Dear Candidates,

The below manuscript and the figures belonging thereto were provided to you by Professor Phantastic from the Great Research Institute in Yahud. In view of the fact that fucosylation can be increased from 0% to any percentage, as shown in Fig. 18, he is sure that his method is different than the prior art. I attach the closest prior art: Kanda 2007.

Please prepare a patent application under Israeli practice with claims that you believe have good chance of acceptance by our Patent Office based on the disclosure and the closest prior art. Also, add a separate set of claims for the application to be filed in the USA.

**GOOD LUCK!**


## Abstract

Antibody-dependent cellular cytotoxicity (ADCC) is one of the critical killing mechanisms for antibodies that bind to ligands on the membranes of the target cells. ADCC reaction is significantly enhanced in the presence of low levels or the absence of fucose on the core carbohydrate chain on Asn-297 of the IgG molecules. We isolated a CHO-S-derived cell line (ITL-F2) that is deficient in de-novo fucose synthesis using a novel and efficient phenotypic selection. These cells enable the synthesis of glycoprotein carbohydrate side chains with adjustable fucose contents, depending on the exogenous fucose concentration in the cell culture medium. The adjustable fucosylation level is an advantage when a certain fucosylation level is desired on a recombinant protein product, e.g., an antibody, or when a certain level is required for various cell functions, e.g., growth or transfection. The cells were generated by incubation with methotrexate and a new, specific negative selection method, which sorts cells with undetectable fucose levels on their surface using specific fucose binding lectin markers.

Genetic analyses revealed that the mRNA for GDP-mannose 4,6-dehydratase (GMD), which is involved in the de novo fucosylation pathway, became inactive due to the use of splice variants with an exon deficiency.

As a test case, ITL-LF2 cells were transfected with an anti-EGFR monoclonal antibody (mAb). The fucose levels on the secreted antibodies correlated with the levels of exogenous fucose added to the culture medium.

In terms of other characteristics, the cells were similar to the parent CHO-S cells.



**Key Terms**

Antibody-dependent cell cytotoxicity, Flow cytometry, Fucosylation, Mutagenesis, Sorting

# Introduction

Recombinant therapeutic antibodies have gained increasing attention for the treatment of a wide variety of diseases.

Fc domain antibodies are responsible for effector functions (Carter 2006; Jefferis 2005; Jefferis 2009; Presta 2008; Strohl 2009a; Strohl 2009b) through the antibody-dependent cellular cytotoxicity (ADCC) mechanism (Cartron et al. 2002; Dall'Ozzo et al. 2004; Gennari et al. 2004; Louis et al. 2004; Miescher et al. 2004; Treon et al. 2005), complement-dependent cytotoxicity (CDC) (Crowe et al. 1992; Idusogie et al. 2000; Idusogie et al. 2001), and the neonatal receptor FcRn (Nezlin and Ghetie 2004; Roopenian and Akilesh 2007).

These effector functions are mediated through the interactions of effector molecules with the hinge and CH2 regions of Fc. The CH2 domain contains an oligosaccharide located on the N glycosylation site at position 297 of the antibody, which is known to play an important role in binding to effector cells (Clark 1997; Morgan et al. 1995; Raju 2008).

ADCC activity is dependent on both the IgG isotype and a specific Fc□R. Although IgG1 and IgG3 may induce this activity, IgG4 does not (Jefferis 2007; Salfeld 2007). The Fc□R that binds IgG and is important for the ADCC activation mechanism is known as the Fc□RIIIa, and it is expressed on NK cells and macrophages (Anolik et al. 2003; Cartron et al. 2002; Gennari et al. 2004; Weng and Levy 2003). In many cases, the ADCC activity achieved upon binding of the NK cell to the target cell is not efficient enough to kill the target cell. The reason may be that the Fc□RIIIa has low affinity for IgG1. In one study, increased ADCC was obtained by manipulating the IgG Fc structure. Computationally designed algorithms were used to engineer and select for high affinity antibodies in high-throughput screening (Lazar et al. 2006), although a decreased thermostability of the mutated IgG1 (IgG1 with the mutations S239D, A330L and I332E) was detected (Oganesyan et al. 2008). Another approach to obtain antibodies with enhanced ADCC is to produce them with few or no fucose residues on the oligosaccharide at position 297. Previously, it was found that fucose residues on this oligosaccharide interfere with Fc binding to Fc□RIIIa (Shinkawa et al. 2003). One method to obtain antibodies with low fucose levels is to harness cells with these natural capabilities, such as rat hybridoma YB2/0 cells (Shinkawa et al. 2003); however, the recombinant proteins produced in these cells are not homogenous in their fucose content. Several mammalian cells are used to produce antibodies with various glycosylation properties in general and enhanced ADCC in particular. Glycotope has created various glycoengineered human cell lines to optimize the glycosylation of bio-therapeutics (http://www.glycotope.com/). Glycart, engineered a cell line producing recombinant antibodies with reduced fucose levels by introducing beta/(1,4)-N-acetylglucosaminyltransferase III (GnTIII), a glycosyltransferase that catalyzes the formation of bisected oligosaccharides that have been implicated in enhanced ADCC(Umana et al. 1999). Additionally, Biowa generated a knockout in the fucosyl transference 8 (Fut8) gene in CHO DG44 cells to decrease fucose levels (Yamane-Ohnuki et al. 2004).

A recent study showed that heterologous expression of the prokaryotic enzyme GDP-6-deoxy-D-lyxo-4-hexulose reductase within the cytosol of mammalian cells could block the conversion of the intermediate

GDP-4-keto-6-deoxymannose into a dead-end product that typically does not occur in vertebrate cells. Thus, CHO cells with this modification secreted antibodies lacking core fucose (von Horsten et al. 2010). Another approach that has been employed is to create lectin-resistant mutants that survive in the presence of a toxic fucose-specific lectin. LEC13 is a CHO-based cell line that was developed by incubating CHO cells in the presence of the toxic pea fucose-specific lectin (Ripka and Stanley 1986). LEC13 is deficient in GDP-mannose 4,6-dehydratase activity (Ohyama et al. 1998; Sullivan et al. 1998),which results in the expression of human IgG1 that is deficient in fucose (Shields et al. 2002).

We used an effective, general approach to select cells expressing a stable phenotype that enabled us to adjust the fucose levels rather than engineering the antibody protein structure. This method enables the selection of cell lines with different required attributes and can be performed on cells that already express recombinant antibodies.

# Materials and Methods

## Incubation of CHO-S Cells in the Presence of Methotrexate (MTX)

CHO-S cells (Gibco BRL, Life Sciences, New York, New York, USA, cat. #11619) in animal component-free medium C6614 (CHO DHFR - medium powder, SAFC Biosciences, Lenexa, Kansas, USA, cat. #C6614) were seeded at $0.2x10^6$ cells/ml with 100 nM MTX (Calbiochem Biochemicals, Darmstadt, Germany, cat. 454125) and propagated until the viability exceeded 90%. Then, the cells were transferred to 200 nM MTX and frozen when they recovered to >90% viability.

## Sorting of Low-Fucose Cells

The MTX-treated cells ($10^8$) were washed in cold PBS + 0.1% pluronic acid (F-68, Sigma, Steinheim, Germany, cat. #P5556), centrifuged, resuspended in 10 ml of the biotinylated fucose-specific lectin AAL biotinylated AAL (Vector, Burlingame, CA, USA, cat. #B-1395) (20 μg/ml) + fluorescently labeled streptavidin (Biolegend, San Diego, CA, USA, cat. # 405203) at a 1:100 mixture in PBS + 0.1% pluronic acid, and transferred to T80 flasks (TCF 80 $cm^2$, Nunc, Kamstruvej, Denmark, cat. #153732) for 30 minutes of incubation at 37 °C with shaking at 45 rpm. The cells were washed twice in cold PBS + 0.1% pluronic acid, resuspended in PBS + 0.1% pluronic acid to obtain a density of $10^7$ cells/ml, and filtered into FACS tubes (Becton Dickinson, Falcon, Canaan, CT, USA, cat. #352054). The cell fractions with the lowest fluorescence (0.2% in the first sort, 0.07% in the second, 1% in the third, and 2% in the fourth) of the population were sorted into 2 ml of Sigma ProCHO5 animal component-free medium (ACFM) (Lonza, Verviers, Belgium, cat. #BE12-766Q) + HT (Biological Industries, Kibbutz Beit Haemek, Israel, cat. #03-085-1C) per tube, centrifuged, resuspended in ProCHO5 + HT, seeded into 24-well plates (Nunc, Roskide, Denmark, cat. #143982) (in the first and second sorts) or a T25 flask (TCF 25 $cm^2$, Nunc, Roskide, Denmark, cat. #136196) (in the third and fourth sorts), and propagated in ProCHO5 medium.

## Analysis of the Fucose Levels on Cell Membranes by Flow Cytometry

Cells ($2x10^6$) were washed in PBS + 0.1% pluronic acid, centrifuged, resuspended in a mixture consisting of 500 μl of biotinylated AAL + SA-PE (diluted to 2 μg/ml) in PBS + 0.1% pluronic acid, and transferred to

24-well plates for a 30 min incubation at 37 °C with shaking. The cells were resuspended thoroughly in PBS + 0.1% pluronic acid and transferred to 15-ml tubes (Becton Dickinson, Falcon, USA, Franklin Lakes, NJ, cat. #2097 and Corning, Oneonta, NY, USA, Cat. #430055). Ten milliliters of PBS + 0.1% pluronic acid were added, and the cells were mixed, centrifuged and washed again. The pellets were resuspended in 0.5 ml of PBS + 0.1% pluronic acid/tube and filtered into FACS tubes. A total of $2x10^6$ cells were labeled with biotinylated AAL + SA-PE and analyzed by flow cytometry.

## Addition of Exogenous Fucose to the ITL-LF2 Cell Culture

Anti-EGFR mAb-transfected and non-transfected ITL-LF2 cells were seeded at a density of $0.2x10^6$ cells/ml in ProCHO5 medium with different concentrations of L-fucose (Sigma, Oakville ON, Canada, cat. # F2252) and incubated at 37 °C on a shaker at 320 rpm with $CO_2$. After 4 days, the cell samples were analyzed by flow cytometry to determine their fucosylation levels.

## Analysis of mRNAs of Genes Involved in Fucosylation Pathways

cDNAs were prepared from total RNAs extracted from CHO-S or ITL-LF2 cells using the Invitrogen SuperScript III Kit (Carlsbad, CA, USA, cat. # 18080-051) and oligo dT primers. PCR was then performed using gene-specific primers. The primers were synthesized at Sigma Aldrich.

The products were analyzed on agarose gels.

The cDNAs were sequenced on the fully automated 16 Capillary ABI Prism 3100 Genetic Analyzer (Foster City, California, USA). The sequences were analyzed in-house utilizing the Sci-Ed general software package (Clone Manager software, version 7.01, and Align Plus 5, version 5.01, Morrisville, NC, USA).

## Construction of DNA Expression Vectors

All vectors were constructed using standard molecular biology techniques, as detailed in the ITL procedures.

## Construction of a Plasmid Containing the Wild Type GMD (pCMV-P-GMD)

The full-length GMD cDNA was created by RT-PCR from the total RNA extracted from CHO-S WT cells. This fragment was sequenced and conformed to the expected full-length GMD cDNA sequence (data not shown).

An additional PCR step was carried out with specific primers to clone this gene into the required vector. The PCR fragment and the plasmid (pCMV-P) were digested and ligated.

The resulting vector contained the GMD gene downstream of the hCMV promoter with the puromycin resistance gene (PAC) as a selection marker on a separate cassette.

