Nonfucosylated Therapeutic IgG1 Antibody Can Evade the Inhibitory Effect of Serum Immunoglobulin G on Antibody-Dependent Cellular Cytotoxicity through its High Binding to FcγRIIa

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Abstract

**Purpose:** Recent studies have revealed that fucosylated therapeutic IgG1s need high concentrations to compensate for FcγRIIa-competitive inhibition of antibody-dependent cellular cytotoxicity (ADCC) by endogenous human plasma IgG. Here, we investigated whether ADCC of nonfucosylated therapeutic IgG1 is also influenced by plasma IgG in the same way as fucosylated IgG1s.

**Experimental Design:** *Ex vivo* ADCC upon CD20⁺ human B cells was induced by incubation of human whole blood with nonfucosylated and/or fucosylated anti-CD20 IgG1 rituximab, and quantified by measuring the remaining CD19⁺ human B cells using flow cytometry.

**Results:** Nonfucosylated anti-CD20 showed markedly higher (over 100-fold based on EC50) *ex vivo* B-cell depletion activity than its fucosylated counterpart in the presence of plasma IgG. The efficacy of fucosylated anti-CD20 was greatly diminished in plasma, resulting in the need for a high concentration (over 1.0 μg/mL) to achieve saturated efficacy. In contrast, nonfucosylated anti-CD20 reached saturated ADCC at lower concentrations (0.01-0.1 μg/mL) with much higher efficacy than fucosylated anti-CD20 in all nine donors through improved FcγRIIa binding. Note-worthy, the high efficacy of nonfucosylated anti-CD20 was inhibited by addition of fucosylated anti-CD20. Thus, the efficacy of a 1:9 mixture (10 μg/mL) of nonfucosylated and fucosylated anti-CD20 was inferior to that of a 1,000-fold dilution (0.01 μg/mL) of nonfucosylated anti-CD20 alone.

**Conclusions:** Our data showed that nonfucosylated IgG1, not including fucosylated counterparts, can evade the inhibitory effect of plasma IgG on ADCC through its high FcγRIIa binding. Hence, nonfucosylated IgG1 exhibits strong therapeutic potential through dramatically enhanced ADCC at low doses in humans *in vivo*.

Monoclonal recombinant antibodies of the human IgG1 isotype, including mouse/human chimeric, humanized, and human IgG1, are commonly used therapeutically. Through their human constant region (Fc), therapeutic antibodies can mediate effector functions of antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity, and direct apoptosis induction (1–3). Recently, therapeutic antibodies have been shown to improve overall survival as well as time to disease progression in a variety of human malignancies, such as breast, colon, and hematologic cancers (4–7). Genetic analysis of leukocyte receptor (FcγR) polymorphisms has clearly shown that ADCC is one of the critical effector functions responsible for the clinical efficacy of therapeutic antibodies (8–12). A superior clinical response of patients carrying the FcγRIIa allotype with higher affinity to anti-CD20 IgG1 rituximab (Rituxan) was observed in contrast to patients with the low-affinity allotype (8–11). Breast cancer patients that responded to anti-HER2 IgG1 trastuzumab (Herceptin) with complete or partial remission were found to have a higher capability to mediate *in vitro* ADCC with trastuzumab (12). ADCC, a lytic attack on antibody-targeted cells, is triggered upon binding of FcγRs to the antibody Fc and is highly dependent on the fucosylation of the N-linked complex oligosaccharides attached to the Fc region (13, 14). Importantly, the removal of fucose from the oligosaccharides is found to dramatically enhance ADCC of the therapeutic antibody through improved FcγRIIa binding (15, 16).

