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EMILIO FERRERO

ASUNTO	Radicación	07-96538
	Trámite	011
	Evento	378
	Actuación	519
	Oficio	
	Folios	004

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Patente de Invención	☐	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (fase nacional)	☐	Cap. I ☐	Cap. II ☐

No. Solicitud Internacional PCT/US2006/005853
Fecha de Presentación Internacional 17 de Febrero 2006

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 153 a 329	18/Septiembre/2007
Reivindicaciones:	Folios 330 a 342	18/Septiembre/2007
	Folios 407 a 413	18/Septiembre/2007
	Folios 415 a 425	18/Septiembre/2007
Secuencias:	Folios 365 a 393	18/Septiembre/2007
Dibujos:	Folios 343 a 364	18/Septiembre/2007
Prioridad:	US 60/654,266	18/Febrero/2005
	US 60/660,431	9/Marzo/2005

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486), y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Modificaciones (Art 34 D 486 y Art 19, 22 y 34 PCT)

Se aceptan las modificaciones a las reivindicaciones en los folios 415 a 425, puesto que no amplían material, y sobre estas se realizará el examen de patentabilidad.

Las modificaciones de las reivindicaciones, folios 407 a 413, no son aceptadas porque se encuentran en un idioma extranjero (inglés), se sugiere presentar la traducción al español.

4. Objeto de la invención

Título de la solicitud: "Anticuerpos monoclonales que carecen de residuos fucosilo

contra antígeno membranal específico de próstata (PMSA)”

El problema planteado en esta solicitud consiste en la necesidad de suministrar anticuerpos útiles para tratar células tumorales e inhibir el crecimiento de PMSA.

Para resolver este problema técnico la solicitud en estudio proporciona un anticuerpo anti-PMSA que carece de residuos fucosil. Los anticuerpos de la invención exhiben aumento una actividad celular citotóxica anticuerpo-dependiente (ADCC por sus siglas en inglés), en comparación con la forma fucosilada. La invención también proporciona células huésped que expresan los anticuerpos anti-PSMA que carecen de los residuos fucosilo, y los métodos de uso de los anticuerpos para inhibir el crecimiento de PSMA.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61P35/00; C07K16/30; C07K16/30P

5. No es una invención (Art 15 D 486 literal (b))

El objeto de las reivindicaciones 6 a 33 no se considera una invención, de acuerdo con el Art citado, ya que corresponde a anticuerpos humanos (ver descripción folio 158 Línea 4 a folio 165 línea 24)

6. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 34 a 40 no es patentable de acuerdo con el Art citado, ya que corresponde a métodos de tratamiento

7. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

- Las Reivindicaciones 1 a 5, no son claras porque definen el anticuerpo reclamado por su EC₅₀, su citotoxicidad, el hecho de ser monoclonal o su fuente de preparación y no por sus características estructurales. Es importante mencionar que un anticuerpo debe caracterizarlo a partir de su estructura completa, mencionando las secuencias que lo conforman en la cadena ligera y cadena pesada, o las secuencias variables de las cadenas ligera y pesada o las secuencias de los 6 CDR's que conforman ambas cadenas.

A pesar que lo reclamado corresponde a materia no patentable, en el estado de la técnica se encuentran divulgados los siguientes documentos que relacionan anticuerpos PSMA, además de las composiciones y moléculas que contienen dichos anticuerpos.

Ítem	Documento	Título	Fecha de Publicación
D1	WO03064606	Human monoclonal antibodies to prostate specific membrane antigen (PSMA)	7/Agosto/2003
D2	WO0109192	Human monoclonal antibodies to prostate specific membrane antigen	8/Febrero/2001

Ítem	Documento	Título	Fecha de Publicación
D3	The journal of biological chemistry, vol. 278, no.5, pp. 3466–3473	The Absence of Fucose but Not the Presence of Galactose or Bisecting N-Acetylglucosamine of Human IgG1 Complex-type Oligosaccharides Shows the Critical Role of Enhancing Antibody-dependent Cellular Cytotoxicity	Enero 31 de 2003
D2	WO03059282	Human monoclonal antibodies against CD30	Julio 24 de 2003

D1 divulga anticuerpos humanos aislados los cuales se unen a PSMA, las composiciones y moléculas que contienen dichos anticuerpos. Estos anticuerpos son producidos por un animal no humano transgénico (por ejemplo un ratón), incluye además los métodos terapéuticos y de diagnóstico en los cuales se pueden utilizar estos anticuerpos (ver Resumen Página 1).
http://worldwide.espacenet.com/searchResults?NUM=WO03064606&DB=EPODOC&locale=en_EP&ST=number&compact=false

D2 divulga anticuerpos monoclonales humanos y pociones de estos que se unen a antígeno, que reconocen específicamente PSMA. Estos anticuerpos pueden ser producidos por un animal transgénico no humano (por ejemplo un ratón). También divulga las composiciones que contienen dichos anticuerpos, los hibridomas para producirlos, y los métodos de diagnóstico y tratamientos relacionados (ver resumen Página 1).
http://worldwide.espacenet.com/searchResults?NUM=WO0109192&DB=EPODOC&locale=en_EP&ST=number&compact=false