## Stable Transfections of ITL-LF2 and CHO-S Cells

ITL-LF2 cells were adapted to C6614 animal component-free medium (ACFM, CHO DHFR - medium powder, SAFC Biosciences, Lenexa, Kansas, USA, cat. #C6614) and CHO-S were cultured in ProCHO5 ACFM (Lonza, Verviers, Belgium, cat. #BE12-766Q) supplemented with 13.61 mg/l hypoxanthine and 3.88 mg/L thymidine (Biological Industries, Kibbutz Beit Haemek, Israel, cat. #03-085-1C). Two days prior to transfection, 100 ml of the cells were seeded at densities of $0.5 \times 10^6$ (ITL-LF2) and $0.2 \times 10^6$ (CHO-S)

cells/ml in 500-ml Erlenmeyer flasks (Corning, Lowell, MA, USA, cat. #WI-431145). The cells were transfected with 20 μg of the linearized PGL3 anti-EGFR vector, which contains the anti-EGFR mAb coding sequence, pCMV-EGFR_HC-BMP/PAC_LC-GS or pCMV-P-GMD with Lipofectamine (Gibco BRL cat. #18324-020). On the day of transfection, the cells (in replicates) were washed and resuspended, and $10^7$ cells were transfected with Lipofectamine (Invitrogen, Carlsbad, CA, USA, cat. #50470) according to the manufacturer's instructions. The cells were allowed to recover in 8 ml of fresh 50% C6614 and 50% C6366 medium (Sigma, Ayrshire, Scotland, UK, cat. #C6366,) supplemented with 13.6 mg/L hypoxanthine, 3.9 mg/L thymidine (Biological Industries, Kibbutz Beit Haemek, Israel, cat. #03-085-1C) and 10 μg/ml L-fucose (Sigma, Oakville ON, Canada, cat. #F2252) (for ITL-LF2) or ProCHO5 supplemented with 0.1 mg/ml dextran sulfate, 27.22 mg/L hypoxanthine and 7.76 mg/L thymidine (HTx1, Biological Industries, Kibbutz Beit Haemek, Israel, cat. #03-085-1B) (for CHO-S) in 125-ml Erlenmeyer flasks with filter caps (Corning, Oneonta, NY, USA, cat. #431143).

The flasks were incubated at 37 °C with shaking at 50 rpm (ITL-LF2) or 125 rpm (CHO-S) for 72 hours. Then, the cells were collected, centrifuged and resuspended in 10 ml of 50% C6614 + 50% C6366 media (supplemented with 10 mg/ml fucose in the case of pCMV-EGFR_HC-BMP/PAC_LC-GS) and 5-10 μg/ml puromycin (InvivoGen, CAYLA, Toulouse, France, cat. #ant-pr-1) and returned to the original T-25 flask (ITL-LF2) or placed in 20 ml of ProCHO5 medium supplemented with 25 μg/ml puromycin (CHO-S). During with the recovery of the transfected ITL-LF2 cells, C6614 medium + 10 μg/ml fucose was added gradually. When the pools recovered completely, the cells were seeded in fresh C6614 medium without fucose. Then, the cells were transferred to ProCHO5 medium without fucose.

## Production of the Anti-EGFR mAb by ITL-LF2 and CHO-S Cells

Cells were seeded at a density of $0.5\text{-}2x10^6$ cells per 200-600 ml of ProCHO5 containing dextran sulfate in 1- or 2-L Erlenmeyer flasks and cultured for 4 days at 37 °C on a shaker incubator at 320 rpm. The harvested cells were centrifuged and filtered through a 0.22-μm filter, and then a protease inhibitor cocktail (Sigma, Saint Louis, MO, USA, cat. #P8340) was added (1 ml per 1 litter of culture) and the samples were frozen at -80 °C until use.

## Purification of the anti-EGFR mAb

The anti-EGFR antibodies produced from the transfected pools were purified on a Protein A Sepharose-MAb Select Xtra pre-packed column (GE Healthcare, Life Sciences, Uppsala, Sweden, cat. #17-5269-07) (5-ml volume) that was pre-equilibrated with 5-6 column volumes (CV) of 50 mM sodium acetate at pH 6.8, with a flow rate of 2.0 ml/min. The clarified crude harvest (~500 ml) containing product from the transfected pool was loaded onto the column at a flow rate of 2.0 ml/min. The column was washed in two steps (7-9 CV): the first wash used 1.5 M NaCl in 50 mM sodium acetate at pH 6.8, and the second wash used 50 mM sodium acetate pH 6.8 at a flow rate of 2.0 ml/min until the baseline was obtained. The product was eluted in one fraction with 20 mM acetic acid at pH 3.2 with a flow rate of 2.0 ml/min. Chromatography was performed at room temperature and monitored by UV spectroscopy at 280 and 215

nm. The pH was adjusted to 6.0 with 1 M Tris. The antibody was purified using the AKTA Explorer system (Uppsala, Sweden).

The product fraction eluted from the Protein A column was dialyzed twice in SnakeSkin dialysis tubing with 10 kDa MW cut-off pore size (Waltham, MA, USA, cat. #68100) against "formulation buffer" (100 mM NaCl, 100 mM glycine, and 10 mM citrate, pH 5.8) overnight, with a volume ratio of approximately 1:100. After dialysis, the sample was concentrated by ultrafiltration on a 10 kDa MW cut-off membrane (Millipore, Darmstadt, Germany, cat. #UFC901024,) in an Amicon concentrator (EMD Millipore, Billerica, MA, USA). These steps were performed at 2-8 °C.

## Analysis of the Fucose Levels on the Anti-EGFR mAb by Flow Cytometry

Antibody samples (100 µl, 25-37 µg/ml) were added to Protein G magnetic beads (New England Biolabs, Ipswich, MA, USA, cat. S1430S). After a washing step, 0.5 ml of the biotinylated AAL-SA-PE mixture (20 µg/ml biotinylated AAL (Vector, Burlingame, CA, USA, cat. #B-1395), and SA-PE (Biolegend, San Diego, CA, USA cat. # 405203), at 0.2 mcg/ml diluted to 2 mcg/ml in PBS + 0.1% pluronic acid) were added and incubated in 24-well plates (Nunc, Roskide, Denmark, cat. 142475) for 30 min at 37 °C in the dark with shaking at 80 rpm. The mixture was resuspended thoroughly and transferred to 15-ml tubes (Corning, Reynosa Tamps, Mexico, cat. 430052) with 10 ml of PBS + 0.1% pluronic acid. The beads were then washed twice in 10 ml of PBS + 0.1% pluronic acid and centrifuged. The pellet was resuspended in 0.5 ml of PBS + 0.1% pluronic acid, filtered through a FACS tube (Falcon, Canaan, CT, USA, cat. 352235), and analyzed by flow cytometry to determine the fucose levels on the antibodies.

## ELISA for the Anti-EGFR Antibodies

An ELISA was used to determine the concentrations of the antibodies in the test samples. The assay was performed as described below.

Microtiter plates (96-well, Waltham, MA, USA) were coated with 2 mcg/ml of goat anti-human IgG (H+L) (Jackson Immuno Research, West Grove, PA, USA, cat. #109-005-088) in 0.1 M carbonate buffer (100 mM $Na_2CO_3$ and $NaHCO_3$, pH 9.6, Darmstadt, Germany) and incubated overnight at 4 °C. The plates were washed four times with washing buffer (0.05% Tween-20 (Merck, Darmstadt, Germany, cat. #8.17072) in PBS). The plates were then blocked with 200 mcl/well blocking buffer (1% BSA (Bovostar, East Keilor, Victoria, Australia, cat. BSAS.01) in 0.05% PBS-T) for 1 hour at RT. After blocking, the plates were washed four times with washing buffer (0.05% Tween-20 in PBS). The samples, standard curve and control samples were added to the plates (100 mcl/well) and incubated for 1 hour at 37 °C. The plates were washed again four times with washing buffer, and 100 mcl of HRP-conjugated goat anti-human IgG Fab (Jackson Immuno Research, West Grove, PA, USA, cat. #109-036-098) diluted to 8 ng/ml in assay buffer (1% skim milk powder) (Skim milk, Fluka, St. Louis, Missouri, USA, cat. #70166) in PBS) was added and incubated for 1 hour at 37 °C. The plates were washed four times with washing buffer, and 100 mcl of chromogenic substrate solution (TMB, Savyon Diagnostics, Ashdod, Israel, cat. #1928) was added and incubated for approximately 10 minutes at RT; then, the reaction was stopped by adding 100 mcl of 1 N HCl to each well. The absorbance

was measured at A450 nm in an ELISA reader (Sunrise TECAN Austria). Magellan 5 software (Ännedorf, Switzerland) was used to calculate the results from the optical data.

## Analysis of the Fucose Levels on Purified Antibody by Octet

NHS-PEG4-Biotin reagent (Thermo, EZ-Link® NHS-PEG4-Biotin, No-Weigh™ Format, Waltham, MA, USA, cat. #21329) was used to biotinylate AAL according to the manufacturer's instructions. The biotin reagent solution (1 mM in 1x PBS, pH 7.2) was added to 1 ml of lectin (1 mg/ml) (1:5 ratio lectin:biotin). The reaction mixture was incubated for 2 hours on ice, followed by dialysis of the sample against PBS at pH 7.2 in a Slide-A-Lyzer cassette (10 kDa MW cut-off pore size, G2 2 kDa, Thermo, Waltham, MA, USA, cat. # 87718).

The buffers, biotinylated lectin and purified product were prepared at various concentrations and transferred to a 96-well plate (250 mcl/well). All steps were performed at 30°C with shaking at 1,000 rpm. First, 1 mcg/ml of biotinylated AAL (AAL-B) was immobilized onto the streptavidin biosensor tip surface for 40 minutes (the loading step). Then, the tips were washed in diluted kinetics buffer (1x KB) (Fortebio, Menlo Park, CA, USA, Cat:18-1092) for 10 minutes (baseline step). For the association step, the purified assayed protein was bound to the biotinylated lectin for 40 minutes, followed by washing in 1x KB buffer for the dissociation step. The data were processed automatically using Octet QK (Fortebio, Menlo park, CA, USA, Software version 6.3).

## Papain Cleavage of the Antibody and Purification of the Fc Dimer

The antibody that was eluted from the Protein A column was dialyzed twice against buffer (0.1 M Tris-HCl, 4 mM EDTA, pH 7.6) and concentrated to 1 mg/ml by ultrafiltration (10 kDa MW cut-off membrane) in an Amicon concentrator. The anti-EGFR antibody was cleaved into Fab and Fc fractions with 1 mg/ml papain (Sigma, Saint Louis, MO, USA, cat. #P3125) in 0.1 M Tris-HCl at pH 7.6, 4 mM EDTA, 5 mM L-cysteine (Sigma, Saint Louis, MA, USA, cat. #C7352), at an antibody:enzyme ratio of 100:1 (w/w). The antibody was digested for 2 hours at 37 °C, and cleavage was stopped by the addition of maleimide (Sigma, Saint Louis, MO, USA, cat. #12.958-5) (33 mM) and chilling on ice. The Fc dimer fraction was purified from the cleavage mixture by Protein A affinity chromatography, as described above. The purified Fc dimer was dialyzed twice against 1x PBS, pH 7.2, and concentrated.

## Isolation of the Fc Monomer

The Fc dimer fraction was reduced and alkylated under denaturing conditions to produce the Fc monomer. The Fc dimer fraction was diluted to 1 mg/ml with a buffer containing 4 M guanidine-HCl (Sigma, Saint Louis, MO, USA, cat. #G3272,), 50 mM Tris-HCl, pH 8.8); then, dithiothreitol (DTT, 5 mM, Sigma, Saint Louis, MO, USA, cat. #D9779) was added, and the reaction mixture was incubated at 75 °C for 5 minutes. The protein solution was then cooled to room temperature, and a 0.5 M iodoacetamide (Sigma, Saint Louis, MO, USA, cat. #I1149) stock solution was added to a final concentration of 15 mM. Alkylation was performed at room temperature for 40 minutes in the dark. Then, a 0.5 M DTT stock solution was added to obtain a 15 mM concentration to quench the alkylation.

### Analysis of the Glycosylation Profile of the Fc Monomer by Mass Spectrometry (MS)

The Fc monomer from the anti-EGFR mAb was isolated from ITL-LF2 cells as described above and sent to the EMD Serono Research Center for further evaluation of the fucose content and glycan structure by mass spectrometry. One hundred fifty micrograms of Fc were diluted in 97.5 mcl of PBS and digested with 2.5 µl of PNGase F (New England Biolabs, Ipswich, MA, USA, cat. #P0705S) at 37 °C for six hours. The digested fractions were buffer-exchanged with 50 mM ammonium bicarbonate using Viva-spin concentrators and volume-adjusted to 100 mcl of DTT (2.5 µl) was added, and the reaction mixture was incubated at 37 °C for 2 hours. The reduced samples were diluted 5-fold with mobile phase A (see composition below), and 10 µl of the diluted sample was injected into the LC-MS system comprised of a Waters Acquity UPLC (Milford, MA 01757 USA) and a Waters QTOF mass spectrometer (Milford, MA 01757 USA). Intact mass analysis was performed. The Q-TOF mass spectrometer was calibrated prior to data acquisition. The samples were resolved on a Jupiter C4 column at a flow rate of 0.25 ml/minutes. Mobile phase A was 0.1% formic acid and 0.01% TFA in water, and mobile phase B was 0.1% formic acid and 0.0085% TFA in acetonitrile. The percentage of mobile phase B was increased from 10% to 35% in 2 minutes and to 60% in seven minutes.