A common feature of therapeutic antibodies used for cancer treatment is that their antitumor efficacy requires high serum concentrations and continued therapy for several months. The treatment cycles thus consume several grams of therapeutic antibody, resulting in a significant amount of drug needed and very high costs. Indeed, administration of a high dose (2-8 mg/kg) of either trastuzumab or rituximab is required to keep the effective serum concentration over 10 μg/mL (17–19). On the
other hand, the maximal in vitro cellular cytotoxicity by ADCC of these therapeutic antibodies can be achieved at antibody concentrations below 10 ng/mL, which are several orders of magnitude below targeted serum concentrations (20, 21). This discrepancy, the low in vivo efficacy of therapeutic antibody in contrast to the high in vitro ADCC, has recently been addressed and found to be mainly due to competition between serum IgG and therapeutic IgG1 for binding to FcγRIIA on natural killer cells. Thus, endogenous serum IgG inhibits therapeutic antibody–induced ADCC (22, 23). This finding leads to new questions about the ADCC efficacy induced by nonfucosylated therapeutic IgG1. First, to what degree is ADCC of nonfucosylated therapeutic IgG1 influenced by human serum IgG? Second, can we expect that clinical treatment with a nonfucosylated therapeutic IgG1 will have greater therapeutic potential than treatment using a fucosylated IgG1? Human serum IgG, as well as currently marketed therapeutic antibodies, are heavily fucosylated (24–26). It has been proposed that these inhibitory effects of serum IgG would be diminished in nonfucosylated therapeutic IgG1 because the binding of nonfucosylated IgG1 to FcγRIIA is shown to be much stronger than that of the fucosylated one (15, 27–29).

To address these issues, we analyzed ex vivo ADCC upon CD20+ human B cells induced by nonfucosylated and/or fucosylated anti-CD20 IgGs having an amino acid sequence equivalent to that of rituximab (Rituxan). Data show that nonfucosylated anti-CD20 against target B cells was 100-fold more potent than fucosylated anti-CD20 (as measured by antibody concentration needed to achieve maximum kill). The in vitro FcγRIIA binding assay revealed that the mechanism for high ex vivo ADCC of nonfucosylated anti-CD20 involved the high binding affinity to FcγRIIA even in the presence of plasma IgG. The high ADCC efficacy of the nonfucosylated anti-CD20 was significantly reduced by the addition of fucosylated anti-CD20 to the reaction mixture. Our findings strongly suggest that nonfucosylated therapeutic IgG1 antibody, not including the fucosylated form, can evade the inhibitory influence of plasma IgG through its improved FcγRIIA binding. Thus, these nonfucosylated IgG1s exhibit strong therapeutic potential through dramatically enhanced ADCC efficacy even when administered in low doses in humans in vivo.

Materials and Methods

Blood donors. Blood donors were randomly selected from healthy volunteers registered at Tokyo Research Laboratories, Koyo Hakko, Co., Ltd. All donors gave written informed consent before the analyses.

Cell lines. Chinese hamster ovary (CHO)/DG44 cell line, in which the dihydrofolate reductase (DHFR) gene locus is deleted, was obtained from Drs. Lawrence Chasin and Gail Urlaub Chasin (Columbia University, New York, NY; ref. 30). FIT8−/− CHO cell lines Ms704 and Ms705 were established from CHO/DG44 by sequential homologous recombination as described previously (15). Human CD20+ B lymphoma cell line Raji, WIL2-S and Ramos, and human HER2+ breast cancer cell line MCF-7 were purchased from the American Type Culture Collection (Manassas, VA).

Antibodies. The mouse/human chimeric anti-CD20 IgG1 expression plasmid, pKANTEX2B8P, was constructed as described previously ([14]. The humanized anti-HER2 IgG1 trastuzumab expression plasmid, pKANTEX4D5-8, was constructed by inserting the VL and VH cDNAs designed on humAb4D5-8 (31) into the humanized IgG1 expression plasmid pKANTEX93 (32). FIT8−/− CHO cell line and parent CHO/DG44 cell line were electroporated with the constructed anti-CD20 or anti-HER2 IgG1 expression plasmids. Stable transfectants were selected in medium lacking hypoxanthine and thymidine. The confluent transfectants were cultured in EX-CELL 301 Medium (IHR Biosciences, Lenexa, KS) for 1 week. IgGs were purified from the culture supernatants using MabSelect (Amersham Biosciences, Piscataway, NJ) and stored in 10 mmol/L citrate buffer (pH 6.0) with 0.15 mol/L NaCl. Protein concentration was measured by absorbance at 280 nm. Rituximab (Rituxan) and trastuzumab (Herceptin), purchased from Genentech, Inc. (South San Francisco, CA), were used as a control.

Analysis of antibody-derived N-linked oligosaccharides. N-linked oligosaccharides were released by digestion of the antibodies with N-glycosidase F (Takara, Shiga, Japan). The released carbohydrates were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with positive ion mode as described previously (33). Monosaccharide composition of IgG1 was characterized by modified high-performance anion exchange chromatography analysis as previously described (14).