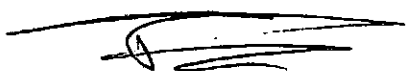
D3 describe la correlación entre la glicosilación de IgG1 humano y ADCC (citotoxicidad celular dependiente de anticuerpos), y demuestra que la fucosa muestra un papel critico en el aumento de ADCC a parte de otros residuos de azúcar reportados previamente.
<http://www.ncbi.nlm.nih.gov/pubmed/12427744>

D4 describe anticuerpos monoclonales anti-CD30 humanos aislados, la producción de los mismos en animales transgénicos, anticuerpos biespecificos, inmunoconjugados, composiciones farmacéuticas, y métodos de tratamiento y diagnostico.
http://worldwide.espacenet.com/publicationDetails/biblio?CC=WO&NR=03059282A2&KC=A2&FT=D&ND=3&date=20030724&DB=EPODOC&locale=en_EP

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante y presentarla por escrito

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha
11.3 JUN. 2012

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Aprobado por: José Luis Salazar López



The Absence of Fucose but Not the Presence of Galactose or Bisecting *N*-Acetylglucosamine 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 column 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 α 1,6-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),¹ 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 mannosyl-chitobiose core (Man3GlcNAc2-Asn) with or without bisecting GlcNAc/L-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 β 1,4-GlcNAc residue transferred to a core β -mannose (Man) residue, and it has been implicated in biological activity of therapeutic antibodies (7). *N*-Acetylglucosaminyltransferase 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 FcγRIIIa 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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‡ The abbreviations used are: ADCC, antibody-dependent cellular cytotoxicity; hIL-5R, human interleukin 5 receptor; Fuc, fucose; Man, mannose; CHO, Chinese hamster ovary; Fc, the constant region of the antibody; PBMNC, peripheral blood mononuclear cell; LCA, *L. culinaris* agglutinin; PHA-E, *P. vulgaris* E; HPLC, high performance liquid chromatography; LTR, long terminal repeat; GnTIII, *N*-acetylglucosaminyltransferase III.

duced by CHO cells. YB2/O-produced IgG1 had lower Fuc content than CHO-produced IgG1. IgG1 containing lower fucosylated oligosaccharides, which was fractionated by *Lens culinaris* agglutinin (LCA) lectin affinity chromatography, showed higher ADCC before separation. In contrast, the addition of bisecting GlcNAc to IgG1 enhanced ADCC much less effectively than defucosylation. The effect of bisecting GlcNAc was only observed in highly fucosylated IgG1. YB2/O cells expressed a lower level of FUT8 (α 1,6-fucosyltransferase gene) mRNA than CHO cells, and overexpression of FUT8 in YB2/O led to the increase of fucosylation of IgG1 and the decrease of ADCC.

EXPERIMENTAL PROCEDURES

Cell Lines. Rat hybridoma YB2/O cells were purchased from the American Type Culture Collection (ATCC; CRL-1662). CHO cell line 244-12, for wild type IgG1 production, was kindly provided by Dr. T. Yoshida (Ch. Ch. Columbia University). LEC10, a variant CHO cell line overexpressing GaTII (β 3), was kindly provided by Dr. Pamela S. Pierce, Albert Einstein College of Medicine.

Expression of IgG1s. For the generation of human IgG1 of human anti-hIL-5R α chain (hIL-5R α -chain) and chimeric anti-CD20, the appropriate humanized or murine VL and VH cDNAs were inserted 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 RituxanTM (VL: GenBankTM accession number AF015962; VH: GenBankTM accession number AR000013). Establishment of anti-hIL-5R humanized IgG1 will be described elsewhere.² Antibody expression vectors were introduced into YB2/O cells or DC44 cells via electroporation and selected for gene amplification in methotrexate-containing medium (14).

Production of IgG1s. The anti-hIL-5R IgG1-producing YB2/O cell line was suspended in GIT medium (Wako, Osaka, Japan) containing 0.5 mg/ml G418 and 200 nM methotrexate to give a density of 3×10^6 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 3×10^5 cells/ml and cultured in 3-l suspension flasks (Asahi Techno Glass, Tokyo, Japan) under agitating at a rate of 100 rpm. The anti-CD20 IgG1-producing YB2/O cell line was suspended in the hybridoma-SFM medium (Invitrogen) containing 5% Daigo's GF21 (Wako) and 200 nM methotrexate to give a density of 1×10^6 cells/ml and dispensed in suspension culture flasks. The flasks were incubated under conditions of 5% CO₂ 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/O cells and CHO cells and anti-CD20 IgG1 from YB2/O cells were clarified by centrifugation and passed through a 0.45- μ m 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 Sepharose 4B 300 (Amersham Biosciences) column. The buffer composition of the YB2/O-produced anti-CD20 IgG1 was changed to that of RituxanTM (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/O- and CHO-produced humanized anti-hIL-5R IgG1s were designated as KM8399 and KM8404, respectively. YB2/O-produced chimeric anti-CD20 IgG1 was designated as KM3065. RituxanTM (chimeric mouse/human anti-CD20 monoclonal antibody derived from the CHO cell line) was purchased from Genentech (South San Francisco, CA; TDEC Pharmaceutical (San Diego, CA).

