The Absence of Fucose but Not the Presence of Galactose or Bisecting N-Acetylgalcosamine of Human IgG1 Complex-type Oligosaccharides Shows the Critical Role of Enhancing Antibody-dependent Cellular Cytotoxicity*

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An anti-human interleukin 5 receptor (hIL-5R) humanized immunoglobulin G1 (IgG1) and an anti-CD20 chimeric IgG1 produced by rat hybridoma YB2/0 cell lines showed more than 50-fold higher antibody-dependent cellular cytotoxicity (ADCC) using purified human peripheral blood mononuclear cells as effector than those produced by Chinese hamster ovary (CHO) cell lines. Monosaccharide composition and oligosaccharide profiling analysis showed that low fucose (Fuc) content of complex-type oligosaccharides was characteristic in YB2/0-produced IgG1s compared with high Fuc content of CHO-produced IgG1s. YB2/0-produced anti-hIL-5R IgG1 was subjected to Lens culinaris agglutinin affinity chromatography and fractionated based on the contents of Fuc. The lower Fuc IgG1 had higher ADCC than the IgG1 before separation. In contrast, the content of bisecting GlcNAc of the IgG1 affected ADCC much less than that of Fuc. In addition, the correlation between Gal and ADCC was not observed. When the combined effect of Fuc and bisecting GlcNAc was examined in anti-CD20 IgG1, only a severalfold increase of ADCC was observed by the addition of GlcNAc to highly fucosylated IgG1. Quantitative PCR analysis indicated that YB2/0 cells had lower expression level of FUT8 mRNA, which codes α1,6-fucosyltransferase, than CHO cells. Overexpression of FUT8 mRNA in YB2/0 cells led to an increase of fucosylated oligosaccharides and decrease of ADCC of the IgG1. These results indicate that the lack of fucosylation of IgG1 has the most critical role in enhancement of ADCC, although several reports have suggested the importance of Gal or bisecting GlcNAc and provide important information to produce the effective therapeutic antibody.

Antibody-dependent cellular cytotoxicity (ADCC), a lytic attack on antibody-targeted cells, is triggered upon binding of lymphocyte receptors (FcγRs) to the constant region (Fc) of the antibodies. ADCC is considered to be a major function of some of the therapeutic antibodies, although antibodies have multiple therapeutic functions (e.g. antigen binding, induction of apoptosis, and complement-dependent cellular cytotoxicity) (1, 2).

One IgG molecule contains two N-linked oligosaccharide sites in its Fc region (3). The general structure of N-linked oligosaccharide on IgG is complex-type, characterized by a mannose-chitobiose core (Man3GlcNAc2-Asn) with or without bisecting GlcNAc and/or fucose (Fuc) and other chain variants including the presence or absence of Gal and sialic acid. In addition, oligosaccharides may contain zero (G0), one (G1), or two (G2) Gal.

Recent studies have shown that engineering the oligosaccharides of IgGs may yield optimized ADCC. ADCC requires the presence of oligosaccharides covalently attached at the conserved Asn297 in the Fc region and is sensitive to change in the oligosaccharide structure. In the oligosaccharide, sialic acid of IgG has no effect on ADCC (4). The relationship between the Gal residue and ADCC is controversial. Boyd et al. (4) have shown that obvious change was not found in ADCC after removal of the majority of the Gal residues. However, several reports have shown that Gal residues enhance ADCC (5, 6).

Several groups have focused on bisecting GlcNAc, which is a β1,4-GlcNAc residue transferred to a core β-mannose (Man) residue, and it has been implicated in biological activity of therapeutic antibodies (7). N-Acetylgalcosaminyltransferase III (GnTIII), which catalyzes the addition of the bisecting GlcNAc residue to the N-linked oligosaccharide (8), has been expressed in a Chinese hamster ovary (CHO) cell line with an anti-neuroblastoma IgG1 and resulted in greater ADCC (9). Moreover, expression of GnTIII in a recombinant CHO cell line has led to the increase in ADCC of the anti-CD20 antibody (10).

Recently, Shields et al. have revealed the effect of fucosylated oligosaccharide on antibody effector functions, including binding to human FcγR, human C1q, human FcRn, and ADCC (11). The Fuc-deficient IgG1s have shown 50-fold increased binding to FcγRIIa and enhanced ADCC. Nevertheless, there are no data on comparison of the effect of Fuc, Gal, and GlcNAc or the combined effect of Fuc and bisecting GlcNAc.

