (0.0132–0.0645)</td>
<td>0.968</td>
<td>0.063 (0.0500–0.0802)</td>
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<td>0.032 (0.0240–0.0416)</td>
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<td>0.018 (0.0157–0.0208)</td>
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<td>467</td>
<td>0.0007 (0.0001–0.0055)</td>
<td>0.957</td>
<td>0.039 (0.0348–0.0442)</td>
<td>0.992</td>
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Fig. 3. Cell surface expression of huCD19 and huCD20 in huCD19/20 double Tg mice. A, live blood mononuclear cells and splenocytes from huCD19/CD20 Tg mice were analyzed for expression of human CD19 and CD20, along with murine CD19, a marker for B cells. B, purified mouse spleen B cells from huCD19/CD20 Tg mice were incubated with serial diluted anti-CD19-2 mAb, anti-huCD20 mAb rituximab, or isotype control mAb for 1 h at 37°C. The cell surface-bound mAbs were then detected with a mouse anti-human IgG1 secondary antibody and measured by FACS. The graph shows one of two experiments with similar results.
mouse model. To explore the mechanism of in vivo B-cell depletion with MEDI-551, huCD19/CD20 Tg mice were treated with the CD19 mAb in the absence of one of the potential effector cell populations or after complement neutralization.

Elimination of NK cells or neutrophils with anti-NK1.1 or anti-GR-1 mAbs, respectively (Gong et al., 2005), did not affect the efficiency of B-cell depletion (data not shown). However, elimination of monocytes/macrophages, by treatment of mice with liposome-encapsulated clodronate (Uchida et al., 2004), almost completely prevented depletion of blood and spleen B cells with MEDI-551 (Fig. 5, A and B). Elimination of monocytes/macrophages had a similar effect on blood B-cell depletion with rituximab. Depletion of splenic B cells with rituximab, however, was less affected. Therefore, we also tested for a potential contribution of complement to the depletion of spleen B cells with CD19 and CD20 mAbs. Treatment of Tg mice with CoVF, which almost eliminated serum C3 levels as determined by enzyme-linked immunosorbent assay (data not shown) (Minard-Colin et al., 2008), significantly reduced spleen B-cell depletion by rituximab (Fig. 5C). Depletion of spleen B cells by MEDI-551 was much less affected by the elimination of complement with CoVF. To further confirm the contribution of macrophages for depletion of mouse B cells, we tested MEDI-551 and rituximab in a phagocytosis assay with mouse peritoneal macrophages. As negative control, we also included a Fc-mutated version of mAb anti-CD19-2, mAb anti-CD19-2-TM, which is devoid of effector function (Oganesyan et al., 2008). As shown in Fig. 5D, the MEDI-551 mAb efficiently mediated phagocytosis of mouse B cells, whereas the effector-less anti-CD19-2-TM mAb was inactive. Although rituximab was also active in the phagocytosis assay as expected, the overall activity was less than with MEDI-551.

In summary, macrophages play an important role for in vivo B-cell depletion in mice with the afucosylated human IgG1 mAb MEDI-551. Although the results do not rule out some contribution of NK cells and neutrophils, especially in the absence of macrophages, these cells seem to play a minor role and are not required for efficient depletion when functional macrophages are present. Furthermore, consistent with the in vitro data, the anti-CD19 mAb does not mediate CDC in vivo. For the depletion of blood B cells, rituximab also largely depends on macrophages. For the depletion of splenic B cells with rituximab, however, CDC seems to also play a role, in accordance with the results from Gong et al. (2005).

Discussion

B-cell depletion with targeted mAbs has proven successful for the treatment of hematologic malignancies of B-cell origin as well as for autoimmune diseases. Given the success of rituximab, a variety of new agents targeting CD20 are now in

Fig. 4. Depletion and recovery of B cells after injection of a single dose of MEDI-551 or rituximab. HuCD19/CD20 Tg mice were treated with MEDI-551 or rituximab at 0.5, 2, or 10 mg/kg. Control animals were treated with PBS only. Mice (six to seven animals/dose group and time point) were sacrificed for analysis of blood and tissue B-cell depletion. The dose-response for B-cell depletion in blood (A) and spleen B cells (B) was analyzed on day 3 after mAb administration. To compare the duration of B-cell depletion between MEDI-551 and rituximab, additional groups of mice treated with 10 mg/kg mAb were sacrificed and analyzed on days 7 and 14 after mAb administration. Shown are depletion of blood (C), spleen (D), and BM (cell number per femur; E) over time. Statistical significance at individual time points between groups treated with MEDI-55 and rituximab is indicated.
preclinical and clinical development (Cheson and Leonard, 2008). Targeting CD20, however, also has limitations, and the antigen can be lost from malignant cells through down-regulation, selection, or both (Davis et al., 1999; Kennedy et al., 2004; Takei et al., 2006). Recent data from clinical trials of autoimmune patients treated with rituximab suggest that the depletion of memory B cells from lymphoid tissues is not always complete. It is important to note that the presence of memory cells, rather than naive immature and mature B cells, at the time of repopulation was generally associated with a poor response to rituximab therapy (Leandro et al., 2004; Pers et al., 2008; Roll et al., 2008). The broader expression along the B-cell lineage and presence on most malignant B cells make CD19 an attractive alternative to CD20 for therapeutic mAb approaches.