ADCC Assay for the Anti-hIL-5R IgG1. An ADCC assay was performed by ⁵¹Cr release assay as reported previously (15). Briefly, target cells (1×10^4) of a murine T cell line CTLL-2 (h5R) expressing hIL-5R α -chain and β -chain (16), were labeled with 3.7 MBq of Na²⁴CrO₄ at 37 °C for 1.5 h. Human effector cells were peripheral blood mononuclear cells (PBMC) purified from healthy donors using Polymorphprep (Nycomed Pharma AS, Roskilde, Norway). Aliquots of the ⁵¹Cr-labeled target cells were dispensed into 96-well U-bottomed plates (1×10^4 /50

μ l) and incubated with serial dilutions of antibodies (50 μ l) in the presence of human effector cells (100 μ l) 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,

$$\% \text{ specific lysis} = 100 \times (E - S)/(M - S) \quad (\text{Eq. 1})$$

where E represents the experimental release (cpm in the supernatant from target cells incubated with antibody and effector cells), S is 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).

ADCC Assay for the Anti-CD20 IgG1. An ADCC 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 (1×10^4 /50 μ l) and incubated with serial dilutions of antibodies (50 μ l) in the presence of human effector cells (100 μ l) 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,

$$\% \text{ specific lysis} = 100 \times (E - S_E - S_T)/(M - S_T) \quad (\text{Eq. 2})$$

where E represents the experimental release (activity in the supernatant from target cells incubated with antibody and effector cells), S_E is the spontaneous release in the presence of effector cells (activity in the supernatant from effector cells), S_T is the spontaneous release of target cells (activity in the supernatant from target cells incubated with medium alone), and M is the maximum release of target cells (activity released from target cells lysed with 9% Triton X-100).

Profiling Analysis of N-Linked Oligosaccharides. Oligosaccharides were prepared from 100 μ g of IgG1 by hydrazinolysis and N-acetylation by the methods reported previously (17). Oligosaccharides were pyridylaminated (17) and analyzed by reverse-phase HPLC using a Shim-pack CLC-ODS column (60 $\times$ 150 mm; Shimadzu, Kyoto, Japan) (18) with slight modifications. Elution was performed at a flow rate of 1.0 ml/min at 55 °C using two solvents, A and B. Solvent A was 10 mM sodium phosphate buffer (pH 3.8), and solvent B was 10 mM sodium phosphate buffer (pH 3.8) containing 0.5% 1-butanol (Sigma). The column was equilibrated with solvent A. After injection of sample, the ratio of solvent B to A was increased with a linear gradient to 60:40 in 30 min. The elution profile was monitored by fluorescence detection with excitation at 320 nm and emission at 400 nm. The oligosaccharide peak assignments were made according to retention time comparison with PA-labeled oligosaccharide standards (TaKaRa Bio, Otsu, Japan). The peak area was used to calculate the percentage of each oligosaccharide, since the relative fluorescence was the same on a molar basis for each component (17). To identify the structure, each oligosaccharide fraction that separated as a peak was collected and evaporated to dryness under vacuum prior to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Monosaccharide Composition of IgG1s. Monosaccharides were released from an aliquoted quantity of IgG1 by heating with 4 M trifluoroacetic acid at 100 °C for 2 h. Monosaccharides were dried under vacuum and reconstituted in water prior to high performance anion exchange chromatography analysis. Monosaccharides were analyzed using a waveform and DX500 system (DIONEX, Sunnyvale, CA) described previously (19). A CarboPac PA-1 column (DIONEX) was used to resolve monosaccharides with a flow rate of 0.8 ml/min at 35 °C. After injection of samples, the monosaccharides were resolved with 18 mM NaOH for 30 min, and the column was regenerated by elution with 500 mM NaOH for 10 min. The column was held 18 mM NaOH for 30 min prior to the next injection.

Lectin Affinity HPLC. LCA or Phaseolus vulgaris E₁ (PHA-E₁) were used as ligands of lectin affinity HPLC for separation of IgG1 based on the content of Fuc or bisecting GlcNAc, respectively. An LA-LCA (4.6 $\times$ 150 mm; HONEN, Tokyo, Japan) column was installed in the LC-6A HPLC system (Shimadzu). Purified antibodies dissolved in 10 mM KH₂PO₄ were applied to the column previously equilibrated with 50 mM Tris-H₂SO₄ (pH 7.3). The column was eluted with a linear gradient of the buffer containing 0.2 M α -methyl-D-mannoside (Nacalai Tesque, Kyoto, Japan) in 60 min. An LA-PHA-E₁ (4.6 $\times$ 150 mm; HONEN) column was installed in the LC-6A HPLC system (Shimadzu). Purified

² M. Koike, E. Shoji-Hosaka, K. Nakamura, A. Furuya, K. Shitara, and N. Hanai, manuscript in preparation.