Here, we describe the correlation between glycosylation of human IgG1 and ADCC and demonstrate that Fuc showed the critical role for enhancing ADCC out of several sugar residues reported previously. We unexpectedly found that human IgG1 produced by rat hybridoma YB2/0 cells showed extremely high ADCC at more than 50-fold lower concentration of those pro-

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duced by CHO cells. YB2/0-produced IgG1 had lower Fuc content than CHO-produced IgG1. IgG1 containing lower fucosylated oligosaccharides, which was fractionated by *Lentilula culinaris* agglutinin (LCA) lectin affinity chromatography, showed higher ADC before separation. In contrast, the addition of bisecting GlcNAc to IgG1 enhanced ADC much less effectively than defucosylation. The effect of bisecting GlcNAc was demonstrated in highly fucosylated IgG1. YB2/0-produced YB2/0 expressed a lower level of FUT8 (α1,6-fucosyltransferase gene) mRNA than CHO cells, and overexpression of FUT8 in YB2/0 led the increase of fucosylation of IgG1 and the decrease of ADC.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Rat hybridoma YB2/0 cells were purchased from the American Type Culture Collection (ATCC; CRL-1662). CHO cell line DG44 (12), for wild type IgG1 production, was kindly provided by Dr. Lawrence Chasin (Columbia University). LEC10, a variant CHO cell line overexpressing GrTIII (13), was kindly provided by Dr. Pamela Stanley (Albert Einstein College of Medicine).

**Expression of IgG1s**—For the generation of human IgG1 of humanized anti-human interleukin-5 receptor (hIL-5R) α-chain and chimeric anti-CD20, the appropriate humanized or murine VL and VH cDNAs were cloned into the previously described pKANTEX93 vector (14). The cDNA coding for the VL and VH region of each antibody was constructed by the PCR-based method (14). In the case of chimeric anti-CD20 antibody, the cDNA sequence of each V region was designed to be the same with that of Rituxan® (VL: GenBank™ accession number EHE0203; VH: GenBank™ accession number AR000413). Establishment of anti-IL-5R humanized IgG1 was described elsewhere. Antibody expression vectors were introduced into YB2/0 cells or DG44 cells via electroporation and selected for gene amplification in methotrexate-containing medium (14).

**Production of IgG1s**—The anti-hIL-5R IgG1-producing YB2/0 cell line was suspended in GIT medium (Waico, Osaka, Japan) containing 0.5 mg/ml G418 and 200 μM methotrexate to give a density of 3 x 10⁵ cells/ml and dispensed in suspension culture flasks (Greiner, Frickenhausen, Germany). The anti-hIL-5R IgG1-producing CHO cell line was suspended in the EX-CELL302 medium (JRH, Kansas City, MO) containing 3 mM l-Gln, 0.5% chemically defined lipid concentrate (Invitrogen), and 0.3% PLURONIC F-68 (Invitrogen) to give a density of 3 x 10⁵ cells/ml and cultured using spinner flasks (Asahi Techno Glass, Tokyo, Japan) under agitation at a rate of 100 rpm. The anti-CD20 IgG1-producing YB2/0 cell line was suspended in the hybridoma-SFM medium (Invitrogen) containing 5% Daigo's GF21 (Waico) and 200 μM methotrexate to give a density of 1 x 10⁶ cells/ml and dispensed in suspension culture flasks. The flasks were incubated under conditions of 37°C in humid air containing 5% CO₂. After 8 or 10 days of incubation, the culture supernatants were recovered.

**Purification of IgG1s**—The culture supernatants containing anti-hIL-5R IgG1 from YB2/0 cells and CHO cells and anti-CD20 IgG1 from YB2/0 and CHO cells were clarified by centrifugation and passed through a 0.2-μm filter. The IgG1 bound to a PROSEP-A (Millipore) column was eluted with 0.1 M citrate buffer (pH 3.5). Then the antibody was subjected to a Sephacryl S-300 (Amersham Biosciences) column. The buffer composition of the YB2/0-produced anti-CD20 IgG1 was changed to that for Rituxan™ (9.0 mg/ml sodium chloride, 7.35 mg/ml sodium citrate dihydrate, 0.7 mg/ml polysorbate 80). The purity of IgG1 was confirmed by SDS-PAGE. YB2/0- and CHO-produced humanized anti-hIL-5R IgG1 were designated as KM3065 and KM8399, respectively. YB2/0-produced chimeric anti-CD20 IgG1 was designated as KM8404. Rituxan® (chimeric mouse/human anti-CD20 monoclonal antibody derived from JCRB, Tokyo, Japan), were distributed into 96-well U-bottomed plates (1 x 10⁵/50 μl) and incubated with serial dilutions of antibodies (50 μl) in the presence of human effector cells (100 μl) at an E/T ratio of 90:1. After 4 h of incubation at 37°C, the plates were centrifuged, and the radioactivity in the supernatants was measured using a γ counter. The percentage of specific cytotoxicity was calculated from the counts of samples according to the formula,

\[
\% \text{ specific lysis } = \frac{(E - S) - (S - T)}{(M - S)} \times 100
\]

where E represents the experimental release (cpm in the supernatant from target cells incubated with antibody and effector cells), S represents the spontaneous release (cpm in the supernatant from target cells incubated with medium alone), and M is the maximum release (cpm released from target cells lysed with 1 mol/liter HCl).