### Cell Propagation and Productivity in ACFM

Cell cultures were maintained in animal component-free medium (ACFM) as follows: cells were seeded in 50-ml tubes at a density of 0.2 x $10^6$ cells/ml and incubated at 37 °C on an orbital shaker at 320 rpm. Twice a week, the cell number and viability were measured. The culture was passaged by centrifugation at 100 g for 5 minutes at 4 °C, and the cell pellet was then re-suspended in fresh pre-warmed ACFM. The population doubling level (PDL), population n doubling time (PDT) and specific productivity (PCD) were determined.

## Results

### Strategy for the Isolation of Fucose-deficient Cells

The strategy used to isolate cells expressing glycoproteins lacking fucose on their carbohydrate side chains was based on the initial generation of random mutations, followed by the selection of cells with the required fucose-deficient phenotype and characterization of their genotype.

The process, which contained several steps, is depicted in Figure 1A. It was initiated with the generation of mutations by incubating CHO-S cells with the mutagenic agent MTX, followed by the isolation of cells displaying little or no binding of the fucose-specific lectin AAL on cell surface. This was achieved using biotinylated AAL and consecutive sorting with a cell sorter after staining with streptavidin-PE (SA-PE). The fucosylation-deficient cell line that was ultimately isolated was designated "ITL-LF2".

### Fucosylation Levels Decrease over the Course of Mutated Cell Sorting

An analysis of the fucose levels on the cell surface over the course of the MTX-mutated cell sorting steps showed a decrease in the fucose levels on the cell membrane at each step. Three sorting rounds resulted in a homogenous population with minimal fucosylation levels. A fourth sort was applied as an additional polishing step (Figure 1B). CHO-S cells that were not treated with MTX and separated with streptavidin

magnetic beads only presented normal fucose profiles (data not shown), suggesting the importance of the mutagenesis step.

## Genetic Analysis of mRNAs in the ITL-LF2 Cells

The fucosylation pathway is comprised of de novo fucose synthesis from D-glucose and the salvage pathway from L-fucose (Figure 2A).

To investigate the mechanism underlying the absence of fucosylation in ITL-LF2 cells in the absence of exogenous fucose, we analyzed the mRNA sizes and sequences of genes that are involved in these fucosylation pathways in ITL-LF2 cells compared with the parent untreated CHO-S cells.

cDNAs of similar sizes were obtained for the GDP-4-keto-6-deoxy-D-mannose epimerase-reductase (Fx) and alpha-1,6-fucosyltransferase (Fut8) genes in both cells (Figure 2B). Sequencing analysis confirmed that the cDNAs for both genes were identical in the two cell lines (data not shown). The level of GDP-beta-L-fucose pyrophosphorylase (GFPP) was under the limit of detection in this assay (Figure 2B).

For the GMD gene, a band at the expected size was detected in the parent CHO-S cells, and sequencing revealed it to be the expected full-length GMD mRNA. However, in ITL-LF2 cells, smaller sized bands were detected compared to the full-length ORF message (Figure 2B). Sequencing revealed the presence of two splice variants: one (denoted splice variant 1 – SV1) contained a deletion of exons 8 and 9, and the second (splice variant 2 – SV2) contained a deletion in exons 3 and 4 (Figure 2C). In addition, a small, ~250-bp band was observed in both the CHO-S and ITL-LF2 cells.

## ITL-LF2 Cells Regain Fucosylation Activity upon Transfection with the WT GMD Gene

To evaluate whether the lack of the full-length GMD protein was the cause of the low-fucose phenotype, ITL-LF2 cells were transfected with a linear plasmid containing the GMD coding sequence (pCMV-P-GMD), followed by a flow cytometry analysis of the fucosylation levels.

The fucose levels on the surface of ITL-LF2 GMD-transfected cells were compared with those on parent CHO-S cells (positive control) and non-transfected ITL-LF2 cells (negative control) (Figure 2D). The results showed that approximately 45% of the ITL-LF2 GMD-transfected population expressed glycoproteins with fucosylation levels similar to those of parent CHO-S cells. The rest of the population (~55%) exhibited lower fucosylation levels, but they were still higher than those of the non-transfected cells. This sub-population may express sufficient levels of the selection marker PAC after transfection, enabling them to grow under puromycin selection pressure, although the GMD levels in these cells are probably low.

## Expression of an Anti-EGFR mAb in ITL-LF2 and CHO-S Cells

An Anti-EGFR mAb was used as a relevant model glycoprotein to test the fucosylation levels on carbohydrate side chains, particularly on Asn 297, when they are expressed in ITL-LF2 cells. ITL-LF2 and CHO-S cells were transfected with a linear plasmid containing the anti-EGFR mAb sequence encoding both the heavy and light chains.

The expression levels were analyzed by ELISA and were only slightly lower in ITL-LF2 cells compared to the transfected CHO-S cells (data not shown).

## Determination of the Fucose Levels on Anti-EGFR mAb by Flow Cytometry

The purified antibodies were bound to Protein G magnetic beads, followed by staining with a fluorescently labeled fucose-specific AAL. The beads with antibody samples were analyzed by flow cytometry to detect the fucose levels (data not shown). The results showed that low fucose levels were observed on the mAb produced by ITL-LF2 cells compared with the mAb produced by the parent CHO-S cells.

## Determination of the Fucose Levels on the Anti-EGFR Fc Monomer by Octet

The Fc monomer domain of the Anti-EGFR mAb, which contains a carbohydrate chain on Asn 297, was isolated as described in the "Materials and Methods". The fucose levels on the Fc monomer were determined by binding to the biotinylated-AAL lectin, which was previously attached to streptavidin-coated biosensors in the Octet QK system. The results showed that the anti-EGFR Fc monomer produced by the ITL-LF2 cells whereas the Fc monomer produced by CHO-S cells contained fucose (Figure 3A).

## Determination of the Fucose Levels on the Anti-EGFR Fc Monomer by Mass Spectrometry

The anti-EGFR Fc samples from the CHO-S and ITL-LF2 cells were analyzed by mass spectrometry at the EMD Serono Research Center (Billerica, USA) to evaluate the fucosylation levels at the conserved Fc region glycosylation site (Asn 297). The observed glycan structures were mainly biantennary with 0, 1 and 2 galactose residues designated as G0-F, G1-F and G2-F, respectively, as shown in Table 1A. The results showed that the IgG Fc domain of the mAb produced by the wild type CHO-S cells was fully fucosylated, while the Fc domain produced by the ITL-LF2 cells was essentially afucosylated.

## Effects of the Addition of Exogenous Fucose to the Cell Culture Medium on the Fucosylation Levels of the anti-EGFR mAb Produced by the Transfected ITL-LF2 Cells

The anti-EGFR mAb-transfected ITL-LF2 cells were seeded and cultured in ProCHO5 medium in the presence of increasing concentrations of L-fucose. After four days of culture, the fucose levels on the carbohydrate side chain of the Fc domain of the secreted mAb were determined, as described in the “Materials and Methods”. The fucosylation level of the recombinant protein produced in parental CHO-S was determined to be 100%. The results, which are depicted in Figure 3B and Table 1B, showed a positive correlation between the exogenous fucose concentration in the culture medium and the fucose levels on the antibodies produced by these cells. This property of the ITL-LF2 cells enabled us to produce glycoproteins with controlled fucosylation rates, ranging from low, undetectable levels to levels as high as those produced by the CHO-S parent cells.

## Effect of exogenous fucose on fucosylation levels of ITL-LF2 cells:

ITL-LF2 cells were seeded in ProCHO5 medium in the presence of increasing concentrations of L-fucose in the culture medium. FACS analysis results show correlation between the exogenously added fucose concentration and the fucosylation level on the cells’ membrane (Fig. 18).

## Discussion and Conclusions

The novel successful approach described in this paper enabled us to isolate a fucose-deficient ITL-LF2 cell line from CHO-S cells to obtain antibodies with enhanced ADCC. This approach is based on two steps: 1) pretreatment of the parent cells with MTX to cause random mutations and 2) efficient sorting of cells that lack fucose residues on the cell membrane by FACS. The mutations and gene amplifications created by incubating cells in the presence of MTX have previously been described in the literature (Coquelle et al. 1997; Schimke 1988; Singer et al. 2000). Following MTX treatment, the mutagenic reagent was removed, and cells deficient in fucosylation activity were isolated by consecutive cell sorting steps. Sorting of such cells was based on monitoring the fucose levels on the cell membrane. This step was performed either by removing the fucose-positive cells with magnetic beads (data not shown) and/or cell sorting using FACS (Figure 1A) after labeling the cells with AAL, which is a fucose-specific lectin with relatively high affinity and specificity for the □Fuc1-6GlcNA residues present on the IgG Fc glycosylation site. We found that the selected cells that lacked fucose residues on the membrane also retained this property on the recombinant antibodies (anti-EGFR mAb) that were produced and secreted by the cells (Figures 2A, B and Tables 1A, B). The anti-EGFR mAb, which served as a model antibody in this study, contains two N-glycosylation sites: one is on the Fab domain, and the other, which is important for ADCC activity, is on the Fc CH2 domain. The fucose levels on the antibody were determined by Octet and mass spectrometry analyses, which were both performed on the intact mAb and the Fc monomer.

The defucosylated phenotype of the ITL-LF2 cells was stable through 370 PDLs (Table 2), which is expected to be sufficient for full commercial clone development and recombinant protein production. Most other characteristics of the ITL-LF2 cell line were similar to the CHO-S cells, as summarized in Table 2, including growth rate, maximum cell concentration, and sialic acid levels on cell membrane proteins and recombinant antibodies expressed in these cells. In addition, the expression levels of the anti-EGFR mAbs were very similar in both CHO-S and ITL-LF2 (Figure 3B, Table 2).

Despite the stable absence of de novo fucose synthesis in the ITL-LF2 cells, fucosylation on the cell membrane and of secreted glycoproteins was still possible, depending on the addition of exogenous fucose to the cell culture medium (Figure 3B and Table 1B). The analysis of the fucosylation pathways (Figure 2A) (Imai-Nishiya et al. 2007) indicated that both the de novo and salvage pathway branches could lead to the generation of GDP-fucose. The observation that the addition of exogenous fucose resulted in the restoration of fucosylation activity implies that the salvage pathway is active in ITL-LF2 cells (Figure 2A). RT-PCR and sequencing analyses of several key enzymes for the de novo and the salvage pathways showed that the size (Figure 2B) and sequence (data not shown) of the fucosyl transferase 8 (Fut8) mRNA was identical in the ITL-LF2 and CHO-S cells. This finding differentiates ITL-LF2 cells from the cells developed by Kyowa Hakko Kogyo Co., Ltd., in which a deficiency in fucosylation activity was achieved by disrupting both FUT8 alleles through sequential homologous recombination in a Chinese hamster ovary CHO DG44 cell line (Kanda et al. 2005; Yamane-Ohnuki et al. 2004).

Similar to Fut8, the size and sequence of GDP-keto-6-deoxymannose-3,5-epimerase,4-reductase (FX) mRNA (Figure 2B), which is involved in the de novo pathway, were intact in ITL-LF2 cells.

On the other hand, the size of the GDP-mannose-4,6-dehydratase (GMD) mRNA in ITL-LF2 cells was shorter than that in the parent CHO-S cells (Figure 2B). The cDNA sequence analysis revealed that there were two splice variants, lacking either exons 3 and 4 or exons 8 and 9 (Figure 2C). The deletions could not be differentiated on the gel because the sizes of the deletions were similar. However, only the short GMD versions (and not the full-length versions) could be detected in ITL-LF2 cells. Neither of the variants could be detected in untreated CHO-S cells, in which only the full-length mRNA was observed (data not shown), indicating that the two deletion isoforms were generated by the MTX pretreatment.