Antigen-binding assay. The antigen-binding activity of anti-CD20 IgG1 was measured by CD20-binding ELISA as described previously (15). The antigen-binding activity of anti-HER2 IgG1 was measured by HER2-binding ELISA with HER2+ MCF-7 cell microsomal fraction as antigen. Immunofluorescence staining of human CD20+ B lymphoma cell line Raji or human HER2+ breast cancer cell line MCF-7 was done using FACSCalibur (BD Biosciences, San Jose, CA) as follows. Cells were adjusted to 4 × 10^6/mL and incubated with 2 µg/mL antibody for 30 minutes in ice-cold stain buffer (PBS containing 1% bovine serum albumin). The cells were then washed and incubated with phycoerythrin-conjugated mouse anti-human IgG (BD Biosciences Pharmingen, San Diego, CA) for 30 minutes to detect antibody binding.

FcγRIIA-binding assay. The effect of human plasma IgG on binding of anti-CD20 IgGs to FcγRIIA was studied by a flow cytometric assay using 6× His-tagged recombinant FcγRIIA (FcγRIIA-His), including both polymorphic variants (Phe158 and Val158; refs. 15, 27). Human CD20+ B lymphoma Ramos cells were adjusted to 2 × 10^6/mL and incubated with serial dilutions of anti-CD20 IgG1 for 30 minutes in ice-cold stain buffer. After washing with the stain buffer, FcγRIIA-His was bound to the cell-bound anti-CD20 IgGs on ice for 1 hour. Cells were then washed and incubated with a fluorescently labeled anti-His antibody, Penta-His Alexa Fluor 488 Conjugate (Qiagen, Valencia, CA). Following incubation on ice for 1 hour, stained cells were washed and resuspended in 100 µL stain buffer with or without heat-inactivated (56°C, 30 minutes) human plasma from healthy donors. After incubation for 30 minutes on ice, Penta-His Alexa Fluor 488 conjugate bound to the cell-bound FcγRIIA-His was detected by FACSCalibur.

In vitro ADCC assay. In vitro ADCC activity was determined by the lactate dehydrogenase release assay as described previously (15). Briefly, human peripheral blood mononuclear cells (PBMC) were used as effector cells, and mixed with human CD20+ B lymphoma cell line WIL2-S or human HER2+ breast cancer cell line MCF-7 at an effector-to-target ratio of 25:1 in the presence of corresponding antibodies. After incubation at 37°C for 4 hours, the supernatant lactate dehydrogenase activity was measured using a nonradioactive cytotoxicity assay kit (Wako, Osaka, Japan), and percentage specific cytolysis was calculated according to the attached instruction.

Ex vivo B-cell depletion assay. Heparinized peripheral blood from healthy donors was dispensed into 24-well culture plates (500 µL/well) and incubated with serial dilutions of anti-CD20 IgG1 (100 µL in PBS) for 4 or 20 hours at 37°C. The aliquots of samples were washed once with the stain buffer, and stained with the anti-CD2-phycoerythrin (clone RPA-2.10; BD Biosciences Pharmingen) and anti-CD19-FITC (clone J4.119; Beckman Coulther, Miami, FL) for 30 minutes at room temperature. Mouse IgG1 labeled with FITC or phycoerythrin (clone MOPC-21; BD Biosciences Pharmingen) was used as isotype control. Stained cells were suspended in FACS lysing solution (BD Biosciences) to lyse erythrocytes, washed twice with the stain buffer, and analyzed on a flow cytometer to quantify the number of B cells as described below.
In vitro B-cell depletion assay. Human PBMC were prepared from healthy donors, and suspended in RPMI medium supplemented with 5% fetal bovine serum at 2 × 10^6/mL without any further purification of certain cell subsets, including effector natural killer cells or target B cells. Then, 250 μL of this PBMC suspension (including 0.5 × 10^6 PBMC), 250 μL of autologous plasma, and serial dilutions of anti-CD20 IgG1 (100 μL in PBS) were dispensed into each well of 24-well culture plates. After incubation at 37°C for 4 hours, the aliquot of samples were washed and stained with the anti-CD2-phycocerythrin and anti-CD19-FITC as described above. The number of B cells was quantified by FACS Calibur as described below.