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 $K_2B_4O_7$ (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 α 1,6-Fucosyltransferase-overexpressing YB2/O cells. A mammalian expression vector, pAGE249, which was derived by insertion of a 2.7-kb *SphI-SphI* fragment containing the dihydrofolate reductase gene expression 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* cDNA (1728 bp) (21) was inserted into pAGE249 under the control of the Mooney murine leukemia virus 3'-LTR promoter to form a construct designated as pAGEmFUT8. Two *FspI* 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-CD20 IgG1-producing YB2/O cells via electroporation, and *FUT8*-overexpressing YB2/O cells, JM8399-72, were selected in 0.5 mg/ml hygromycin-B (Sigma)-containing medium.

Competitive RT-PCR Analysis of *FUT8*. Total RNA was isolated from 1 × 10⁶ YB2/O cells, *FUT8*-overexpressing YB2/O cells, or CHO cells (22) using the RNeasy minikit (Qiagen, Tokyo, Japan) and incubated at 37°C for 10 min with 20 units of RNase-free DNase (RQ1, Promega, Madison, WI) to remove 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 cDNA 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* cDNA was used as a standard DNA and a 772-bp fragment deleting a 202-bp *HindIII* (blunt)-*HindIII* (blunt) fragment from standard DNA was used as a competitor. Standard DNA was amplified from single-stranded cDNAs of YB2/O cells by PCR using primers 5'-ACTGATCTTGGATCTCAGAAATGG-3' and 5'-CTTGACCGTTTCTATCTTCTCTCT-3'. PCRs for detection of *FUT8* were carried out by heating at 94°C for 3 min and subsequent 32 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min in 20 µl of reaction mixture containing 5 µl of the 50-fold diluted single-stranded cDNA, 10 fg of linearized competitors, 50 pmol of *FUT8*-specific primers, 4 nmol of dNTP mixture, 5% dimethyl sulfoxide, and ExTaq polymerase (Takara Bio). The sense primer 5'-GTC CATGGTGCATCTGCAGTGTGG-3' and antisense primer 5'-CACTCAATGATATCTTCAGGTTCC-3' were employed to amplify *FUT8* transcripts. Aliquots of PCR products (7 µl) were subjected to electrophoresis in 1.7% agarose gel and stained with SYBR Green I (Molecular Probes, Eugene, OR). The amount of product was quantified by measuring luminescence intensity using a fluorimeter SL 4000 (Amersham Biosciences), calculated from standard curves, and converted into molar numbers. To normalize the synthesis of any of test-strand cDNAs, the amount of β -actin transcripts was also quantified by competitive RT-PCR in which a 1128-bp partial fragment of rat β -actin cDNA was used as a standard DNA and a 948-bp fragment deleting a 180-bp *DraIII* (blunt)-*DraIII* (blunt) fragment from standard DNA as a competitor were employed. Standard DNA was amplified from single-stranded cDNAs of YB2/O cells by PCR using primers 5'-ATTTAAGGTACCGAAGCATTTGCGGTGCACGATGGAG-3' and 5'-AAGTATAAGCTTACATGGATGACGATATCGCTGCG-3'. PCRs for detection 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 2 min in 20 µl of reaction mixture containing 5 µl of the 50-fold single-stranded cDNAs, 1 pg of linearized competitors, 10 pmol of β -actin-specific primers, 4 nmol of dNTP mixture, 5% dimethyl sulfoxide, and ExTaq polymerase (Takara Bio). The sense primer 5'-CACTATCTGCTCCCTCGCTCTGAC-3' and antisense primer 5'-CACTAAGGAGGCTGGAGAGAGGC-3' were designed to amplify an 80-bp fragment. Aliquots of PCR products (7 µl) were subjected to electrophoresis in 1.7% agarose gel for analysis.

RESULTS

ADCC of YB2/O- and CHO-produced IgG1.—The purified humanized anti-hIL-5R IgG1 antibodies, KM8399 (YB2/O-produced) and KM8404 (CHO-produced), were compared for their ability to induce ADCC against a murine T cell line CTLL-2 (hIL-5R) expressing hIL-5R α -chain and β -chain. Human periph-