Another research group used a different phenotype selection approach for the generation of lectin-resistant mutants in CHO cells (Lec13) (Ripka and Stanley 1986). In this approach, the cells were incubated with a toxic pea fucose-specific lectin, and the resistant cells expressed human IgG1 that was deficient in fucose attached to the Asn(297)-linked carbohydrate (Shields et al. 2002). Further work revealed that the fucose levels in these cells increased after a relatively short time, indicating that the low fucose phenotype was not stable, and some active GDP-fucose synthesis protein was synthesized through the de novo pathway (Kanda et al. 2006). In another recent paper, Kanda and co-workers (Kanda et al. 2007) showed that Lec13 cells express a GMD mRNA of the same size as the mRNA expressed in CHO DG44 cells using RT-PCR analysis. However, the ITL-LF2 cells present a stable defucosylation phenotype, likely due to the complete deficiency of the active GMD form (Figure 2A). Genotypic analysis of CHO-SM cells (Kanda et al. 2007), which is another lectin (*Lens culinaris* agglutinin-LCA)-resistant cell line, by RT PCR, sequencing and southern blotting revealed that this cell line expresses a mutated GMD mRNA that lacks exons 5, 6 and 7, which encode domains that are critical for activity (Somoza et al. 2000; Webb et al. 2004). Our results indicate that exons 3 and 4, as well as exons 8 and 9, are also critical for GMD activity, as was demonstrated in ITL-LF2 cells. Therefore, we concluded that the GMD profile of ITL-LF2 cells is different from both Lec13 and CHO-SM cells. Moreover, we showed a linear correlation between the external fucose levels in the cell culture medium and the fucosylation levels on the glycoproteins produced by these cells (Figure 3B). The general approach employed to obtain these cells involved the initial generation of random mutations, followed by the isolation of afucosylated mutants by either magnetic beads and/or flow cytometry. The adjustable fucosylation levels are an advantage over other methods in cases where fucose is needed at a certain level for various cell functions and to maintain normal fucose levels in the same cell line. In principle, this experimental approach can potentially be exploited as a platform for the isolation of cells with different repertoires of desired characteristics, such as various post-translational modifications and growth properties. The approach to select cells with a desired characteristic can be performed with cells that already express a recombinant protein, thus saving the time required for clone development.

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**Fig. 1 Strategy for the isolation of fucose-deficient ITL-LF2 cells and flow cytometry analysis of the sorted ITL-LF2 cells**

CHO-S cells were incubated with the mutagenic agent MTX, followed by four rounds of cell labeling with biotinylated fucose-specific lectin (biotinylated AAL) + fluorescent streptavidin and sorting of the cells exhibiting the lowest fluorescence levels by flow cytometry (A). Distribution profiles of the fucose levels on the cells from the consecutive sorting steps during the process used to isolate fucose-deficient cells (B). The low-fluorescence cells were sorted by FACS and were further propagated in cell culture. This procedure was repeated four times. Samples of cells from each cycle were analyzed by FACS to determine the fucose levels on the cell membrane. The analyses shown here were performed by labeling the cells with biotinylated AAL and fluorescent streptavidin. Unlabeled MTX-treated CHO-S cells (□),MTX-treated CHO-S cells (○),MTX-treated CHO-S cells after the first sort (Δ),MTX-treated CHO-S cells after the second sort (◊),MTX-treated CHO-S cells after the third sort (▼),MTX-treated CHO-S cells after the fourth sort (■).

**Fig. 2 Analyses of the fucosylation pathways**

Potential enzymes involved in glycoprotein fucosylation (A) (Imai-Nishiya et al. 2007). The mRNAs of the enzymes marked in boxes were selected for analysis (rectangles).

mRNA analysis of fucosylation pathway genes (B). Total RNAs were isolated from the parent CHO-S (CH) and ITL-LF2 (LF) cells and subjected to RT-PCR utilizing polydT primers, followed by gene-specific primers. The resulting products were run on an agarose gel and detected under UV light by SyberSafe® staining. Expected sizes: Fut8 – 1.7 Kb; FX – 1.2 Kb; GFPP – 1.7 Kb; GMD – 1.1 Kb; M – DNA size marker.

Comparison of the protein sequences between full-length GMD and the splice variants observed in ITL-LF2 cells (C). cDNAs were prepared by RT-PCR from RNAs that were extracted from CHO-S and ITL-LF2 cells and were run on gels, isolated and sequenced. The DNA sequences were then translated into protein sequences with CloneManager® software. The protein sequences of splice variant 1 (SV1 panel A) and splice variant 2 (SV2 panel B) were compared to the full-length GMD gene. The dashed lines denote areas of deletion. The text indicates the exons that were deleted in each splice variant.

ITL-LF2 cells regain fucosylation activity upon transfection with the wild type GMD cDNA (D).

ITL-LF2 cells were transfected with a vector containing the GMD cDNA (pCMV-P-GMD) in C6614 medium. The transfected cells were transferred to ProCHO5 medium without fucose. The fucose levels on the cell surface were analyzed by FACS after the cells were labeled with the biotinylated fucose-specific lectin AAL and fluorescent streptavidin. Unlabeled CHO-S cells (□), CHO-S cells (○), ITL-LF2 cells (Δ), GMD-transfected ITL-LF2 cells (◊).

**Fig. 3 Fucosylation of a recombinant protein produced in ITL-LF2 cells in the absence and presence of different fucose concentrations**

Analysis of the fucose levels on the purified anti-EGFR Fc monomer produced by ITL-LF2 and CHO-S cells (A). The Fc monomers of the anti-EGFR mAb from CHO-S cells and from ITL-LF2 cells were produced as described in the "Materials and Methods" section; 40 μg/ml of monomer as determined by the O.D. at 280, was bound to biotinylated AAL (1 μg/μl) that was previously attached to streptavidin pre-coated Octet biosensors. The graph represents the association step of the kinetic analysis by the Octet QK system. Each curve represents a specific sample, which is marked by an arrow.

The fucosylation levels on the anti-EGFR mAb produced by the transfected ITL-LF2 cells are dependent on the external fucose concentrations (B). The fucosylation level of the recombinant protein produced in the parental CHO-S cells was determined to be 100%.

The anti-EGFR mAb-transfected ITL-LF2 cells were seeded at a density of $0.2x10^6$ cells/ml in ProCHO5 medium with the indicated concentrations of L-fucose and incubated in a 37 °C incubator with 5% $CO_2$ for 4 days with shaking at 320 rpm. The anti-EGFR mAb was purified from the cells, bound to Protein G-containing beads, and the fucose levels were analyzed by FACS after the cells were labeled with the fluorescently labeled fucose-specific lectin AAL (for details, see the “Materials and Methods” section).

**Table 1: Glycan profiles (*) of the anti-EGFR Fc fractions produced in CHO-S and ITL-LF2 cells in the absence (A) or presence (B) of 3.5 mg/ml fucose in the cell culture medium (**)**

A

| Sample | Glycan Structure | Relative abundance (%) | Fucosylation (%) |
|---|---|---|---|
| **Normal Fucose CHO-S WT** | G0-F | 25.5% | 100 |
| | G1-F | 56.8% | |
| | G2-F | 17.8% | |
| **Low Fucose ITL-LF2** | G0 | 45.9% | 0% |
| | G1 | 49.0% | |
| | G2 | 5.1% | |

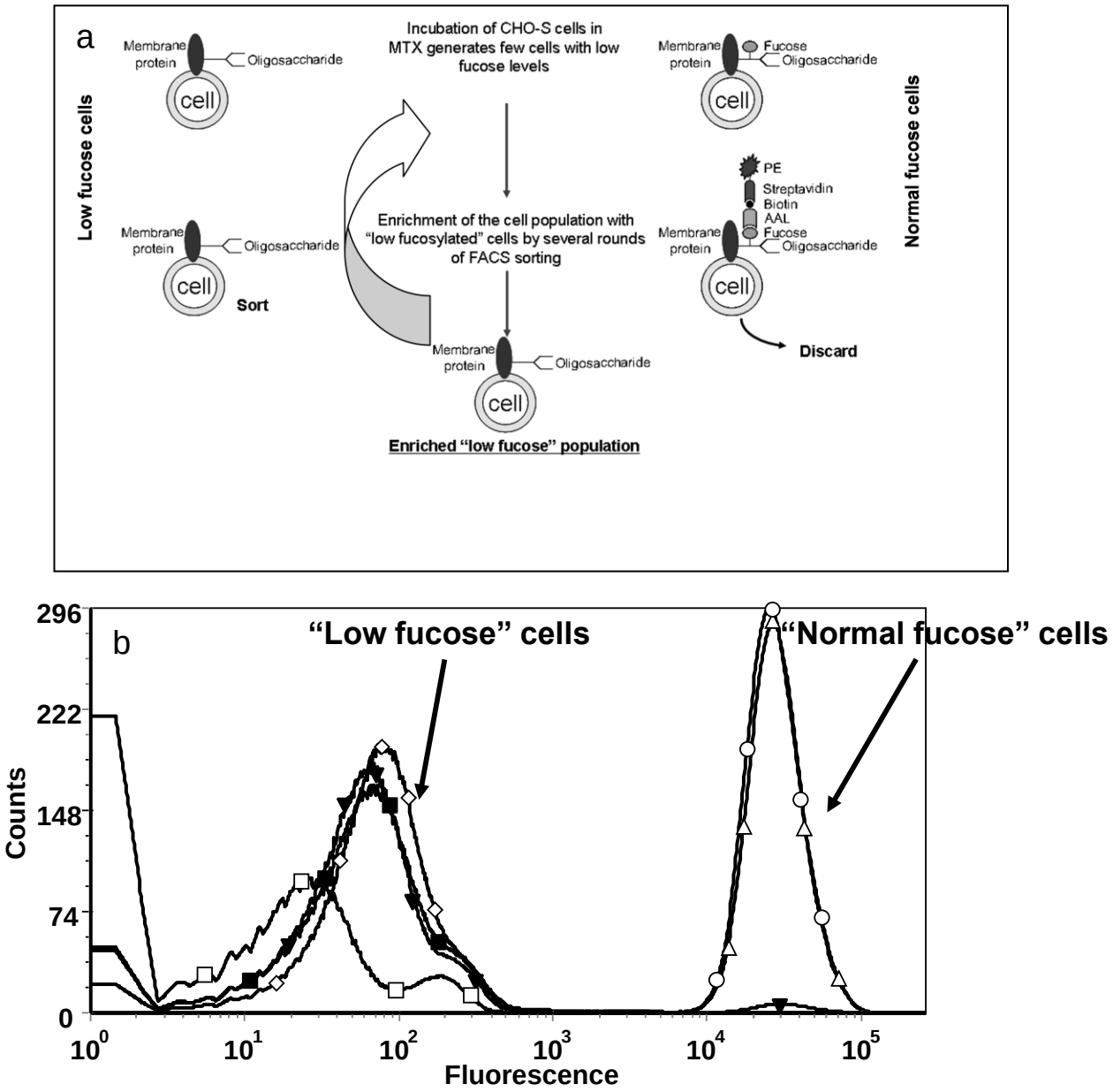
B

| **Possible Glycan Mass** | **1298** | **1444** | **1460** | **1606** | **1623** | **1769** | **1509** | |
|---|---|---|---|---|---|---|---|---|
| **Structure** | **G0 (%)** | **G0f (%)** | **G1 (%)** | **G1f (%)** | **G2 (%)** | **G2f (%)** | **Unknown (%)** | **Total Fucosylation (%)** |
| **Batch ID** | | | | | | | | |
| WT | | 30.10 | | 60.70 | | 9.20 | | 100 |
| 0 fucose | 49.80 | | 40.80 | | 5.10 | | 4.30 | 0 |
| 3.5 mcg/ml (1) | 41.70 | 6.50 | 37.60 | 7.70 | 6.40 | | | 14.20 |
| 3.5 mcg/ml (2) | 37.50 | 7.10 | 38.80 | 9.60 | 7.00 | | | 16.70 |
| 3.5 mcg/ml (3) | 37.20 | 7.10 | 38.70 | 10.70 | 6.30 | | | 17.80 |
| 3.5 mcg/ml (4) | 36.70 | 5.50 | 37.90 | 8.10 | 6.20 | | 5.50 | 13.60 |
| 3.5 mcg/ml (5) | 38.20 | 6.50 | 39.70 | 9.00 | 6.50 | | | 15.50 |

* The carbohydrate structures were analyzed by mass spectrometry, as described in the Materials and Methods.

** ITL-LF2 cells expressing the anti-EGFR mAb were cultured in five batches in parallel for 4 days in the presence of 3.5 mcg/ml of fucose. The anti-EGFR mAb samples were purified from the five

batches and cleaved by papain, followed by reduction and alkylation to isolate the Fc monomer fraction as described in the “Materials and Methods” section. The carbohydrate structures on the Fc monomers were analyzed by mass spectrometry, as described in the “Materials and Methods” section. The observed glycan structures were mainly biantennary with 0, 1 and 2 galactose residues designated as G0, G1 and G2, respectively. G0f, G1f and G2f represent the fucosylated glycan forms, as described in Table 1B. The results show that the addition of 3.5 mcg/ml of fucose to the medium induces an average of 15.6% +/-1.7 fucosylation. These results correlate with the data presented in Figure 7, which show ~15% fucosylation at this fucose concentration in the culture medium. WT CHO-S and ITL-LF2 cells without the addition of fucose served as controls.