Flow cytometric analysis of B-cell depletion. A FACSCalibur was used for acquisition and analysis of all samples. The B-cell depletion activity of anti-CD20 IgG1 was determined by the following gating strategy as reported previously (22, 34). The lymphocyte population was gated on the forward and side scatter dot plot. Using events in a selected gate, fluorescence intensity dot plots were displayed for CD19 and CD2 markers. Fluorescently labeled isotype controls were used to determine the respective cutoff points for CD19+ and CD2+ cells. The percentage of CD19+CD2+ B cells in total lymphocytes was calculated according to the following formula: %B cells = 100 /C0 /C2

where %B cells treated with PBS) The number of CD19+CD2+ B cells in the nontreated PBMC fraction varied from 64.0 to 33.0.

Results

Generation of nonfucosylated and fucosylated antibodies. Monoclonal recombinant mouse/human chimeric anti-CD20 IgG1 and humanized anti-HER2 IgG1s were generated using FUT8−/− cell line Ms704 or Ms705 and parent CHO/DG44 cells as host cells. The anti-CD20 and anti-HER2 have amino acid sequences equivalent to those of rituximab and trastuzumab, respectively, which are widely used for either the treatment of B-cell disorders or breast cancers, and thus showed identical binding activities to the specific antigens in the antigen-binding ELISA. Flow cytometric analysis also revealed that there was no significant difference in the antigen-specific cell surface binding between these generated IgG1s and each control (rituximab or trastuzumab; data not shown). Oligosaccharide analysis of the generated IgG1s confirmed that the majority of the attached oligosaccharides were of a complex biantennary type, and the IgG1s generated by FUT8−/− cell lines contained no fucose residue (Table 1). Monosaccharide composition analysis also confirmed that the fucose content was the only significant difference between the N-linked oligosaccharide core structures of the generated IgG1s and controls. Consistent with previous reports (13–16), the nonfucosylated IgG1s showed over 100-fold higher in vitro ADCC than the fucosylated ones (the products by parent CHO/DG44 cells and controls; Fig. 1).

Ex vivo ADCC activity of anti-CD20 IgG1s. To analyze the ex vivo ADCC activity of each anti-CD20 IgG1, whole blood samples from healthy volunteers were incubated with serial dilution of nonfucosylated or fucosylated anti-CD20 at 37°C, and the remaining CD19+CD2+ B cells in the PBMC fraction were counted by flow cytometry. In nine individuals, the ratios of CD19+CD2+ B cells in the nontreated PBMC fraction varied

Table 1. Oligosaccharide analysis of the mouse/human chimeric anti-CD20 IgG1 and the humanized anti-HER2 IgG1

| Host (specificity) | Relative composition of oligosaccharides (%) |
|-------------------|-------------------------------------------|
|                   | F0G0 | F0G1 | F0G2 | F1G0 | F1G1 | F1G2 | Fu(−) |
| FUT8−/− Ms704 (CD20) | 64.0 | 33.0 | 3.0  | ND  | ND  | ND   | 100   |
| CHO/DG44 (CD20)     | ND   | ND   | ND   | 41.9 | 51.1 | 7.0  | ND    |
| Rituximab (CD20)    | ND   | ND   | ND   | 56.1 | 40.8 | 4.1  | ND    |
| FUT8−/− Ms705 (HER2) | 63.3 | 33.7 | 3.0  | ND  | ND  | ND   | 100   |
| CHO/DG44 (HER2)     | ND   | 2.5  | ND   | 53.6 | 39.8 | 4.1  | 2.5   |
| Trastuzumab (HER2)  | 9.1  | ND   | ND   | 61.9 | 29.0 | ND   | 9.1   |

Abbreviation: ND, not detected.

*Each value of composition is the relative amount in total complex type oligosaccharides detected.
†Relative amount of nonfucosylated oligosaccharides.
‡Less than 2.0%.