**ADC Assay for the Anti-CD20 IgG1**—An ADC assay was performed by a lactate dehydrogenase release assay. Aliquots of target cells, a human B lymphoma cell line Raji (number 9012, purchased from JCRB, Tokyo, Japan), were distributed into 96-well U-bottomed plates (1 x 10⁵/50 μl) and incubated with serial dilutions of antibodies (50 μl) in the presence of human effector cells (100 μl) at an E/T ratio of 25:1 or 20:1. Human effector cells were PBMC purified from healthy donors using lymphoprep (Axis Shield, Dundee, UK). After a 4-h incubation at 37°C, the plate was centrifuged, and the lactate dehydrogenase activity in the supernatants was measured using a nonradioactive cytotoxicity assay kit (Promega, Madison, WI). The percentage of specific cytotoxicity was calculated from the activities of samples according to the formula,

\[
% \text{ specific lysis } = \frac{(E - S) - (S - T)}{(M - S)} \times 100
\]
antibodies were applied to the column, previously equilibrated with 50 mM Tris-HCl, pH 8.0 (A buffer). The column was eluted with the buffer containing 0.1 M K$_2$B$_4$O$_7$ (B buffer). Elution was followed by linear gradients from 0 to 58% B buffer in 35 min and then 100% B buffer for 5 min. The column was equilibrated with 100% A buffer for 20 min before the next injection. Each chromatography was performed at room temperature and a flow rate of 0.5 mL/min.

Establishment of α1,6-Fucosyltransferase-overexpressing YB2/0 Cells—A mammalian expression vector, pAGE249, which was derived by excision of a 2.7-kb SphI-SphI fragment containing the dihydrofolate reductase gene cassette from pAGE249 (20), was employed. This plasmid contained a hygromycin-resistant gene driven by the herpes simplex virus thymidine kinase gene promoter. The murine FUT8 sense DNA (1729 bp) (21) was inserted into pAGE249 under the control of the Moloney murine leukemia virus 3'-LTR promoter to form a plasmid designated as pAGEmFUT8. Two EsfI restriction sites located within the plasmid backbone enabled linearization of the expression vector construct, prior to transfection of cells. FUT8 expression vector, pAGEmFUT8, was introduced into anti-c-CD20-IgG1-producing YB2/0 cells via electroporation, and FUT8-overexpressing YB2/0 cells, 3065f8–72, were selected in 0.5 mg/mL hygromycin-B (Sigma)-containing medium.

Competitive RT-PCR Analysis of FUT8—Total RNA was isolated from 1.0 × 10$^6$ YB2/0 cells, FUT8-overexpressing YB2/0 cells, or CHO/DO44 cells using the RNeasy minikit (Qiagen, Tokyo, Japan) and incubated for 1 h at 37°C with 20 units of RNase-free DNase (RQI, Promega) to degrade genomic DNA. After DNA digestion, the total RNA was purified again using the RNeasy minikit. The single-strand cDNA was synthesized from 3 μg of each total RNA using the Superscript first strand synthesis system for RT-PCR (Invitrogen). The 50-fold diluted reaction mixture was used as a template for the following competitive RT-PCR. Quantification of FUT8 transcripts was carried out using competitive RT-PCR in which a 979-bp partial fragment of rat FUT8 product was quantified by measuring luminescence intensity using a FluorImager SI (Amersham Biosciences), calculated from standard products, and 50-fold diluted single-stranded cDNA, 10 μg of linearized competitors, 5 pmol of FUT8-specific primers, 4 nmol of dNTP mixture, 5% dimethyl sulfoxide, and ExTaq polymerase (Takara Bio). The sense primer 5'-GCTCATGTGATCCTGCAGTGTGG-3' and antisense primer 5'-CACCAATGATATCTCCAGGTTCC-3' were employed to amplify a FUT8 fragment. Aliquots of PCR products (7 μL) were subjected to electrophoresis in 1.75% agarose gel and stained with SYBR Green I nucleic gel stain (Molecular Probes, Inc., Eugene, OR). The amount of amplified cDNA was quantified by measuring luminescence intensity using a FluorImager SI (Amersham Biosciences), calculated from standard curves, and converted into molar numbers. To normalize the synthesis efficiency of first-strand cDNAs, the amount of 18S-actin-specific primers, 4 nmol of dNTP mixture, 5% dimethyl sulfoxide, and ExTaq polymerase (Takara Bio). The sense primer 5'-GCTCATGTGATCCTGCAGTGTGG-3' and antisense primer 5'-CACCAATGATATCTCCAGGTTCC-3' were employed to amplify an 180-bp DraIII (blunt)-DraIII (blunt) fragment from standard DNA as a competitor. Standard DNA was amplified from single-stranded cDNAs of YB2/0 cells by PCR using primers 5'-ATTATGACTGAAGCCGAGTTGCCGCAAGCATGAGGG-3' and 5'-AATGATAAAGCTTCACTAGATTGACAATCCTGGCGCC-CTCGCC-3' after digestion of β-actin were carried out by heating at 94°C for 3 min and subsequent 17 cycles of 94°C for 30 s, 65°C for 1 min, and 72°C for 1 min in 20 μL of reaction mixture containing 5 μL of the 50-fold single-stranded cDNAs, 1 μg of linearized competitors, 10 μmol of β-actin-specific primers, 4 nmol of dNTP mixture, 5% dimethyl sulfoxide, and ExTaq polymerase (Takara Bio). The sense primer 5'-GCTCATGTGATCCTGCAGTGTGG-3' and antisense primer 5'-GAAGGGAAGGACCTGGAGAGACC-3' were designed to amplify a β-actin fragment. Aliquots of PCR products (7 μL) were subjected to electrophoresis in 1.75% agarose gel for analysis.