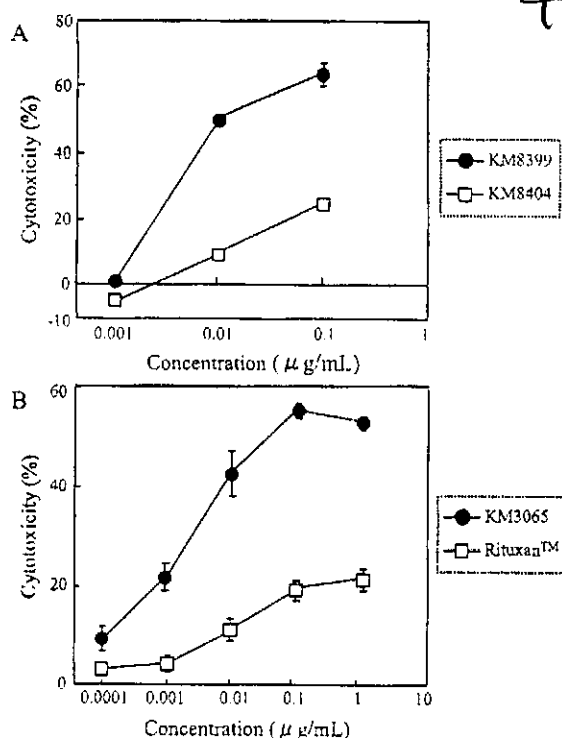


Fig. 1. Evaluation of ADCC of anti-hIL-5R IgG1 (A) and anti-CD20 IgG1 (B). Lysis (%) of target cells by human PBMC as effector cells (E/T ratio of 90:1 (A) or 25:1 (B)) in the presence of antibody at the indicated concentrations was measured via release of ⁵¹Cr (A) or lactate dehydrogenase (B). The percentage of cytotoxicity was calculated as described under "Experimental Procedures." A, filled circles, KM8399; open squares, KM8404. B, filled circles, KM3065; open squares, RituxanTM.

eral 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 ~50-fold higher than that of KM8404 at the concentration of antibody of 25% cytotoxicity that was the maximum activity of KM8404 (Fig. 1A), indicating that YB2/O-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. RituxanTM was CHO-produced chimeric anti-CD20 IgG1 approved as a therapeutic agent in non-Hodgkin's lymphoma. We originally established YB2/O-produced chimeric anti-CD20 IgG1, KM3065, that had the same V region amino acid sequences as RituxanTM. Both chimeric RituxanTM 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 RituxanTM at the concentration of antibody of 20% cytotoxicity that was the maximum activity of RituxanTM (Fig. 1B). Moreover, the maximum cytotoxicity of YB2/O-produced KM8399 and KM3065 was 2–3-fold higher than that of CHO-produced KM8404 and RituxanTM, respectively (Fig. 1, A and B).

Oligosaccharide Analysis.—To elucidate the molecular basis of the difference of ADCC between YB2/O- 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.

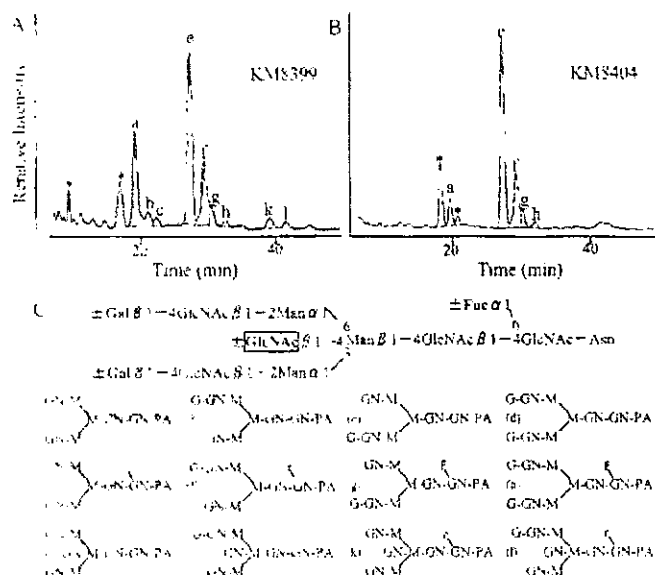


Fig. 2. Oligosaccharide profiles of anti-hIL-5R IgG1. Oligosaccharides derived from IgG1s produced by YB2/0 (A) and CHO (B) cell lines were pyridylaminated and applied to reverse-phase HPLC as described under "Experimental Procedures." The alphabetical peak labels correspond to those of oligosaccharide structure in C. A conserved oligosaccharide core, linked to the Asn, is composed of three Man and β -1,4-GlcNAc monosaccharide residues. Additional GlcNAcs are normally α 1-linked to the α 6 Man and α 3 Man (α 6 and α 3 arms, respectively), and the monosaccharide residues in *boldface type*, Gal, Fuc, and GlcNAc (β 1,4-GlcNAc), can be present or absent. Peaks indicated by *boldface type* correspond to the facts of pyridylamino derivatives (33). The percentage of nonfucosylated oligosaccharides was calculated by the following equation: $(\text{total peak area of a, b, c, d, f, and j} / \text{total peak area}) \times 100$.