![Fig. 1. In vitro ADCC of nonfucosylated [Fu(−)] and fucosylated [Fu(+)] IgG1s. Cytotoxicity against CD20+ human B lymphoma cell line WILO-2-S cells (left) and HER2+ human breast cancer cell line MCF-7 cells (right) were induced by corresponding anti-CD20 IgG1s or anti-HER2 IgG1s. All experiments were done by a 4-hour lactate dehydrogenase release assay using human PBMC as effector cells at an effector-to-target ratio of 25:1. Points, mean cytotoxicity (%) values of triplicates; bars, SD.](image-url)
from 7.5% to 21.0%. The representative results of 20-hour treatment with anti-CD20 are shown in Fig. 2A (donors 1 and 2). Fucosylated anti-CD20 reduced B cells to 40% of the anti-CD20 nontreated control at 10 μg/mL in donor 1, but could barely decrease B cells in donor 2, which was consistent with the results of treatment with rituximab (data not shown). The EC50 for fucosylated anti-CD20-mediated B-cell depletion was estimated to be ~2 μg/mL in donor 1. In contrast, nonfucosylated anti-CD20 showed marked activity to decrease target B cells in both individuals, with EC50 values of 0.01 μg/mL (in donor 1) and 0.03 μg/mL (in donor 2).

Next, we measured the ex vivo ADCC activity over a different time course to determine whether the added anti-CD20s would show their maximal efficacy. Using the same volunteer blood sample (donor 3), ex vivo B-cell depletion activity of 4- or 20-hour treatment with anti-CD20 was measured (Fig. 2B). Consistent with the reports of Vugmetster et al. (22, 34) that target B cells were sufficiently depleted from human whole blood just after 1-hour treatment with rituximab at 10 μg/mL, the added anti-CD20s effectively depleted B cells and showed the saturable efficacy at antibody concentrations over 1 μg/mL regardless of the incubation time. The greatly enhanced ex vivo ADCC of nonfucosylated anti-CD20 was clearly observed even after 4 hours of incubation.

The ex vivo ADCC activity of nonfucosylated CD20 was further investigated in six more volunteers (donors 4-9), resulting in detection of >100-fold potent B-cell depletion activity with much higher efficacy than fucosylated anti-CD20 in all individuals (Fig. 2C). Nonfucosylated anti-CD20 showed enough potential to deplete over 50% of the target B cells in all nine donors. Fucosylated anti-CD20, however, did not show such a high efficacy except in the case of donor 1, and mostly failed to show the B-cell depletion activity in donor 4.

Inhibitory effect of human plasma on ADCC of anti-CD20 IgG. To investigate how the B-cell depletion activity of anti-CD20s is influenced by human plasma IgG, we did an in vitro B-cell depletion assay with purified PBMC in the presence or absence of autologous plasma using two volunteers (donors 8 and 9). In this assay, B-cell depletion activity was shown as cytotoxicity (%) with the following formula: 100 − %B cells for easy comparison with the following experiments (Fig. 3A). In the absence of autologous plasma, fucosylated anti-CD20 induced higher ADCC at lower dose than ex vivo in both individuals (Fig. 3B). On the other hand, autologous plasma strongly inhibited the ADCC of fucosylated anti-CD20 with respect to both maximal cell lysis and EC50 values. In contrast, the ADCC activity of nonfucosylated anti-CD20 was less affected by plasma (Fig. 3C). Importantly, the dose cytotoxicity curves of nonfucosylated anti-CD20 in vitro with plasma just shifted to approximately one order of magnitude higher antibody concentration than the profiles in vitro without plasma.

Inhibitory effect of human plasma on FcγRIIIa binding. The influence of human plasma on binding of anti-CD20 to

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**Fig. 2.** Ex vivo B-cell depletion activity of anti-CD20 IgGs in a whole blood matrix. Whole blood samples were incubated with nonfucosylated (Fu−) and fucosylated (Fu+) anti-CD20 at the concentrations indicated in the X axis. Cytotoxicity against human normal B cells was quantitated by flow cytometry as described in Materials and Methods. B-cell depletion activities of anti-CD20 IgGs are represented as percentage of B cells in two different individuals (donors 1 and 2) after 20-hour treatment (A), in the same individual (donor 3) after 4- and 20-hour treatment (B), and in six different individuals (donors 4, 5, 6, 7, 8, and 9) after 4-hour treatment (C). Dashed lines (A-C), 50% of B cells in the PBS-treated sample.
FcγRIIIa was investigated. To this end, a cell-based binding assay was done as described in Materials and Methods. Nonfucosylated anti-CD20 exhibited markedly stronger binding activity for both polymorphic variants of FcγRIIIa than the fucosylated anti-CD20 (Fig. 3D). It is worth noting that plasma from donors 8 and 9 almost abolished the binding of fucosylated anti-CD20 to FcγRIIIa-Val158, and completely abolished the binding of fucosylated anti-CD20 to FcγRIIIa-Phe158. In contrast, nonfucosylated anti-CD20 still retained high binding activity for both polymorphic variants of FcγRIIIa even in the presence of plasma.