RESULTS

ADCC of YB2/0- and CHO-produced IgG1—The purified humanized anti-hIL-5R IgG1 antibodies, KM8399 (YB2/0-produced) and KM8404 (CHO-produced), were compared for their ability to induce ADCC against a murine T cell line CTLL-2 (h5R) expressing hIL-5R α-chain and β-chain. Human peripheral blood mononuclear cells were used as effector cells for ADCC. Both humanized KM8399 and KM8404 showed high affinity to the soluble hIL-5R α-chain antigen and had no differences in antigen binding in enzyme-linked immunosorbent assay (data not shown). In contrast, the ADCC of KM8399 was less than that of KM8404 at the concentration of antibody that was the maximum activity of KM8404 (Fig. 1A), indicating that YB2/0-produced IgG1 promoted killing of IL-5R-positive cells at an 50-fold lower concentration than the CHO-produced IgG1.

To confirm the reproducibility of this result, we assessed the ADCC of another antibody, chimeric anti-CD20 IgG1. Rituxan™ was CHO-produced chimeric anti-CD20 IgG1 approved as a therapeutic agent in non-Hodgkin’s lymphoma. We originally established YB2/0-produced chimeric anti-CD20 IgG1, KM3065, that had the same V region amino acid sequences as Rituxan™. Both chimeric Rituxan™ and KM3065 exhibited the same antigen binding activity in flow cytometric analysis using CD20-positive cell lines (data not shown). On the other hand, the ADCC of KM3065 was at least 100-fold higher than that of Rituxan™ at the concentration of antibody that was the maximum activity of KM8404 (Fig. 1A), indicating that YB2/0-produced IgG1 killed 50-fold higher than that of CHO-produced KM8404 and Rituxan™, respectively (Fig. 1, A and B).

Oligosaccharide Analysis—To elucidate the molecular basis of the difference of ADCC between YB2/0- and CHO-produced IgG1, we analyzed the protein portion and oligosaccharide portion of KM8399 and KM8404. There was no significant difference in SDS-PAGE, peptide mapping, and CD (data not shown), suggesting the importance of oligosaccharides for controlling ADCC.

![Fig. 1. Evaluation of ADCC of anti-hIL-5R IgG1 (A) and anti-CD20 IgG1 (B).](image-url)
Absence of Fuc of IgG Enhances ADCC

Hydrazinolysis-derived oligosaccharides were labeled with 2-amino pyridine and separated by HPLC (Fig. 2, A and B). As shown in Fig. 2, A and B, peak patterns and content of oligosaccharides between YB2/0-produced KM3065 and CHO-produced KM3065 were quite different. KM3065 contained nine major oligosaccharides (peaks a, b, c, e, f, g, h, k, and l); in contrast, KM3064 contains five major oligosaccharides (peaks a, e, f, g, and h). The oligosaccharide structure of each peak is shown in Fig. 2C, and all structures have been found in human IgG N-linked oligosaccharides as natural structures (22, 23).

To clear the difference of oligosaccharides found in Fig. 2, quantitative monosaccharide compositions of IgGs were determined (Table I). The contents of Fuc, Gal, and GlcNAc were different between YB2/0-produced IgGs and CHO-produced IgGs. KM3899 and KM3065 contained 0.8- and 0.9-fold lower content of Fuc than KM8404 and Rituxan™ (CHO-produced), respectively. In contrast, the two YB2/0-produced IgGs showed a higher content of GlcNAc than the two CHO-produced IgGs. The difference in the content of Gal between YB2/0-produced IgGs and CHO-produced IgGs was not consistent. The results suggest the difference of ADCC between YB2/0-produced IgGs and CHO-produced IgGs is caused by that of oligosaccharide structure, especially Fuc-and/or GlcNAc-containing oligosaccharides.