Several different mechanisms, including ADCC, CDC, and induction of apoptosis, can lead to target cell elimination by therapeutic mAbs, such as rituximab (Glennie et al., 2007). Although CDC may play a role in B-cell depletion with rituximab, the contribution of direct apoptosis to in vivo B-cell depletion is controversial (Glennie et al., 2007).

In contrast to CDC and apoptosis, the relevance of ADCC and antibody-mediated phagocytosis for in vivo activity is now well established (Desjarlais et al., 2007; Glennie et al., 2007). The results from this study demonstrate that MEDI-551 has potent ADCC activity but does not mediate CDC. Although potentially contributing to overall activity of CDC-competent mAbs, such as rituximab, activation of complement may also be involved in infusion-related reactions, a side effect often observed with therapeutic mAbs. Using different forms of a mAb against HLA-DR in preclinical animal models, Tawara et al. (2008) demonstrated that infusion reactions were correlated to the ability of the mAbs to mediate CDC. It will have to be determined in the clinic whether the absence of CDC in MEDI-551 results in fewer infusion reactions compared with other CDC-competent mAbs.

The ADCC activity of therapeutic mAbs can be enhanced by increasing the affinity of the mAb Fc for activating Fcγ receptors, in particular FcγRIIA (Desjarlais et al., 2007). This can be achieved by the introduction of point mutations in the Fc or by modification of the Fc carbohydrate. The removal of fucose from the Fc of the anti-CD19 mAb anti-CD19-2 resulted in ~9-fold increased affinity to FcγRIIA. It is interesting that the increase in ADCC activity was much greater than the fold affinity increase to FcγRIIA. Not only did the EC_{50} drop by approximately 20-fold but also the maximal level of cytotoxicity was significantly greater for the afucosylated mAb MEDI-551 compared with the fucosylated mAb anti-CD19-2 (Fig. 1A; data not shown). Because the activity and potency of the anti-CD20 mAb rituximab have been well characterized, we included this mAb as control in this study. The results from ADCC assays with B-cell lines as well as primary B cells demonstrate that MEDI-551 is more effective at low mAb concentrations than rituximab (Figs. 1A and 2; data not shown). In addition, in vivo, using huCD19/CD20 double Tg mice as a model system, MEDI-551 effectively depleted blood and tissue B cells. At high doses MEDI-551 and rituximab achieved comparable depletion in blood and secondary lymphoid organs. However, MEDI-551 was more effective in eliminating B cells at lower mAb doses. The longer duration of B-cell depletion with MEDI-551 is probably a result of the greater impact MEDI-551 has on BM B cells. CD20 is expressed by immature and mature BM B cells, but, in contrast to CD19, it is not yet present in earlier stages of B-cell development (Levesque and St Clair, 2008). Given that rituximab was almost as effective as MEDI-551 in depleting B cells from blood and spleen when used at high doses, the relatively incomplete depletion of B cells from the
BM of Tg mice is probably a result of the differential expression of the two antigens along the B-cell lineage. It has been noted that in RA patients treated with rituximab clinical benefit often lasts for the duration of B-cell depletion (Dörner et al., 2010). Thus, more complete depletion of early B cells from the BM could provide additional benefit in this indication and possibly other autoimmune diseases.

Consistent with previous reports, the removal of fucose selectively increased the affinity of the IgG1 anti-CD19 mAbs to human FcγRIIIA, which is expressed on NK cells, monocytes/macrophages, and neutrophils (Shields et al., 2002; Nimmerjahn and Ravetch, 2008). Among mouse Fcγ receptors tested, the affinity increase was limited to FcγRIV. In contrast to human FcγRIIIA, however, mouse FcγRIV is not expressed on NK cells. This has potential consequences for the in vivo activity when testing the afucosylated mAb in a murine model. Analysis of the effector mechanisms engaged in the mouse model by MEDI-551 clearly demonstrates an important contribution by macrophages for in vivo B-cell depletion. Elimination of neutrophils or NK cells on their own had no effect on the ability of MEDI-551 to deplete B cells from blood and lymphoid tissues (data not shown). MEDI-551 also efficiently depleted splenic B cells in the absence of complement, although a minor, but statistically significant difference was observed between CoVF-treated and untreated animals (Fig. 5C). In previous studies carried out in huCD19 Tg animals, elimination of complement by CoVF had no noticeable effect on depletion of blood or spleen B cells by MEDI-551 (data not shown). Overall, the findings are consistent with the results from the in vitro CDC assay (Fig. 1B). The absence of CDC activity is probably the result of the inability of the anti-CD19 mAb to mobilize the target antigen into lipid rafts, a phenomenon required for anti-CD20 mAbs to mediate CDC (data not shown; Cragg et al., 2003). Complement, however, was required to achieve maximal depletion of spleen B cell with rituximab (Fig. 5C). In addition, using a huCD20 Tg mouse model, Gong et al. (2005) investigated the mechanisms of B-cell depletion with anti-huCD20 mAbs. Consistent with the findings presented here, neutralization of complement reduced the efficiency of B-cell depletion in the spleen. It is interesting that the marginal zone B cells were selectively dependent on complement for anti-CD20-mediated depletion (Gong et al., 2005). Using wild-type mice and anti-mouse-CD20 mAbs, however, Minard-Colin et al. (2008) did not observe a significant contribution of complement to depletion of spleen B cells. It is possible that these different observations reflect differences in potency of the mAbs used. In the huCD19/CD20 Tg model used here, marginal zone and follicular B cells were equally susceptible to depletion with MEDI-551, despite the lack of CDC activity (data not shown). Thus, the combined in vivo data demonstrate that the afucosylated human IgG1 mAb behaves most similarly to a mouse IgG2a mAb, which has the highest affinity to FcgRIV and largely relies on macrophages for target cell depletion in mouse models (Uchida et al., 2004; Gong et al., 2005; Yazawa et al., 2005).