**Fucosylated IgG1 inhibits the enhanced ADCC efficacy of nonfucosylated IgG1.** To address the reason why nonfucosylated anti-CD20 showed a higher saturable ex vivo ADCC efficacy than fucosylated anti-CD20, we explored the influence of fucosylated anti-CD20 on ADCC of nonfucosylated anti-CD20. A series of mixtures composed of nonfucosylated and fucosylated anti-CD20s with different ratios was prepared to add serial 3-fold amounts of fucosylated anti-CD20 to a certain amount (0.11 μg/mL) of nonfucosylated anti-CD20, and then their ex vivo B-cell depletion activities were measured (Fig. 4A). The concentration of 0.11 μg/mL was chosen to be the minimum dose showing the saturable ex vivo efficacy in the donor blood (donor 7). There was no significant change observed by the addition of nonfucosylated anti-CD20 because the initial dose already reached to the saturable effective dose. On the other hand, surprisingly, the addition of fucosylated anti-CD20 dramatically reduced the saturable ex vivo B-cell depletion activity of nonfucosylated anti-CD20.
depletion efficacy of nonfucosylated anti-CD20 in a dose-dependent manner. The efficacy of an anti-CD20 mixture of the 0.11 μg/mL nonfucosylated and 9 μg/mL fucosylated forms was >60% of that of 0.11 μg/mL nonfucosylated anti-CD20 alone, although ~90-fold total amount of anti-CD20 was included in the mixture.

To confirm that the inhibitory effect of fucosylated anti-CD20 on the ex vivo ADCC activity of nonfucosylated anti-CD20 is generally observed, we checked the ex vivo B-cell depletion efficacy of a 1:9 mixture of nonfucosylated and fucosylated anti-CD20s at total antibody concentrations of 0.1, 1, and 10 μg/mL in one more individual (donor 6). Nonfucosylated anti-CD20 started to show its ex vivo activity at a concentration of 0.01 μg/mL and showed higher efficacy at concentrations of 1 and 10 μg/mL in donor 6 (Fig. 4B). However, the ADCC activity of each mixture prepared was inferior to that of nonfucosylated anti-CD20 alone, although a 1,000-fold total amount of anti-CD20 was included in the mixture of 10 μg/mL.

Next, the effect of fucosylated IgG1 on in vitro ADCC of nonfucosylated IgG1 was investigated using anti-CD20 and anti-HER2. A series of IgG1 mixtures composed of nonfucosylated and fucosylated IgG1s in different ratios was prepared to add serial 3-fold amounts of IgG1s to a certain amount (3.7 ng/mL) of nonfucosylated IgG1, and then their in vitro ADCC activities were measured (Fig. 5A). The inhibitory effect of fucosylated IgG1 on the in vitro ADCC of nonfucosylated IgG1 was observed in a dose-dependent manner. The treatment with nonfucosylated IgG1 alone at an initial dose of 3.7 ng/mL showed much stronger in vitro ADCC activity than a mixture of 3.7 ng/mL of the nonfucosylated and 300 ng/mL of the fucosylated form, although the mixture contained ~90-fold total amount of the antibody. Moreover, measurement of the in vitro ADCC of a 1:9 mixture of nonfucosylated and fucosylated IgG1s at total antibody concentrations of 0.01, 0.1, and 1 μg/mL showed that every mixture did not have superior in vitro ADCC activity to a 1,000-fold dilution (1 ng/mL) of the nonfucosylated form alone (Fig. 5B). Thus, the inhibitory effect of fucosylated IgG1 on in vitro ADCC of nonfucosylated IgG1 was also reproducibly observed.

Discussion

Clinical studies have shown that the use of therapeutic monoclonal antibodies is a promising approach for the treatment of human malignancies, as exemplified by the success of breast, colon, and hematologic cancer treatments (4–7). However, it is also important to acknowledge the shortcomings of therapeutic antibodies, especially in terms of their recognition of surface tumor antigens. Thus far, only low in vivo efficacy of the specific antitumor activity has been achieved; to date, numerous clinical trials using such antibodies have concluded with insufficient results (35, 36). Even in the case of cancer patients treated with the effective therapeutic antibody, multiple high-dose administrations are required to maintain an effective serum concentration for several months.