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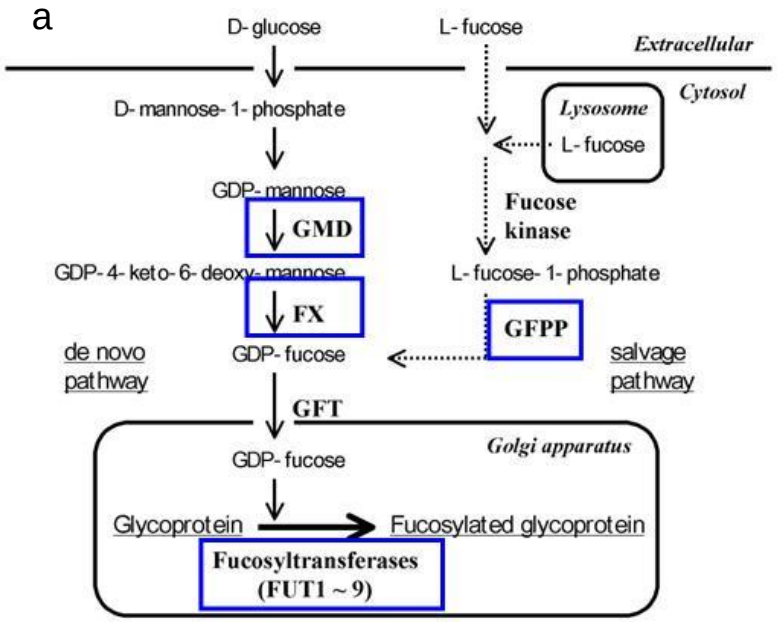
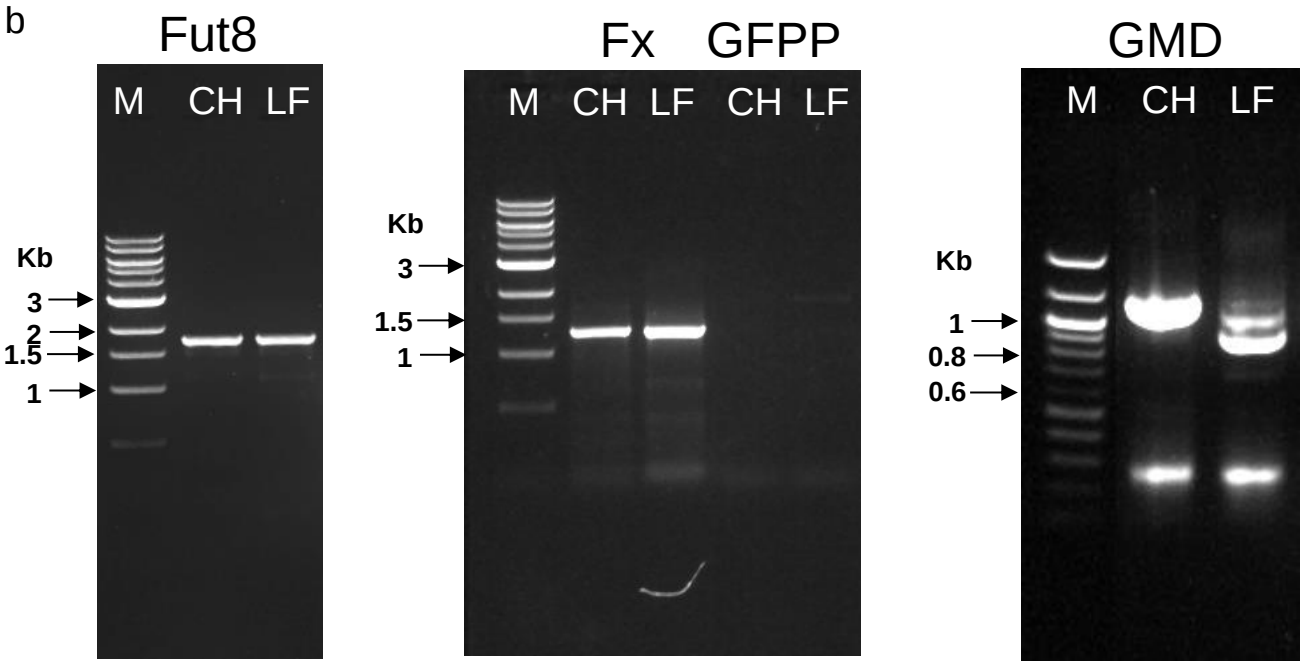
CHO-S cells were incubated with the mutagenic agent MTX, followed by four rounds of cell labeling with biotinylated fucose-specific lectin (biotinylated AAL) + fluorescent streptavidin and sorting of the cells exhibiting the lowest fluorescence levels by flow cytometry (A). Distribution profiles of the fucose levels on the cells from the consecutive sorting steps during the process used to isolate fucose-deficient cells (B). The low-fluorescence cells were sorted by FACS and were further propagated in cell culture. This procedure was repeated four times. Samples of cells from each cycle were analyzed by FACS to determine the fucose levels on the cell membrane. The analyses shown here were performed by labeling the cells with biotinylated AAL and fluorescent streptavidin. Unlabeled MTX-treated CHO-S cells (□),MTX-treated CHO-S cells (○),MTX-treated CHO-S cells after the first sort (∆),MTX-treated CHO-S cells after the second sort (◊),MTX-treated CHO-S cells after the third sort (▼),MTX-treated CHO-S cells after the fourth sort (■).

a


D-glucose
L-fucose
Extracellular
Cytosol
D-mannose-1-phosphate
Lysosome
L-fucose
GDP-mannose
GMD
Fucose kinase
GDP-4-keto-6-deoxy-mannose
L-fucose-1-phosphate
FX
GFPP
de novo pathway
GDP-fucose
salvage pathway
GFT
Golgi apparatus
GDP-fucose
Glycoprotein
Fucosylated glycoprotein
Fucosyltransferases (FUT1 ~ 9)

b


Fut8
M CH LF
Kb
3
2
1.5
1
Fx GFPP
M CH LF CH LF
Kb
3
1.5
1
GMD
M CH LF
Kb
1
0.8
0.6

C

## SV1

```
GMD (Full)        1 mahapascpssrnsgdgdkgkprkvalitgitgqdgsylaefllekgyevhgivrrsssfntgriehlyknpqahiegnm
GMD SV1           1 mahapascpssrnsgdgdkgkprkvalitgitgqdgsylaefllekgyevhgivrrsssfntgriehlyknpqahiegnm

GMD (Full)       81 klhygdltdstclvkiinevkpteiynlgaqshvkisfdlaeytadvdgvgtlrlldaiktcglinsvkfyqastselyg
GMD SV1         241 klhygdltdstclvkiinevkpteiynlgaqshvkisfdlaeytadvdgvgtlrlldaiktcglinsvkfyqastselyg

GMD(Full)       161 kvqeipqkettpfyprspygaaklyaywivvnfreaynlfavngilfnhesprrganfvtrkisrsvakiylgqlecfsl
GMD SV1         481 kvqeipqkettpfyprspygaaklyaywivvnfreaynlfavngilfnhesprrganfvtrkisrsvakiylgqlecfsl

GMD (Full)      241 gnldakrdwghakdyveamwlmlqndepedfviatgevhsvrefveksfmhigktivwegknenevgrcketgkihvtvd
GMD SV1         721 gnldakrdwghakdyve----------------------------------------------------------------
```

***Deletion of Exon 8 and Exon 9 in SV1***

```
GMD (Full)      321 lkyyrptevdflqgdcskaqqklnwkprvafdelvremvqadvelmrtnpna
GMD SV1         772 ---------dflqgdcskaqqklnwkprvafdelvremvqadvelmrtnpna
```

## SV2

```
GMD (Full)        1 mahapascpssrnsgdgdkgkprkvalitgitgqdgsylaefllekgyevhgivrrsssfntgriehlyknpqahiegnm
GMD SV2           1 mahapascpssrnsgdgdkgkprkvalitgitgqdgsylaefllekgye------------------------------
```