Oligosaccharide profiling analysis also showed that content of nonfucosylated oligosaccharides of KM3899 (34%, Table II) and KM3065 (91%, Table IV) were higher than those of KM8404 (9%, data not shown) and Rituxan™ (6%, data not shown). Fuc composition of four IgGs (KM3899, KM8404, KM3065, and Rituxan™), which was calculated from oligosaccharide profiling analysis (0.71, 0.91, 0.09, and 0.94, respectively) coincided well with the result of monosaccharide analysis (0.76, 0.91, 0.08, and 0.94, respectively) (Table I). Based on these results, we selected the oligosaccharide profiling analysis for further study.

 Lectin Affinity Chromatography—To analyze the effect of Fuc-containing and bisecting GlcNAc-containing oligosaccharides on ADCC, YB2/0-produced anti-hIL-5R IgG1 KM3899 was fractionated based on the content of Fuc or bisecting GlcNAc by two lectin affinity chromatographies, LCA (Fig. 3) or PHA-E4 (Fig. 4). KM3899 was fractionated by LCA lectin affinity chromatography to the fraction I with lower Fuc content and the fraction II with higher Fuc content before separation (Fig. 3A and Table II). The contents of nonfucosylated IgG1 of the fraction I, II, and unseparated fraction were 100, 15, and 34%, respectively. As shown in Fig. 3B, ADCC of the fraction I was enhanced 10-fold before separation; nevertheless, that of the fraction II was decreased 10-fold. These results indicated that Fuc content is inversely correlated to ADCC of anti-hIL-5R IgG1.

Several groups have focused on the relationship between ADCC and bisecting GlcNAc, although KM3899 had few contents of bisecting GlcNAc-binding oligosaccharides (4%, Table II). To examine the relationship, KM3899 was separated to fractions III and IV based on the content of bisecting GlcNAc by PHA-E4 column (Fig. 4A) following by sequential separation of fraction III and IV by LCA chromatography (Fig. 4, B and C) to reduce the effect of nonfucosylated oligosaccharides. As shown in Table III, fraction III contained no bisecting GlcNAc-binding oligosaccharides (0%), and fraction IV had more content of bisecting GlcNAc-binding oligosaccharides (30%) than before separation (4%). On the other hand, the content of Fuc of each fraction was consistent (fraction III: 88%; fraction IV: 90%). As shown in Fig. 4D, fractions III and IV showed no significant difference in ADCC, suggesting bisecting GlcNAc at least under 30% content in the oligosaccharides has little effect in ADCC of anti-hIL-5R IgG1.

YB2/0-produced KM3065 contained bisecting GlcNAc-binding nonfucosylated oligosaccharides with a relatively high percentage of 16%. Although oligosaccharides of such structure are quite small in quantity (less than 1%), those oligosaccharides are detectable in serum IgG derived from normal human (22, 24). To analyze the combined effect of Fuc and bisecting GlcNAc, KM3065 was separated to four fractions, V, VI, VII, and VIII, based on the content of bisecting GlcNAc by PHA-E4 lectin affinity chromatography (Fig. 5A). As shown in Table IV, each fraction had a different content of bisecting GlcNAc-binding oligosaccharides (from 0 to 45%); however, the content of nonfucosylated oligosaccharides was not significantly different. All four fractions showed almost the same ADCC to unseparated fraction KM3065 (Fig. 5B), indicating that the content of bisecting GlcNAc at least under 45% did not affect the ADCC of anti-CD20 chimeric IgG1, which contained around 90% content of the nonfucosylated oligosaccharides.

To verify the combined effect of bisecting GlcNAc with Fuc, LEC10 cells, a variant CHO cell line overexpressing GnTIII (12), were used to produce chimeric anti-CD20 monoclonal IgG1. In oligosaccharide analysis, bisecting GlcNAc-binding fucosylated oligosaccharides were the majority on LEC10-produced anti-CD20 IgG1 (74% of bisecting GlcNAc and 100% of Fuc; data not shown), whereas bisecting GlcNAc-nonbinding
Absence of Fuc of IgG1 Enhances ADCC

**TABLE II**

Oligosaccharide composition of IgGs

Pyridylaminated oligosaccharides were analyzed for their compositions by reverse phase HPLC as described under “Experimental Procedures.” The percentages of the total oligosaccharide are given on a molar basis. The structures of each oligosaccharide are represented in Fig. 2. ND, not detected. Fuc(−), total percentage of nonfucosylated oligosaccharides. Bi(−), total percentage of bisecting GlcNAc-binding oligosaccharides. G0, G1, and G2, total percentage of nongalactosylated, monogalactosylated, and digalactosylated oligosaccharides, respectively.