Fig. 4. Influence of fucosylated anti-CD20 on the ex vivo ADCC induction by nonfucosylated anti-CD20. A, nonfucosylated anti-CD20 was added to the whole blood sample from donor 7 at 0.11 μg/mL. Nonfucosylated or fucosylated anti-CD20 was further added to the reaction as indicated. B, whole blood sample from donor 6 was treated with 0.01 to 1 μg/mL of nonfucosylated anti-CD20, or with anti-CD20 IgG1 composition prepared by mixing nonfucosylated anti-CD20 with fucosylated anti-CD20. The values plotted as the abscissa show the concentration of nonfucosylated anti-CD20, added fucosylated anti-CD20, and the total anti-CD20 antibody concentration, respectively, from the upper row. In both experiments, B-cell depletion after 4-hour treatment is represented as cytotoxicity (%) as described in Materials and Methods.

Fig. 5.
Although the reasons for the low in vivo efficacy in contrast to the high in vitro efficacy of therapeutic antibodies are not well understood, numerous efforts to improve the efficacy of monoclonal therapeutic antibodies are currently under way. These include modification of N-linked Fc oligosaccharides (15, 16, 37–39), introduction of amino acid mutations in the Fc region (39, 40), and the coadministration of CpG as an adjuvant (41, 42), or of cytokines such as interleukin-2 (43) and granulocyte macrophage colony-stimulating factor (44). These strategies either aim at improving FcγR binding of IgG1 or at enhancement of effector cell function by costimulating signal activation. It is very important to evaluate whether each strategy has the potential to overcome the low in vivo efficacy problem. Thus, we focused on the potentiality of nonfucosylated IgG1 as a recently introduced, next-generation therapeutic antibody.

Nonfucosylated IgG1 is a normal component of natural human serum IgG (24, 25) and is proven to have greatly enhanced ADCC than fucosylated IgG1 through its improved FcγRIIIa binding in vitro (13–16, 27–29, 45) and in vivo in a human PBMC-engrafted mouse model (46). In the current study, we further investigated the ADCC of nonfucosylated IgG1 using a human ex vivo model (22, 34) by monitoring rituximab-induced B-cell depletion from human whole blood. In this human ex vivo model, nonfucosylated anti-CD20s were simply added to individual whole blood samples, and the number of remaining B cell was counted after incubation. B cells are ADCC sensitive, and complement-dependent cytotoxicity and apoptosis resistant (47). ADCC is also known to be mediated by natural killer cells (48). This model is thought to be very close to the intravascular environment of humans in vivo because no artificial operations, such as purifying effector cells from individuals, mixing large number of the purified effector cells and low number of target cells, and incubating the mixture in nonhuman serum without any human plasma components, were conducted (49). Actually, the magnitude of the recruitment of effector cells to target cells in vivo is considered to be less than that used in the in vitro assay, and the human serum-induced inhibition of the ADCC of therapeutic antibodies is also absent in the in vitro assay (22, 23). This human ex vivo experiment, therefore, was used as a nonclinical feasibility study to show the efficacy against non–Hodgkin’s disease in developing rituximab (49).

Here, we showed the ex vivo ADCC of nonfucosylated anti-CD20 IgG1 rituximab upon CD20+ B cells as target cells using blood samples from nine healthy human volunteers. Fucosylated anti-CD20 depleted over 40% of B cells at 10 μg/mL in half of the volunteers with individual differences, which is