Hydrazinolysis-derived oligosaccharides were labeled with 2-aminopyridine and separated by HPLC (Fig. 2, A and B). As shown in Fig. 2, A and B, peak patterns and content of oligosaccharide between YB2/0-produced KM8399 and CHO-produced KM8404 were quite different. KM8399 contained nine major oligosaccharides (peaks a, b, c, e, f, g, h, k, and l); in contrast, KM8404 contains five major oligosaccharides (peaks a, b, 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 clarify the difference of oligosaccharides found in Fig. 2, representative mono-saccharide compositions of IgG1s were determined (Table I). The contents of Fuc, Gal, and GlcNAc were different between YB2/0-produced IgG1s and CHO-produced IgG1s. KM8399 and KM3065 (YB2/0-produced) contained 0.8- and 0.09-fold lower content of Fuc than KM8404 and RituxanTM (CHO-produced), respectively. In contrast, the two YB2/0-produced IgG1s showed a higher content of GlcNAc than the CHO-produced IgG1s. The difference in the content of Gal between YB2/0-produced IgG1s and CHO-produced IgG1s was not consistent. These results suggest the difference of ADCC between YB2/0-produced IgG1s and CHO-produced IgG1s 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 KM8399 (34%, Table II) and KM3065 (91%, Table IV) were higher than those of KM8404 (9%, data not shown) and RituxanTM (6%, data not shown). Fuc composition of four IgG1s (KM8399, KM8404, KM3065, and RituxanTM), 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

TABLE I
Monosaccharide composition of IgG1s

IgG1	Cell line	Fuc	Gal	GlcNAc	Man ^a
KM8399	YB2/0	0.72	1.01	4.51	3
KM8404	CHO	0.91	0.27	3.80	3
KM3065	YB2/0	0.08	0.31	4.41	3
Rituxan TM	CHO	0.94	0.54	3.98	3

^a Molar ratios calculated versus 3 mannoses.

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 KM8399 was fractionated based on the content of Fuc or bisecting GlcNAc by two lectin affinity chromatographies, LCA (Fig. 3) or PHA-E₄ (Fig. 4). KM8399 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 KM8399 had few contents of bisecting GlcNAc-binding oligosaccharides (4%, Table II). To examine the relationship, KM8399 was separated to fractions III and IV based on the content of bisecting GlcNAc by PHA-E₄ column (Fig. 4A) followed 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 oligosaccharide (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 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

TABLE II
Oligosaccharide composition of IgG1s

Quantitatively estimated oligosaccharides were analyzed for their compositions by reverse phase HPLC as described under "Experimental Procedures." The percentage 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. Bis, 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	Bis	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	15	1	1	ND	49	21	2	5	5	3	100	15	8	67	28	5

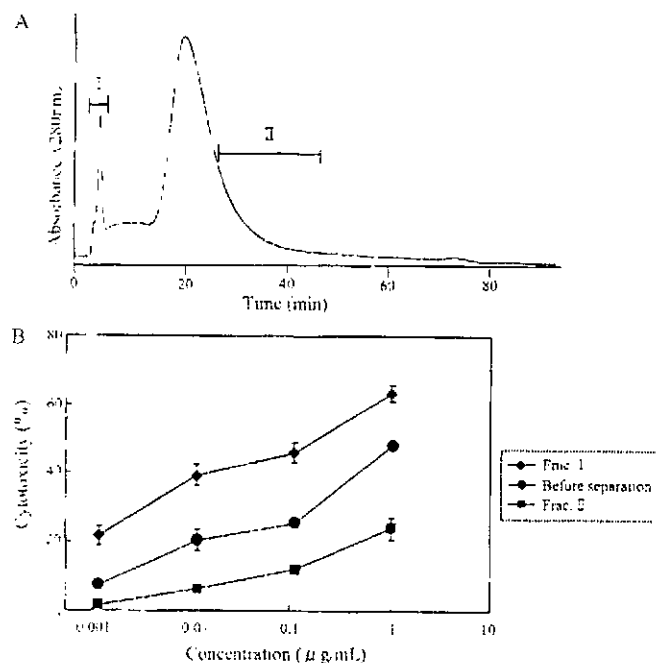


Fig. 3. LCA lectin affinity chromatography and ADCC assay of KM8399. **A**, purified KM8399 was applied on an LA-LCA column. The column was eluted with a linear gradient of 0.5 M α -methyl-D-mannoside as described under "Experimental Procedures." Fractions I and II were analyzed in Table II. **B**, lysis (%) 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 ^{51}Cr . Filled circles, KM8399 before separation, filled diamonds, fraction I; filled squares, fraction II.

Separated oligosaccharides were the majority of RituxanTM (91% of bisecting GlcNAc and 94% of Fuc; data not shown). In the ADCC assay, LEC10-produced IgG1 showed only several-fold enhancement of ADCC compared with RituxanTM (Fig. 3B). In addition, YB2/0-produced KM3065 with low Fuc content (19% of bisecting GlcNAc and 9% of Fuc; Table IV) exhibited 100-fold higher ADCC than LEC10-produced IgG1. These results suggest that a relatively high content of bisecting GlcNAc (74%) improves ADCC when IgG1 has a low content of fucosylated oligosaccharides, although the impact of the content of bisecting GlcNAc in ADCC is much less than that of Fuc.