| IgG1 | a | b | c | d | e | f | g | h | k | l | Total | Fuc(−) | Bi(−) | G0 | G1 | G2 |
|------|---|---|---|---|---|---|---|---|---|---|------|-------|-------|---|---|---|
| KM8399 | 28 | 4 | 2 | ND | 39 | 15 | 5 | 3 | 3 | 1 | 100 | 34 | 4 | 70 | 27 | 3 |
| Fraction I | 75 | 10 | 15 | ND | ND | ND | ND | ND | ND | ND | 100 | 100 | ND | 75 | 25 | ND |
| Fraction II | 13 | 1 | 1 | ND | 49 | 21 | 2 | 5 | 5 | 3 | 100 | 15 | 8 | 67 | 28 | 5 |

Fig. 3. LCA lectin affinity chromatography and ADCC assay of KM8399. A, purified KM8399 was applied on an LA-LCA column. The column was eluted with a linear gradient of 0.5 M α-methyl-D-mannoside as described under “Experimental Procedures.” Fractions I and II were analyzed in Table II. B, lysis (%) of target cells by human PBMC as effector cells (E/T ratio of 90:1) in the presence of antibody at the indicated concentrations was measured via release of 51Cr. Filled circles, KM8399 before separation; filled diamonds, fraction I; filled squares, fraction II.

Expression Levels of α,1,6-Fucosyltransferase in YB2/0 Cells and CHO Cells—FUT8 is considered to be the only gene coding α,1,6-fucosyltransferase, which catalyzes the transfer of Fuc from GDP-Fuc to GlcNAc in α,1,6-linkage of complex-type oligosaccharides, because no homologous gene has been found (25). To elucidate the mechanism of lower Fuc content of YB2/0-produced IgG1 than that of CHO-produced IgG1, we examined expression levels of FUT8 mRNA in each cell line.

Quantification of FUT8 transcripts was performed using competitive RT-PCR. To normalize the synthesis efficiency of first-strand cDNAs, the amounts of β-actin transcripts were also quantified by competitive RT-PCR. These PCR analyses, which were performed independently three times, revealed that an expected FUT8 fragment from YB2/0 cells has poor intensity compared with CHO cells (Fig. 6A). The expression level of FUT8 transcripts in YB2/0 cells was shown to be 0.1% relative to β-actin transcripts, whereas CHO cells have 2.0% FUT8 transcripts to β-actin transcripts. There was no significant difference observed when we performed PCR analysis using primers and competitor DNAs specific for Chinese hamster FUT8 and β-actin. These results suggest that lower expression of FUT8 mRNA is the cause for lower content of Fuc of IgG1 produced by YB2/0 cells.
A

Overexpression of \( \alpha, \beta, \gamma\)-fucosyltransferase in YB2/0 Cells—
To confirm the effects of FUT8 expression on the ADCC of IgG1 produced by YB2/0 cells, FUT8-overexpressing YB2/0 cells were established by transfection of murine FUT8 cDNA to anti-CD20 IgG1-producing YB2/0 cells. The most highly FUT8-expressing clone that showed 145.5% FUT8 transcripts relative to \( \beta\)-actin transcripts was selected and designated as 3065ft8-72 (Fig. 6A). The ADCC of anti-CD20 IgG1 produced by 3065ft8-72 was 100-fold lower than that produced from the original YB2/0 cells and equivalent to the commercially available anti-CD20 IgG1 Rituxan\textsuperscript{TM} produced by CHO cells (Fig. 6A). Monosaccharide analysis of each IgG1 showed that there was no significant difference between the IgG1 from FUT8-overexpressing cells and the original YB2/0 cells except that a higher amount of Fuc (81%; data not shown) was detected in the IgG1 from FUT8-overexpressing cells. These findings strongly suggest that FUT8 acts as a key gene in YB2/0 cells to affect the Fuc content as well as ADCC of IgG1.


discussion
In this study, we analyzed the molecular basis of extremely high ADCC of recombinant IgG1 produced by rat hybridoma YB2/0 cells, which produced IgG1 with at least 50-fold higher ADCC than that produced by CHO cells, one of the most widely used host cell lines for production of recombinant antibodies (Fig. 1). Our conclusion of the present study is that nonfucosylated oligosaccharide of YB2/0-produced IgG1 has a more critical role in enhancing ADCC than Gal-binding or bisecting GlcNAc-binding oligosaccharides according to the following evidence. First, monosaccharide composition and oligosaccharide profiling analysis showed that high content of nonfucosylated complex-type oligosaccharides were characteristic in YB2/0-produced anti-hIL-5R IgG1 (91%, Table II) and anti-CD20 IgG1 (91%, Table IV) compared with low content of those in CHO-produced anti-hIL-5R IgG1 (9%, data not shown) and anti-CD20 IgG1 (6%, data not shown). Second, ADCC assay of the anti-hIL-5R IgG1 separated by LCA affinity chromatography demonstrated that Fuc content of IgG1 was inversely correlated with ADCC (Fig. 3, A and B). Third, quantitative PCR analysis indicated that YB2/0 cells had a 10-fold lower expression level of FUT8 mRNA than CHO cells (Fig. 6A). Fourth, overexpression of FUT8 in YB2/0 cells increased the content of fucosylated oligosaccharides and also decreased ADCC of anti-CD20 IgG1 (Fig. 6B).