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**Fig. 5.** Influence of fucosylated antibodies on the in vitro ADCC induced by nonfucosylated antibodies. A, nonfucosylated anti-CD20 (left) or nonfucosylated anti-HER2 (right) was added to the culture at 3.7 ng/mL. The corresponding nonfucosylated antibody (hatched columns), fucosylated antibody (black columns), or PBS alone (open columns) was further added to the reaction at the indicated concentrations. B, ADCC of anti-CD20s (left) and anti-HER2s (right) was induced by nonfucosylated antibody alone (open columns), or by antibody composition prepared by mixing nonfucosylated antibody with fucosylated antibody (black columns). The values plotted as the abscissa show the concentration of the nonfucosylated antibody, added fucosylated antibody, and the total antibody concentration, respectively, from the upper row. All experiments were done by a 4-hour lactate dehydrogenase release assay using human PBMC as effector cells at an effector-to-target ratio of 25:1. Columns, mean cytotoxicity (%); bars, SD. *, *P < 0.05; **, **P < 0.01, statistically significant differences between nonfucosylated antibody (A, 3.7 ng/mL; B, 1 ng/mL) alone and antibody mixture–mediated ADCC as determined by paired t test.
consistent with the previous results reported using 19 volunteers (34). In contrast, nonfucosylated anti-CD20 exhibited more effective activity than fucosylated anti-CD20, depleting >50% of B cells when treated at a concentration of 0.01 to 1 μg/mL in each of the nine volunteers. This contrasting result clearly shows the advantage of antibody therapy with the nonfucosylated IgG1 form of rituximab. Nonfucosylated rituximab might be an effective therapeutic agent against non–Hodgkin’s disease irrespective of individual patient differences, with higher efficacy than fucosylated rituximab even when administrated in low doses (e.g., from 10- to 1,000-fold dilution).

Recently, the inhibition of the ADCC of therapeutic IgG1 antibodies by endogenous human serum, which has necessitated the use of high doses in the clinical setting, has been shown to be mediated by a competition with the therapeutic antibody for binding to FcyRIIa on natural killer cells (23). Human serum IgG does not affect antigen binding by therapeutic IgG1 antibody but significantly impairs interaction of the antibody with FcyRIIa. In our experiments, we confirmed that the binding of fucosylated anti-CD20 to FcyRIIa was markedly reduced by human plasma and that the ADCC was also reduced by human plasma in the in vitro and ex vivo B-cell depletion assays. FcyRIIa binding of fucosylated anti-CD20 in vitro was almost abolished in FcyRIIa-Val158 and completely abolished in FcyRIIa-Phε158 in the presence of human plasma. Even under this severe condition, nonfucosylated anti-CD20 had a tendency to be less sensitive to the inhibitory effect of human plasma and retained the much higher FcyRIIa binding than the fucosylated anti-CD20. These results suggested that nonfucosylated anti-CD20 could induce high B-cell depletion activity even in the presence of human serum through high FcyRIIa binding. Therefore, a strategy aimed at improving FcyRIIa binding of therapeutic antibodies, such as nonfucosylated IgG1, to overcome the low in vivo efficacy problem of antibody therapy would seem to be promising.

Interestingly, fucosylated anti-CD20 inhibited the activity of nonfucosylated anti-CD20 in a dose-dependent manner. Nonfucosylated anti-CD20 existing in the anti-CD20 mixtures, composed of nonfucosylated and fucosylated forms, did not show activity equivalent to that of equal amounts of nonfucosylated anti-CD20 alone. Thus, this phenomenon seems to reflect our finding that the maximum B-cell depletion activity of fucosylated anti-CD20 was never as high as that by nonfucosylated anti-CD20 in any of the volunteers, even when high doses of the fucosylated antibody were used. The inhibition of the ADCC of nonfucosylated antibody by fucosylated form was also confirmed by testing another different therapeutic IgG1 antibody, trastuzumab. One of the major mechanisms by which the ADCC induced by the nonfucosylated therapeutic IgG1 antibody is inhibited by the fucosylated form is considered to be through the competition of the two forms for the antigens on target cells. The density of the nonfucosylated form on the target cells is thus reduced by fucosylated form occupation, which seems to yield a similar effect to shed the target antigens from capture by the therapeutic agent having high ADCC activity even in plasma.

To overcome the interference of human plasma IgG, it is important to coat the target cells with therapeutic antibodies having higher binding affinity to FcyRIIa than plasma IgG, such as nonfucosylated IgG1. We believe that antibody therapy consisting of nonfucosylated IgG1 alone, not including the fucosylated form, can provide new benefits for patients through its improved efficacy at a reduced dose, which will have a great effect on human cancer therapy.

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Nonfucosylated Therapeutic IgG1 Antibody Can Evade the Inhibitory Effect of Serum Immunoglobulin G on Antibody-Dependent Cellular Cytotoxicity through its High Binding to Fc γRIIIa

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