***Deletion of Exon 3 and Exon 4 in SV2***

```
GMD (Full)       81 klhygdltdstclvkiinevkpteiynlgaqshvkisfdlaeytadvdgvgtlrlldaiktcglinsvkfyqastselyg
GMD SV2         148 ------------------------------------isfdlaeytadvdgvgtlrlldaiktcglinsvkfyqastselyg

GMD (Full)      161 kvqeipqkettpfyprspygaaklyaywivvnfreaynlfavngilfnhesprrganfvtrkisrsvakiylgqlecfsl
GMD SV2         283 kvqeipqkettpfyprspygaaklyaywivvnfreaynlfavngilfnhesprrganfvtrkisrsvakiylgqlecfsl

GMD (Full)      241 gnldakrdwghakdyveamwlmlqndepedfviatgevhsvrefveksfmhigktivwegknenevgrcketgkihvtvd
GMD SV2         523 gnldakrdwghakdyveamwlmlqndepedfviatgevhsvrefveksfmhigktivwegknenevgrcketgkihvtvd

GMD (Full)      321 lkyyrptevdflqgdcskaqqklnwkprvafdelvremvqadvelmrtnpna
GMD SV2         763 lkyyrptevdflqgdcskaqqklnwkprvafdelvremvqadvelmrtnpna
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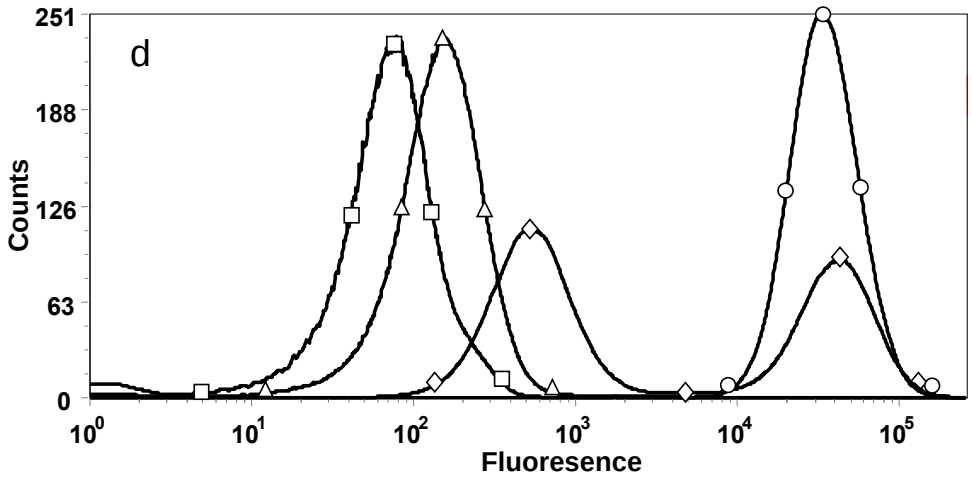



**Figure 2 Analyses of the fucosylation pathways**

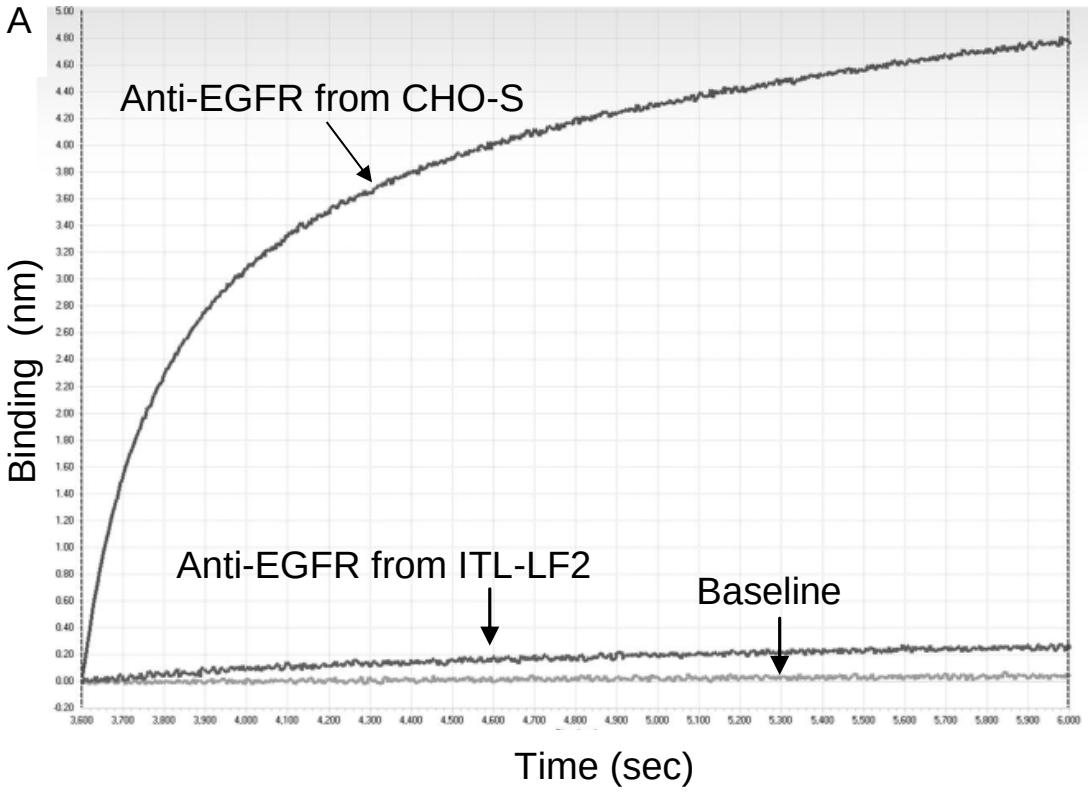
Potential enzymes involved in glycoprotein fucosylation (A). The mRNAs of the enzymes marked in blue boxes were selected for analysis (blue rectangles).

mRNA analysis of fucosylation pathway genes (B). Total RNAs were isolated from the parent CHO-S (CH) and ITL-LF2 (LF) cells and subjected to RT-PCR utilizing polydT primers, followed by gene-specific primers. The resulting products were run on an agarose gel and detected under UV light by SyberSafe® staining. Expected sizes: Fut8 – 1.7 Kb; FX – 1.2 Kb; GFPP – 1.7 Kb; GMD – 1.1 Kb; M – DNA size marker.

Comparison of the protein sequences between full-length GMD and the splice variants observed in ITL-LF2 cells (C). cDNAs were prepared by RT-PCR from RNAs that were extracted from CHO-S and ITL-LF2 cells and were run on gels, isolated and sequenced. The DNA sequences were then translated into protein sequences with CloneManager® software. The protein sequences of splice variant 1 (SV1 panel A) and splice variant 2 (SV2 panel B) were compared to the full-length GMD gene. The dashed lines denote areas of deletion. The text indicates the exons that were deleted in each splice variant.

ITL-LF2 cells regain fucosylation activity upon transfection with the wild type GMD cDNA (D). ITL-LF2 cells were transfected with a vector containing the GMD cDNA (pCMV-P-GMD) in C6614 medium. The transfected cells were transferred to ProCHO5 medium without fucose. The fucose levels on the cell surface were analyzed by FACS after the cells were labeled with the biotinylated fucose-specific lectin AAL and fluorescent streptavidin. Unlabeled CHO-S cells (□), CHO-S cells (○), ITL-LF2 cells (Δ), GMD-transfected ITL-LF2 cells (◊).

A


Anti-EGFR from CHO-S
Anti-EGFR from ITL-LF2
Baseline
Binding (nm)
Time (sec)

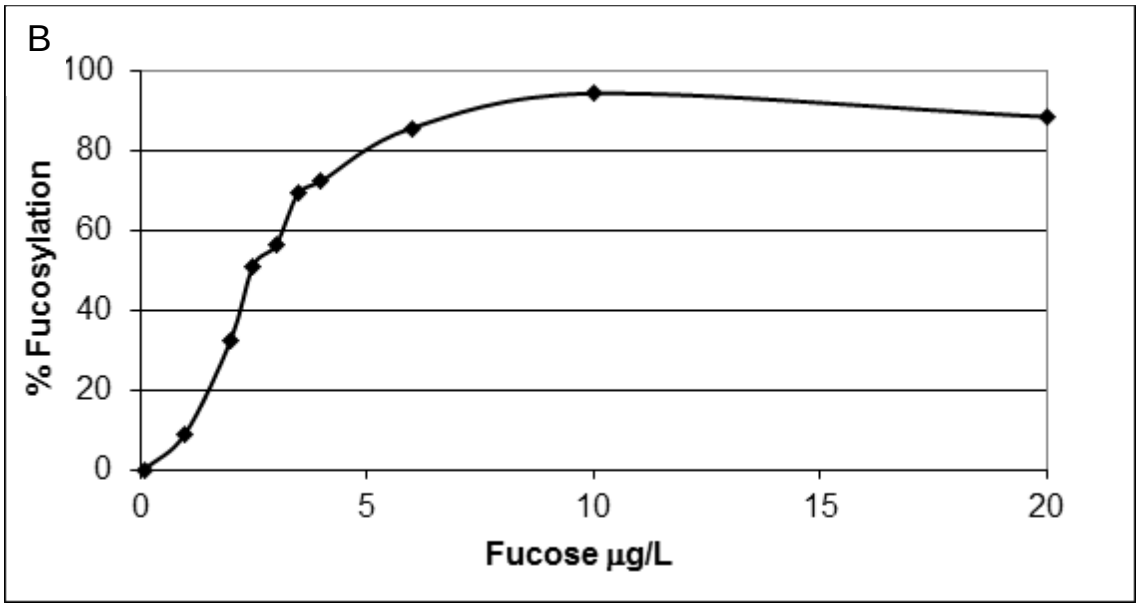



**Figure 3: Fucosylation of a recombinant protein produced in ITL-LF2 cells in the absence and presence of different fucose concentrations.**

Analysis of the fucose levels on the purified anti-EGFR Fc monomer produced by ITL-LF2 and CHO-S cells (A). The Fc monomers of the anti-EGFR mAb from CHO-S cells and from ITL-LF2 cells were produced as described in the "Materials and Methods" section; 40 µg/ml of monomer as determined by the O.D. at 280, was bound to biotinylated AAL (1 µg/ml) that was previously attached to streptavidin pre-coated Octet biosensors. The graph represents the association step of the kinetic analysis by the Octet QK system. Each curve represents a specific sample, which is marked by an arrow.

The fucosylation levels on the anti-EGFR mAb produced by the transfected ITL-LF2 cells are dependent on the external fucose concentrations (B). The fucosylation level of the recombinant protein produced in the parental CHO-S cells was determined to be 100%. The anti-EGFR mAb-transfected ITL-LF2 cells were seeded at a density of $0.2 \times 10^6$ cells/ml in ProCHO5 medium with the indicated concentrations of L-fucose and incubated in a 37 °C incubator with 5% $CO_2$ for 4 days with shaking at 320 rpm. The anti-EGFR mAb was purified from the cells, bound to Protein G-containing beads, and the fucose levels were analyzed by FACS after the cells were labeled with the fluorescently labeled fucose-specific lectin AAL (for details, see the “Materials and Methods” section).

**Table 1: Glycan profiles (*) of the anti-EGFR Fc fractions produced in CHO-S and ITL-LF2 cells in the absence (A) or presence (B) of 3.5 mg/ml fucose in the cell culture medium (**).**

A

| Sample | Glycan Structure | Relative abundance (%) | Fucosylation (%) |
|---|---|---|---|
| **Normal Fucose CHO-S WT** | G0-F | 25.5% | 100 |
| | G1-F | 56.8% | |
| | G2-F | 17.8% | |
| **Low Fucose ITL-LF2** | G0 | 45.9% | 0% |
| | G1 | 49.0% | |
| | G2 | 5.1% | |

B

| **Possible Glycan Mass** | **1298** | **1444** | **1460** | **1606** | **1623** | **1769** | **1509** | |
|---|---|---|---|---|---|---|---|---|
| **Structure** | **G0 (%)** | **G0f (%)** | **G1 (%)** | **G1f (%)** | **G2 (%)** | **G2f (%)** | **Unknown (%)** | **Total Fucosylation (%)** |
| **Batch ID** | | | | | | | | |
| WT | | 30.10 | | 60.70 | | 9.20 | | 100 |
| 0 fucose | 49.80 | | 40.80 | | 5.10 | | 4.30 | 0 |
| 3.5 mcg/ml (1) | 41.70 | 6.50 | 37.60 | 7.70 | 6.40 | | | 14.20 |
| 3.5 mcg/ml (2) | 37.50 | 7.10 | 38.80 | 9.60 | 7.00 | | | 16.70 |
| 3.5 mcg/ml (3) | 37.20 | 7.10 | 38.70 | 10.70 | 6.30 | | | 17.80 |
| 3.5 mcg/ml (4) | 36.70 | 5.50 | 37.90 | 8.10 | 6.20 | | 5.50 | 13.60 |
| 3.5 mcg/ml (5) | 38.20 | 6.50 | 39.70 | 9.00 | 6.50 | | | 15.50 |

* The carbohydrate structures were analyzed by mass spectrometry, as described in the Materials and Methods.

** ITL-LF2 cells expressing the anti-EGFR mAb were cultured in five batches in parallel for 4 days in the presence of 3.5 mcg/ml of fucose. The anti-EGFR

mAb samples were purified from the five batches and cleaved by papain, followed by reduction and alkylation to isolate the Fc monomer fraction as described in the “Materials and Methods” section. The carbohydrate structures on the Fc monomers were analyzed by mass spectrometry, as described in the “Materials and Methods” section. The observed glycan structures were mainly biantennary with 0, 1 and 2 galactose residues designated as G0, G1 and G2, respectively. G0f, G1f and G2f represent the fucosylated glycan forms, as described in Table 1B. The results show that the addition of 3.5 mcg/ml of fucose to the medium induces an average of 15.6% +/-1.7 fucosylation. These results correlate with the data presented in Figure 7, which show ~15% fucosylation at this fucose concentration in the culture medium. WT CHO-S and ITL-LF2 cells without the addition of fucose served as controls.

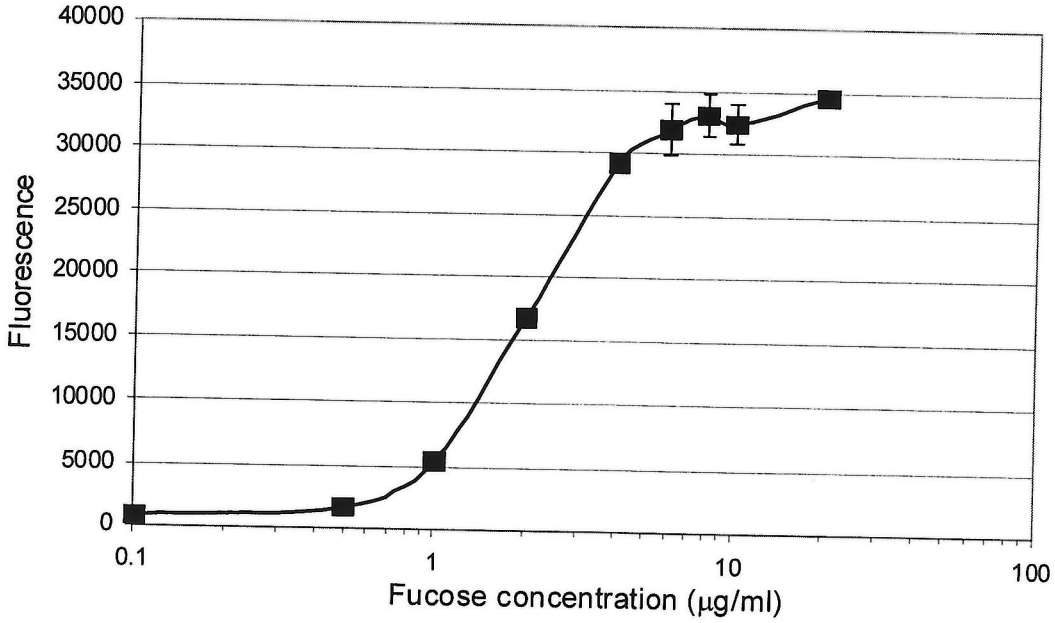
**Table 2: Summary of the characteristics of the ITL-LF2 cells**

| Characteristic | Result |
|---|---|
| Fucose level in the absence of fucose | Below detection level |
| Fucose level in the presence of fucose in the culture medium | Adjustable |
| Transfectability | Similar to CHO-S cells |
| Stability of the low fucose phenotype | At least 370 PDLs* |
| Growth rate (PDT) | 15-20 hours, similar to CHO-S cells** |
| Maximum cell density | $8.4x10^6$ cells, similar to CHO-S cells** |
| Sialylation level | Similar to CHO-S cells** |
| Transient expression | ~42-75% of the expression in CHO-S cells** |
| Genetic modification | Alternative splicing in GMD |
| Stable expression | Similar to CHO-S cells |

PDL – Population doublings

** Data not shown

FIG 18


40000
35000
30000
25000
20000
15000
10000
5000
0
Fluorescence
0.1
1
10
100
Fucose concentration (µg/ml)

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# Establishment of a GDP-mannose 4,6-dehydratase (*GMD*) knockout host cell line: A new strategy for generating completely non-fucosylated recombinant therapeutics

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**Abstract**

Currently, removal of core fucose from the Fc oligosaccharides of therapeutic antibodies is widely recognized as being of great importance for the effector function of antibody-dependent cellular cytotoxicity, and α-1,6-fucosyltransferase (*FUT8*) knockout cells have been generated as an ideal host cell line for manufacturing such therapeutics. Here, we attempted to identify genes other than *FUT8* that could be targeted for the manufacture of non-fucosylated therapeutics. Loss-of-function analyses using siRNAs against three key genes involved in oligosaccharide fucosylation in Chinese hamster ovary (CHO) cells revealed that there was a positive correlation between the Fc oligosaccharide fucosylation and the mRNA expression through the origin in the cases of both GDP-fucose 4,6-dehydratase (*GMD*) and *FUT8*, but not for the GDP-fucose transporter, suggesting that there is no functional redundancy in *GMD* and *FUT8*. *GMD* knockout CHO/DG44 cells were successfully established, and were confirmed to be devoid of intracellular GDP-fucose and to produce completely non-fucosylated antibodies. *GMD* knockout cells recovered their fucosylation capability through the salvage pathway upon addition of L-fucose into the culture medium, and exhibited equable morphology, growth kinetics and recombinant protein productivity, demonstrating that loss of oligosaccharide fucosylation has no impact on these cellular phenotypes. Our results demonstrate that *GMD* knockout is a new strategy applicable to the manufacture of non-fucosylated therapeutic antibodies, and completely *O*-fucose-negative therapeutics as well.