Fuc residues in an \( \alpha, \beta, \gamma\)-linkage to the GlcNAc of the reducing end (“core Fuc”) are relatively common in mammalian N-linked oligosaccharide. FUT8, considered to be the only gene that codes \( \alpha, \beta, \gamma\)-fucosyltransferase, catalyzes the transfer of Fuc from GDP-Fuc to GlcNAc of the reducing end. Therefore, we focused on FUT8 as a key gene controlling the low Fuc content of IgG1 produced by YB2/0 and indicate that the cells produce low Fuc content IgG1 simply due to the low expression level of FUT8. Since biosynthesis of N-linked oligosaccharides is controlled by a number of glycosyltransferases, their acceptors and substrates, etc., it remains to be determined whether there is possible involvement of the other factor in biosynthesis of nonfucosylated IgG1 in YB2/0 cells.

The importance of nonfucosylated oligosaccharide on ADCC has been reported very recently by Shields et al. (11). They have shown that nonfucosylated anti-Her2 humanized IgG1 and anti-IFN humanized IgG1 produced by a variant of CHO cells, Lec13, had enhanced ADCC relative to fucosylated IgG1s produced by normal CHO cells. However, they have only focused on Fuc, because no appreciable differences in the content of the other sugar residues have been found in Lec13-produced IgG1 and normal CHO-produced IgG1. Until now, the effects of Fuc, Gal, or bisecting GlcNAc on ADCC have been analyzed independently (4–7, 9–11); therefore, comparison of the effect of each sugar residue or the combined effect of each sugar residue has not yet been reported.

In this report, we could not find any correlation between the content of Gal and ADCC. A difference in the content of Gal between YB2/0-produced IgG1s and CHO-produced IgG1s was not correlated to ADCC (Table I). As a result of the separation of KM8399 using LCA lectin affinity chromatography, the compositions of G0, G1, and G2 of fraction II were very similar to that of KM8399 (Table II); nevertheless, ADCC of fraction II and KM8399 was quite different (Fig. 3B). Our results show a good coincidence with the report of Boyd et al. (4), in which obvious change was not found in ADCC after removal of the majority of the Gal residues of anti-CDw52 IgG1 produced by

\begin{table}[h]
\centering
\caption{Oligosaccharide composition of PHA-LCA-separated fractions}
\begin{tabular}{cccccccccccc}
\hline
IgG1 & a & b & c & d & e & f & g & h & k & l & Total & Fuc (\%) & Bis (\%) \\
\hline
Fraction III & 12 & ND & ND & ND & 76 & 8 & 4 & ND & ND & 100 & 12 & ND & 88 & 12 & ND \\
Fraction IV & 9 & 1 & ND & ND & 36 & 17 & 4 & 3 & 10 & 20 & 100 & 10 & 30 & 55 & 42 & 3 \\
\hline
\end{tabular}
\end{table}
Absence of Fuc of IgG1 Enhances ADCC

TABLE IV

| IgG1 | a | b | c | d | e | f | i | j | k | l | Total | Fuc(−) | Bis(+) | G0 | G1 | G2 |
|------|---|---|---|---|---|---|---|---|---|---|-------|-------|-------|----|----|----|
| KM3065 | 54 | 11 | 8 | 2 | 1 | 12 | 1 | 4 | 2 | 1 | 100 | 91 | 19 | 73 | 25 | 2 |
| Fraction V | 77 | 6 | 11 | ND | 6 | ND | ND | ND | 1 | ND | 100 | 94 | ND | 85 | 17 | ND |
| Fraction VI | 60 | 16 | 7 | 2 | 5 | 2 | 7 | 1 | ND | ND | 100 | 93 | 8 | 72 | 26 | 2 |
| Fraction VII | 32 | 12 | 16 | 3 | 4 | ND | 29 | ND | 4 | ND | 100 | 92 | 33 | 69 | 28 | 3 |
| Fraction VIII | 30 | 10 | 6 | 5 | 4 | ND | 16 | 23 | 2 | 4 | 100 | 90 | 45 | 52 | 43 | 5 |

Fig. 6. Competitive PCR analysis and ADCC assay of FUT8 expression in YB2/0 cells and CHO/DG44 cells. A, single-stranded cDNAs prepared from each cell line or standard FUT8 DNAs (0.1, 1, 10, 100, and 1,000 fg) were subjected to PCR with 10 fg of competitor DNA as described under “Experimental Procedures.” Arrows, the amplified products with the expected size. B, lysis of Raji human B by human PBMC at a target/effector ratio of 1:20 in presence of different concentrations of antibodies was quantified by detecting lactate dehydrogenase activity. The percentage of cytotoxicity is calculated relative to a total lysis control, after subtraction of the signal in the absence of antibodies. The results are presented as the mean ± S.D. of triplicate samples.