As mentioned, the affinity of mAbs for Fcγ receptors can be increased by point mutations in the Fc (Desjarlais et al., 2007). Although a selective increase in FcγRIIIA binding seems difficult, several mAbs with significantly enhanced ADCC have been reported previously (Bowles et al., 2006; Lazar et al., 2006). One such mAb is XmAb5574, an anti-CD19 mAb that has been engineered for enhanced ADCC by introducing two point mutations in the Fc (Horton et al., 2008). These mutations result in a significant increase in affinity not only to FcγRIIIA but also to FcγRIIa and the inhibitory receptor FcγRIIB. It is, however, difficult to compare the in vitro activity of XmAb5574 with the activity of MEDI-551, due to differences in the assay systems used. Using rituximab as a template, Masuda et al. (2007) compared the effect of removal of fucose to Fe mAbs on Fcγ receptor binding and ADCC activity. In their study, the afucosylated mAb had ~10-fold better binding to FcγRIIIA, whereas the affinity of the Fe mutant versions of rituximab was improved up to ~90-fold. When tested in vitro for ADCC activity, however, the afucosylated and Fe mutant mAbs were equipotent over a range of effector cell/target cell ratios (Masuda et al., 2007). The results suggest the existence of a threshold effect with regard to the affinity of the mAb Fe to FcγRIIIA. Furthermore, point mutations in the Fe can potentially result in undesired effects, such as increased immunogenicity or decreased stability. Removal of fucose, however, does not affect mAb stability and is unlikely to change the immunogenicity in vivo.

While this manuscript was in preparation, Cardarelli et al. (2009) reported another afucosylated anti-CD19 mAb, MDX-1342. Although the same approach for ADCC enhancement was used in the generation of MEDI-551 and MDX-1342, it is difficult to compare the activity in vitro and in vivo from the published data. MDX-1342 cross-reacts with CD19 from cynomolgus monkeys and was compared with rituximab for in vivo B-cell depletion. When given as a single dose, extent and duration of blood B-cell depletion with the two mAbs was comparable, although the onset of depletion with rituximab appeared more rapid (Cardarelli et al., 2009). In addition to Fe modifications, the ADCC potency of mAbs is also determined by other factors. The anti-CD19 mAb anti-CD19-2 was generated by humanization and affinity maturation, which improved the binding characteristics with decreased internalization rate, and prolonged residence time on the cell surface, features favorable for an ADCC-dependent mechanism (D. C. Rowe, G. P. Sims, and R. Herbst, unpublished). Ghetie et al. (1990) have shown that some, but not all, CD19 mAbs can inhibit B-cell proliferation, suggesting that binding properties and/or the particular epitope recognized play an important role for this effect. Here, we show that the CD19 mAb anti-CD19-2 (the fucosylated version of MEDI-551) also reduces proliferation of B cells; thus, over time, the mAb could also affect B cells in vivo, independently of ADCC effector function. To test this, we also generated an Fc-mutated version of anti-CD19-2, anti-CD19-2-TM, that is unable to bind to Fcγ receptors (Oganeyan et al., 2008) and compared the effect to MEDI-551 in SCID mouse human lymphoma xenograft models. Although less potent than MEDI-551, anti-CD19-2-TM resulted in noticeable tumor growth inhibition, which is probably the result of the antiproliferative effect of the mAb (E. Ward and R. Herbst, unpublished data).

In summary, MEDI-551 is a new glycoengineered anti-CD19 antibody, optimized for ADCC effector function. The removal of fucose resulted in a selective increase in affinity to human FcγRIIIA and mouse FcγRIV and enhanced potency in vitro and in vivo. Furthermore, the in vitro activity of MEDI-551 with lymphoma cells and primary human B cells...
compared to the anti-CD20 mAb rituximab, which was included as positive control in our experiments. Given the broad expression of CD19 in B-cell malignancies and continued expression on late stage memory B cells and plasmablasts, B-cell depletion with MEDI-551 has therapeutic potential in the treatment of B-cell malignancies as well as in autoimmune disease. Clinical studies with MEDI-551 have already been initiated.

References


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