*Keywords:* Antibody-dependent cellular cytotoxicity (ADCC); GDP-fucose synthesis; GDP-mannose 4,6-dehydratase (*GMD*); *GMD* knockout; Non-fucosylated therapeutics production; Oligosaccharide fucosylation pathway

## 1. Introduction

Recombinant protein expression technology in mammalian cell culture is the principal means of commercial production of glycosylated biopharmaceuticals. In fact, all approved recombinant therapeutic antibodies as well as erythropoietin (EPO) have been manufactured in mammalian cells (Wurm, 2004), and comprise the majority of recombinant therapeutics currently used in clinics. The control of the oligosaccharide structures is now widely recognized as being of great importance to the efficacy of such therapeutics; The sialylation of the non-reducing end of EPO oligosaccharides greatly affects the *in vivo* efficacy (Tsuda et al., 1990) and the core fucosylation of the innermost *N*-acetylglucosamine (GlcNAc) of *N*-linked Fc oligosaccharide of therapeutic antibodies is critical to the effector function of antibody-dependent cellular cytotoxicity (ADCC) (Shields et al., 2002; Shinkawa et al., 2003; Satoh et al., 2006). ADCC is now known to be closely related to the clinical efficacy in humans *in vivo*, especially in anti-cancer antibodies such as anti-CD20 IgG1 Rituxan® (rituximab) and anti-HER2 IgG1 Herceptin® (trastuzumab) (Clynes et al., 2000; Cartron et al., 2002; Gennari et al., 2004). One of the most common mammalian host cell lines used for the manufacture of these therapeutics is the Chinese hamster ovary (CHO) cell line. Because the characteristics of CHO cells, which include case of genetic manipulation, high cloning efficiency, good proliferation in large-scale suspension culture, easy adaptability

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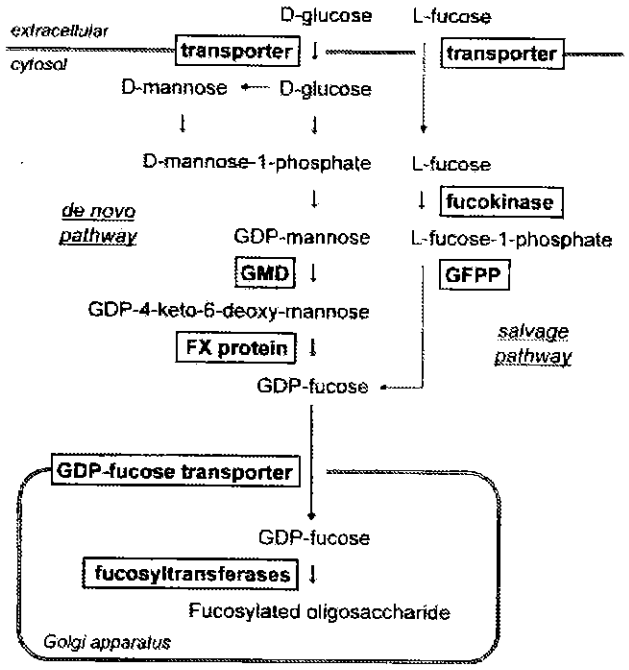


Fig. 1. Oligosaccharide fucosylation and GDP-fucose synthesis in mammalian cells. Oligosaccharide fucosylation is catalyzed by Golgi-localized fucosyltransferases using GDP-fucose as a substrate of fucose donor. GDP-fucose is synthesized through two distinct pathways of *de novo* and salvage pathways.

to serum- and protein-free media, and high productivity and stability, are the characteristics necessary for industrial application, the robust commercial production processes using this cell line have been developed. However, the control of the desired oligosaccharide structures of biopharmaceuticals still remains a challenging issue, and efforts have been made to achieve the control of oligosaccharide fucosylation, including the application of either a variant CHO cell line Lec13 (Shields et al., 2002) or a rat hybridoma cell line YB2/0 (Shinkawa et al., 2003) as host cells, the introduction of a small interfering RNA (siRNA) against the *FUT8* gene (Mori et al., 2004), and co-expression of β-1,4-*N*-acetylglucosaminyltransferase III (*GnT-III*) and Golgi α-mannosidase II (*ManII*) (Ferrara et al., 2006). Among these efforts in mammalian systems, α-1,6-fucosyltransferase (*FUT8*) knockout is currently the only strategy for manufacturing completely non-fucosylated recombinant therapeutics (Yamane-Ohnuki et al., 2004; Kanda et al., 2006a).

In mammalian cells, GDP-fucose, an essential substrate for oligosaccharide fucosylation, is synthesized in the cytoplasm through both the *de novo* and salvage pathways shown in Fig. 1. Most of the intracellular GDP-fucose is derived from D-glucose that enters the cytoplasm via the *de novo* pathway, and free L-fucose from extracellular or lysosomal sources is also reutilized via the salvage pathway (Becker and Lowe, 2003). In the *de novo* pathway, GDP-mannose originating from the D-glucose taken into the cytoplasm is converted to GDP-fucose via three enzymatic reactions catalyzed by two proteins, i.e., GDP-mannose 4,6-dehydratase (GMD) and GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase (FX protein) (Tonetti et al., 1996). In the salvage pathway, free L-fucose is phosphorylated by fucokinase and then converted to GDP-fucose by GDP-fucose pyrophosphorylase (GFPP) (Pastuszak et al., 1998). The import of intracellular GDP-fucose from the cytosol to the Golgi-lumen is mediated by a GDP-fucose transporter (GFT) anchored at the Golgi membrane (Handford et al., 2006). The concentrated GDP-fucose in the Golgi-lumen is finally transported to oligosaccharides of glycoproteins by Golgi-localized fucosyltransferases such as FUT8 (Hirschberg, 2001). Thus, it appears that there may be key enzymes other than *FUT8* which would enable us to control the oligosaccharide fucosylation of biopharmaceutical products. In this study, among the genes involved in the oligosaccharide fucosylation pathway, we attempted to identify target genes industrially applicable to the manufacture of completely non-fucosylated therapeutics. As a result, we established *GMD* knockout CHO cells as ideal host cells for such manufacture.

## 2. Materials and methods

### 2.1. Cell lines

The dihydrofolate reductase-deficient CHO cell line, CHO/DG44 (Urlaub and Chasin, 1980), was obtained from Dr. Lawrence Chasin, Columbia University, NY. The variant CHO cell line deficient in endogenous *GMD*, Pro-Lec13.6A (Lec13) (Ripka et al., 1986), was provided by Dr. Pamela Stanley, Albert Einstein College of Medicine, Yeshiva University, NY. The *FUT8* knockout CHO cell line, Ms704 (Yamane-Ohnuki et al., 2004), was established in our laboratory. All the CHO cell lines were cultured in IMDM medium (Invitrogen, Carlsbad, CA) containing 10% (v/v) dialyzed fetal bovine serum (dFBS; Invitrogen), 0.1 mM hypoxanthine and 16 μM thymidine using a tissue culture flask (Greiner, Frickenhausen, Germany). Recombinant mouse/human chimeric IgG1-producing clones, Ms704 1A7-15, CHO/DG44 32-05-12, and 1H5, generated as described previously (Mori et al., 2004; Kanda et al., 2006a), were cultured in IMDM medium containing 10% (v/v) dFBS and 500 nM methotrexate (MTX; Sigma–Aldrich, St. Louis, MO).

### 2.2. Construction of siRNA expression plasmids

As a *FUT8* siRNA expression plasmid, U6_FUT8_R_puro (Mori et al., 2004), comprising a puromycin resistance gene as a selection marker and a *FUT8* siRNA tandem expression cassette controlled by the U6 promoter, was employed. The siRNA expression plasmid against *GMD*, U6_GMD_B_puro, was constructed by replacing the sense and antisense sequences of U6_FUT8_R_puro with synthetic double-strand DNAs encoding the *GMD* sense sequence with transcriptional termination (T5) (5′-CGTATAAGAA TCCACAGGCT CATATTGAAG GCTTTTTG-3′) and the *GMD* antisense sequence with T5 (5′-CGCCTTCAAT ATGAGCCTGT GGATTCTTAT ACTTTTTT-3′), respectively. Likewise, the siRNA expression plasmid against *GFT*, U6_GFT_H_puro, was constructed by replacing the sense and antisense sequences with synthetic double-

strand DNAs encoding the *GFT* sense sequence with T5 (5′-CGCGCCTAAC CTTCTATAAC TTTTTG-3′) and the *GFT* antisense sequence with T5 (5′-CGTTATAGAA GGTTAG-GCGC TTTTTT-3′), respectively.

### 2.3. RT-PCR analyses for GMD

Total RNA was isolated from $1.0 \times 10^6$ cells using an RNeasy minikit (Qiagen, Hilden, Germany) and incubated for 1 h at 37 °C with 20 units of RNase-free RQI DNase (Promega, Madison, WI) to degrade genomic DNA. Single-strand cDNA was synthesized from 3 μg total RNA using a Superscript™ first strand cDNA synthesis system for RT-PCR (Invitrogen). A 50-fold diluted reaction mixture was used as a template for *GMD* RT-PCR analyses using the following two sets of primers: one set of 5′-AGGAAGGTGG CGCTCATCAC GGGC-3′ and 5′-TAAGGCCACA AGTCTTAATT GCATCC-3′ for a partial sequence of *GMD* cDNA and another set of 5′-ATGGCTCACG CTCCCGCTAG CTGC-3′ and 5′-TCAGGCGTTG GGGTTG-GTTC TCATG-3′ for the full length sequence of *GMD* cDNA. PCR was carried out by heating at 94 °C for 2 min followed by 35 cycles of 98 °C for 15 s, 60 °C for 30 s, and 74 °C for 1 min in 50 μl of reaction mixture containing 1 μl of the diluted single-strand cDNA, 15 pmol of primers, 10 nmol of dNTP mixture, 50 nmol of $MgSO_4$, and 1 μl of KOD-plus DNA polymerase (TOYOBO, Osaka, Japan) using a GeneAmp PCR System 9700 (Perkin-Elmer, Wellesley, MA). RT-PCR products were analyzed by 0.9% (w/v) agarose gel electrophoresis and stained with SYBR Green I (Molecular Probes, Inc., Eugene, OR), or cloned into pCR-Blunt II TOPO (Invitrogen) for sequence analysis using an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA).

### 2.4. Real-time PCR analyses for GMD, FUT8 and GFT

In order to quantify the amounts of *GMD*, *FUT8* and *GFT* mRNA in cells, real-time PCR analyses were employed using a SYBR *Premix Ex Taq*™ kit (TaKaRa, Shiga, Japan) and the following four sets of primers: 5′-GTCCATGGTG ATCCT-GCAGT GTGG-3′ and 5′-CACCAATGAT ATCTCCAGGT TCC-3′ for *FUT8*, 5′- AGGAAGGTGG CGCTCATCAC GGGC -3′ and 5′- TAAGGCCACA AGTCTTAATT GCATCC-3′ for *GMD*, 5′-TCTGGCGCCT GACTTTCTAT AACAA-3′ and 5′-ACCATCATGT TGCTCGTCCA CCAGA-3′ for *GFT*, and 5′-GATATCTGCT GCGCTCGTCG TCGAC-3′ and 5′-CAGGAAGGAA GGCTGGAAGA GAGC-3′ for β-actin as an internal standard gene. PCR was carried out by heating at 95 °C for 10 min followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s in 20 μl of reaction mixture containing 10 μl of SYBR *Premix Ex Taq*™ (TaKaRa), 2 μl of the diluted single-strand cDNA, and 4 pmol of primers using an ABI PRISM 7700 sequence detection system (Applied Biosystems) according to the manufacturer's instructions. After PCR amplification, data acquisition and analyses were performed using the GeneAmp 7700 sequence detection system version 1.7 (Applied Biosystems).