CHO cells. In contrast, Kumpel et al. (5, 6) reported that highly galactosylated anti-D-antigen IgG1s have higher ADCC, although that effect was only 2–3-fold.

Two groups independently reported that increasing the level of bisecting GlcNAc of anti-neuroblastoma IgG1 and anti-CD20 IgG1 could enhance ADCC (9, 10). GnTIII-transfected CHO cells produced anti-CD20 IgG1 with a high content of bisecting GlcNAc (48–71%), which showed a 10–20-fold enhancement of ADCC compared with that with no content of bisecting GlcNAc (0%). In the present study, we carefully examined the effect of bisecting GlcNAc in ADCC in comparison with that of Fuc. We prepared anti-hIL-5R IgG1 with different content of bisecting GlcNAc (0–30%). To avoid the effect of fucosylation, nonfucosylated IgG1s were depleted by LCA lectin affinity chromatography. To our surprise, we could not detect any correlation between ADCC and content of bisecting GlcNAc. One possible explanation of the discrepancy with the results of Umana et al. (9) and Davies et al. (10) might be that 30% content of bisecting GlcNAc is not enough to enhance ADCC. We next produced anti-CD20 IgG1 by LEC10 cells, a variant CHO cell that over-expressed GnTIII. The resultant IgG1 (74% bisecting GlcNAc and 100% Fuc) had shown only severalfold higher ADCC than normal CHO-produced IgG1 (0% bisecting GlcNAc and 94% Fuc); in contrast, YB2/0-produced IgG1 (19% bisecting GlcNAc and 91% non-Fuc) had 100-fold higher ADCC than LEC10-produced IgG1 (Fig. 5B). These results suggest that an extremely high content of bisecting GlcNAc (74%) has a relatively weak effect for enhancing ADCC. More importantly, nonfucosylated oligosaccharide was shown to have a prominent effect in enhancement of ADCC of IgG1 compared with bisecting GlcNAc-containing oligosaccharide. We further evaluated the combined effect of bisecting GlcNAc with nonfucosylated oligosaccharides. YB2/0-produced anti-CD20 IgG1 was separated based on the content of bisecting GlcNAc-binding oligosaccharides (0, 8, 33, and 45%), which contained the same content of nonfucosylated oligosaccharides (around 90%). These four fractions did not show any significant difference in ADCC, indicating that the presence of bisecting GlcNAc-binding oligosaccharides, at least under 45%, does not have any additional effect in ADCC of highly nonfucosylated IgG1 (90%). To our knowledge, this is the first report that shows the effect of Fuc, Gal, and bisecting GlcNAc simultaneously and also shows the combined effect of Fuc and bisecting GlcNAc.

The ADCC have been believed to be a result of specific killing of antigen-positive cells by natural killer cells through binding of the IgG Fc domain to FcγRIIIa. Recently, Shields et al. (11) have revealed that binding of the Fuc-deficient IgG1 (produced by Lec13 cells) to FcγRIIIa was enhanced up to 50-fold. They have shown that improved binding to FcγRIIIa has translated into improved ADCC in vitro, using PBMC or natural killer cells. These results suggest that Fuc-deficient IgG1 may require a lower concentration of antibody on the surface of the target cell to activate an effector cell. There are a few possible explanations of why the antibodies with nonfucosylated oligosaccharides give rise to stronger binding to FcγRIIIa than those in which the glycoforms are absent. A core Fuc has been shown to influence the conformational flexibility of biantennary oligosaccharides (26, 27). The oligosaccharides of IgG appear to be largely sequestered between the CH2 domains and may help to stabilize the CH2 domain (28). In the co-crystal structure of IgG Fc:FcγRIII,b, Fuc is orientated away from the interface and making no specific contacts with the receptor (29); nevertheless, Harris et al. (30, 31) have supposed that Fuc could have influence on the binding by the receptor. We speculated that the absence of Fuc provided a more suitable conformation for the binding of IgG1 to FcγRIII than the presence of bisecting GlcNAc or Gal, although structural analyses of a series of IgG1 with or without Fuc, bisecting GlcNAc, or Gal are needed for further discussion.

Several recombinant monoclonal antibodies are being used as human therapeutics. Some of these are blocking monoclonal antibodies to receptors or soluble ligands and therefore may function without utilizing antibody effector functions. However, ADCC is still considered to be one of the most important anti-tumor mechanisms of clinically effective anti-Her2 hu-
Absence of Fuc of IgG1 Enhances ADCC