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 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

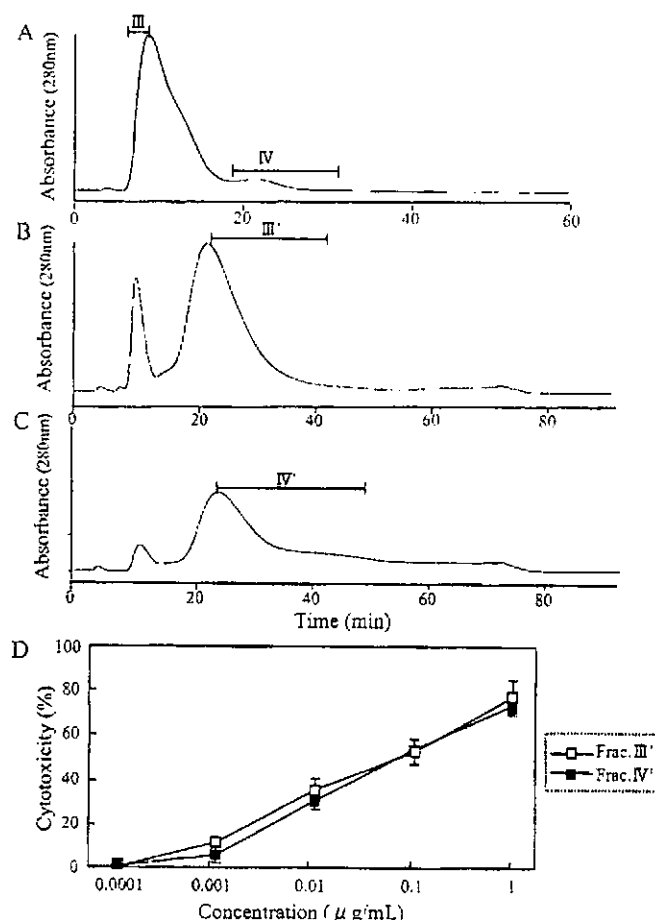


Fig. 4. Serial lectin affinity chromatography and ADCC assay of KM8399. **A**, purified KM8399 was applied on an LA-PHA-E₄ column. The column was eluted with a linear gradient of 0.1 M $\text{K}_2\text{B}_4\text{O}_7$ as described under "Experimental Procedures." Fractions III and IV (**B**) and (**C**) were then applied to the LA-LCA column. The column was eluted with linear gradient of 0.2 M α -methyl-D-mannoside as described under "Experimental Procedures." Fraction III' and IV' were analyzed in Table III. **D**, 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 ^{51}Cr . Open squares, fraction III'; filled squares, fraction IV'.

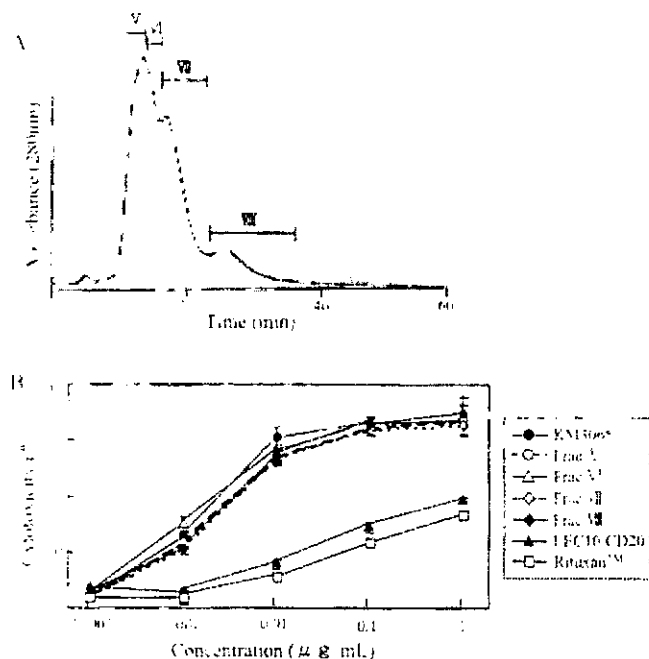
also quantified by competitive RT-PCR. These PCR analyses, which were performed independently three times, revealed that an expected FUT8 fragment from YB2/0 cells has poor intensity compared with CHO cells (Fig. 6A). The expression level of FUT8 transcripts in YB2/0 cells was shown to be 0.1% relative 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.

485

TABLE III
Oligosaccharide composition of PHA-LCA-separated fractions

ND, not detected; Fuc(-), total percentage of nonfucosylated oligosaccharides; Bisi(+), 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(-)	Bisi(+)	G0	G1	G2
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Fraction III	12	ND	ND	ND	76	8	4	ND	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



PHA-E₄ lectin affinity chromatography and ADCC assay of KM3065. A purified KM3065 was applied on an LA-PHA-E₄ column. The column was eluted with a linear gradient of 0.1 M K₂HPO₄. Fractions were analyzed in Table IV. B, lysis (%) of target cells by human PBMC as effector cells (E/T ratio of 20:1) in the presence of antibody at the indicated concentrations was measured via release of ⁵¹Cr-thymidine. Filled circles, KM3065 before separation, open circles, fraction V, open triangles, fraction VI; open diamonds, fraction VII; filled triangles, fraction VIII; filled squares, anti-CD20 IgG1; open squares, RituxanTM.

Overexpression of α 1,6-Fucosyltransferase in YB2/O Cells—To confirm the effects of FUT8 expression on the ADCC of IgG1 produced by YB2/O cells, FUT8-overexpressing YB2/O cells were established by transfection of murine FUT8 cDNA to anti-CD20 IgG1-producing YB2/O cells. The most highly FUT8-expressing clone that showed 145.5% FUT8 transcripts relative to β -actin transcripts was selected and designated as 3065ft8-72 (Fig. 6A). The ADCC of anti-CD20 IgG1 produced by 3065ft8-72 cells was 100-fold lower than that produced from the original YB2/O cells and equivalent to the commercially available anti-CD20 IgG1 RituxanTM produced by normal CHO cells (Fig. 6B). Monosaccharide analysis of each IgG1 showed that there was no significant difference between the IgG1 from FUT8-overexpressing cells and the original YB2/O 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/O 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/O 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/O-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/O-produced anti-hIL-5R IgG1 (34%, 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/O cells had a 10-fold lower expression level of FUT8 mRNA than CHO cells (Fig. 6A). Fourth, overexpression of FUT8 in YB2/O cells increased the content of fucosylated oligosaccharides and also decreased ADCC of anti-CD20 IgG1 (Fig. 6B).

L-Fuc residues in an α 1,6-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 α 1,6-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/O 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/O 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-IgE 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/O-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

TABLE IV
Oligosaccharide composition of PHA-E₁-separated fractions

ND, not detected. Fuc, total percentage of nonfucosylated oligosaccharides. Bisect(+), total percentage of bisecting GlcNAc-binding oligosaccharides. G0, G1, and G2, total percentage of nongalactosylated, monogalactosylated, and digalactosylated oligosaccharides, respectively.

igG	a	b	c	d	e	f	g	h	i	j	k	l	Total	Fuc(+)	Bisect(+)	G0	G1	G2
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
KM3065	54	11	8	2	5	1	12	4	2	1	1	100	91	19	73	25	2	
Fraction V	77	6	11	ND	6	ND	ND	ND	ND	ND	ND	100	94	ND	83	17	ND	
Fraction VI	60	16	7	2	5	2	7	1	ND	ND	ND	100	93	8	72	26	2	
Fraction VII	32	12	16	3	4	ND	29	ND	4	ND	ND	100	92	33	69	28	3	
Fraction VIII	46	10	6	5	4	ND	16	23	2	4	ND	100	90	45	52	43	5	

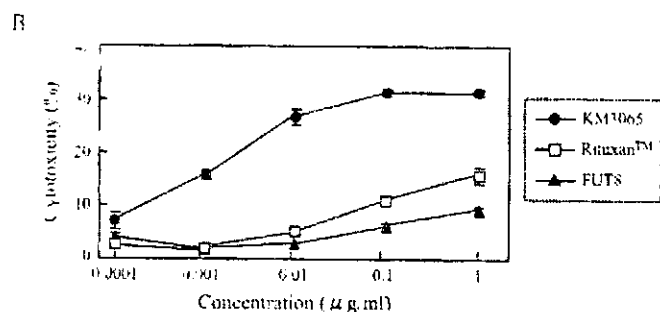
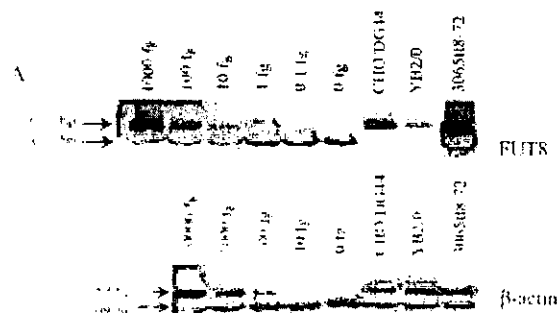


Fig. 5. Competitive PCR analysis and ADCC assay of FUT8 expression in YB2/0 cells and CHO/DG44 cells. A, single-stranded PCR products prepared from each cell line or standard FUT8 DNAs (0.1, 1, 10, 100, 1,000 ng) were subjected to PCR with 10 ng of competitor DNA. The products were subjected to agarose gel electrophoresis under "Experimental Procedures." Arrows, the amplified products with the expected size. B, lysis of Raji human B by human anti-CD20 IgG1. A target-to-effector ratio of 1:20 in presence of different concentrations of antibody was quantified by detecting lactate dehydrogenase activity. The percentage of cytotoxicity is calculated relative to a control (lysis control, after subtraction of the signal in the absence of antibody). Symbols: KM3065, open squares; Rituxan™, filled squares; anti-CD20 IgG1 derived from FUT8-overexpressed YB2/0 cells.

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.

Five groups independently reported that increasing the level of bisecting GlcNAc of anti-neuroblastoma IgG1 and anti-CD20 IgG1 could enhance ADCC (9, 10). GnTHII-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 GnTHII. 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 IgG1 Fc:FcγRIIIb, 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-

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