### 2.5. Measurement of intracellular GDP-fucose concentration

Cells ($1.0 \times 10^8$) were harvested and washed three times in cold Dulbecco's phosphate buffered saline (DPBS; Invitrogen), then resuspended in 1.0 ml of cold DPBS and frozen at −80 °C overnight. After thawing, the supernatant was recovered by centrifugation at $10{,}000 \times g$ for 1 h at 4 °C. The amount of GDP-fucose in the supernatant was measured using high-performance liquid chromatography (HPLC). Samples were injected onto an anion-exchange column Partisil 10SAX (Sumitomo, Osaka, Japan) with 0.1 M $KH_2PO_4$ (pH 3.2) as a mobile phase at a flow rate of 1 ml $min^{-1}$. The eluate was monitored with a UV detector at 254 nm. The standard curve was made by using a serial dilution of chemically synthesized GDP-fucose reagent (Sigma–Aldrich).

### 2.6. Analyses of antibody-derived N-linked Fc oligosaccharides

Recombinant antibodies were purified from the serum-free culture supernatant by Protein A-affinity chromatography using MabSelect™ (Amersham Biosciences) and stored in 10 mM citrate/0.15 M NaCl (pH 6.0). The concentration of the purified antibodies was measured by absorbance at 280 nm. The monosaccharide composition of each purified IgG1 was characterized by modified high-performance anion exchange chromatography (HPAEC) as previously described (Shinkawa et al., 2003). The oligosaccharide profile of each purified antibody was characterized by modified matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in a positive ion mode as described previously (Kanda et al., 2006b).

### 2.7. Biological activity analyses of antibodies

An ADCC assay for each anti-CD20 IgG1 ($10^{-5}$ to 10 μg $ml^{-1}$ in 10-fold dilutions, $n = 3$) was performed using Raji cells (American Type Culture Collection, Manassas, VA) as target cells and human peripheral blood mononuclear cells (PBMC) from healthy donors as effector cells at an E:T ratio of 20:1 as described previously (Shinkawa et al., 2003). The antigen-binding activity of the antibodies was measured by CD20-binding ELISA (Yamane-Ohnuki et al., 2004). Anti-human CD20 mouse/human chimeric IgG1 rituximab purchased from Genentech, Inc. (South San Francisco, CA)/IDEC Pharmaceuticals (San Diego, CA) was employed as a control.

### 2.8. Isolation of LCA-resistant clones transformed by siRNA expression vectors

An IgG1 antibody-producing clone CHO/DG44 32-05-12 ($1.6 \times 10^6$ cells) was transformed by electroporation with 10 μg of each siRNA expression vector linearized at the *Fsp* I site. Transfectants were selected in 12 μg $ml^{-1}$ puromycin (Sigma–Aldrich) for 6 days, and then the drug-

resistant clones were subjected to 7-day selection with 500 μg ml$^{-1}$ *Lens culinaris* agglutinin (LCA; Vector Laboratories, Burlingame, CA). The resultant LCA-resistant clones was isolated and expanded in IMDM medium containing 10% (v/v) dFBS, 500 nM MTX, and 12 μg ml$^{-1}$ puromycin for further real-time PCR and antibody oligosaccharide analyses.

### 2.9. Establishment of GMD knockout CHO/DG44 cells

CHO/DG44 cells ($8 \times 10^4$ cells ml$^{-1}$) were suspended in IMDM medium containing 5% (v/v) dFBS, 0.1 mM hypoxanthine, 16 μM thymidine and 300 μg ml$^{-1}$ LCA, and inoculated onto 96-well tissue culture plates (Greiner). The plates were incubated at 37 °C under 5% $CO_2$ (v/v) for 17 days, during which time the medium was exchanged for fresh medium whenever it turned yellow. The surviving LCA-resistant clones were isolated and expanded in IMDM medium containing 5% (v/v) dFBS, 0.1 mM hypoxanthine and 16 μM thymidine. Among these clones, those showing an LCA reactivity equivalent to that of the *FUT8* knockout CHO/DG44 cell line Ms704 were selected using LCA-FACS staining as described previously (Kanda et al., 2006a). Cells stained with fluorescein isothiocyanate (FITC)-labeled LCA (Vector Laboratories) were analyzed using FACSCalibur (BD Biosciences, San Jose, CA). In selected clones, *GMD* knockout was confirmed by further analyses of RT-PCR and Southern blot for *GMD*.

### 2.10. Establishment of GMD knockout CHO/DG44 antibody-producing cells

Twenty micrograms of the recombinant mouse/human chimeric IgG1 antibody expression vector of either pKANTEX2B8P (Shinkawa et al., 2003) or pKANTEX2160 (Niwa et al., 2004) was transfected into *GMD* knockout CHO/DG44 cells ($3.2 \times 10^6$) via electroporation, and the transfectants were selected on the basis of antibody production with stepwise gene amplification in medium containing MTX at 0, 50, 200, or 500 nM. To analyze the produced antibodies, the serum-free culture supernatants of the 500 nM MTX-resistant transformants were recovered after 7-day cultures of the confluent cells with serum-free medium EX-CELL™ 301 (JRH Biosciences, Lenexa, KS). Single cell cloning by limiting dilution was performed to establish high producers, and then adapted to serum-free medium EX-CELL™ 302 (JRH Biosciences) supplemented with 6 mM L-glutamine and MTX. One cell line transformed by pKANTEX2160 with high productivity was selected, and designated as CHO SM 3G1.

### 2.11. Serum-free fed-batch culture of GMD knockout CHO/DG44 antibody-producing cells

Fed-batch cultures were carried out at 35 °C under 5% $CO_2$ (v/v) in 250 ml Erlenmeyer Flasks (Corning, NY) with 100 rpm agitation for 13 days without pH and dissolved oxygen (DO) controls. Cells were inoculated at $3.0 \times 10^5$ cells ml$^{-1}$ in serum-free medium EX-CELL™ 302 (JRH Biosciences) supplemented with 6 mM L-glutamine, and fed on days 3, 6, 9 and 11 with serum-free IMDM-based feeding medium containing 5.3 μM human recombinant insulin (Invitrogen) to maintain a glucose content of approximately 5.0 g l$^{-1}$. Culture aliquots were drawn on days 3, 6, and 9 prior to adding the feeding medium and on 11 and 13 days. Viable cell density and cell viability were measured with a Vi-CELL™ XR automatic cell counter (Beckman Coulter, Fullerton, CA) using trypan blue exclusion. The antibody concentration in the culture supernatant was measured by an enzyme-linked immunosorbent assay (ELISA) specific for human IgG1 as previously described (Nakamura et al., 2000).

## 3. Results

### 3.1. Correlation of fucosylation of the produced antibodies with expressions of FUT8, GMD and GFT in the producing cells

Three constitutive siRNA expression vectors, specifically against CHO *FUT8*, *GMD*, and *GFT*, were generated and introduced into IgG1 antibody-producing CHO/DG44 cells to evaluate the contribution of each of the target genes to fucosylation of the products. Puromycin-resistant clones appeared 6 days after transfection with a transformation efficiency of approximately 900 per $1.6 \times 10^6$ electroporated cells. There was no significant difference in transformation efficiency among the vectors. Subsequent selection of LCA-resistant clones, however, yielded clear differences in the survival colony appearance frequency. The ratios of LCA-resistant clones to puromycin-resistant clones transformed by the three siRNA expression vectors against *FUT8*, *GMD*, and *GFT* were 1.0%, 1.2%, and 0.2%, and thus yielded 9, 10, and 2 independent clones, respectively. The resultant LCA-resistant clones were expanded in the medium in the absence of LCA, and the oligosaccharide structure of the produced IgG1s and the mRNA expression of *FUT8*, *GMD*, and *GFT* in these clones were quantitatively analyzed. The results showed that there was a positive correlation between the Fc oligosaccharide fucosylation and the *FUT8* mRNA expression, i.e., the fucosylation level was linearly proportional to the mRNA expression of *FUT8* roughly through the origin (0, 0) to the 100% point (100, 100) (Fig. 2A), which was consistent with the previous result (Mori et al., 2004). A similar positive correlation of the Fc oligosaccharide fucosylation with the *GMD* mRNA expression was observed, though the regression line did not pass through the 100% point (Fig. 2B). More strikingly, in the LCA-resistant clones from the *GFT* siRNA-introduced transformants, there was only a 20–40% reduction of the Fc oligosaccharide fucosylation, although a greater than 90% reduction of the *GFT* mRNA expression was observed in these clones (Fig. 2C). Indeed, the clone in which the *GFT* mRNA was almost completely knocked down (98%) still produced IgG1s with highly fucosylated (60%) Fc oligosaccharides.

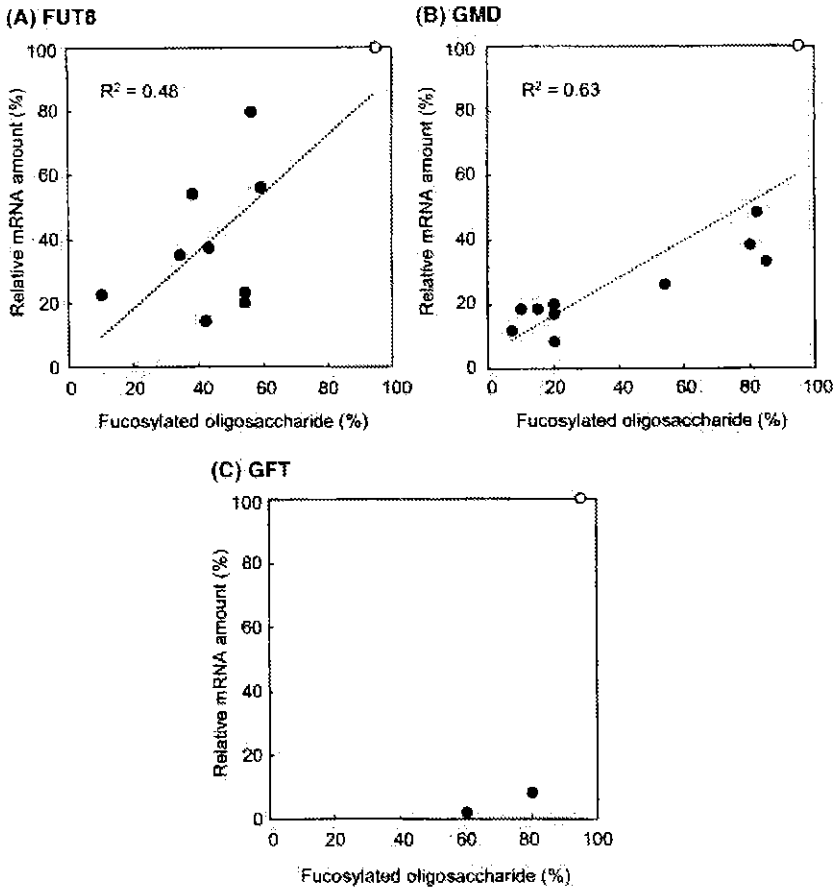


Fig. 2. The expression of *FUT8*, *GMD* and *GFT* in the siRNA-introduced antibody-producing cells. The relative mRNA amount of *FUT8* (A), *GMD* (B), and *GFT* (C) was quantitated in the siRNA-introduced antibody-producing cells (closed circles) to the amount in the parental CHO/DG44-derived clone 32-05-12 (open circles). The plot of the relative mRNA amount against the oligosaccharide fucosylation level of the products was analyzed for conducting simple linear regression analysis in *FUT8* and *GMD*.

### 3.2. Establishment of GMD knockout CHO/DG44 cells

LCA-resistant clones appeared from CHO/DG44 cells after cultivation in the medium containing 300 $\mu g\,ml^{-1}$ LCA with a colony appearance frequency of $10^{-6}$. Approximately $1.0 \times 10^8$ cells were subjected to the selection, and the LCA reactivity of each resultant LCA-resistant clone was analyzed by LCA-staining using flow cytometry. The LCA reactivity of almost all clones was lower than that of the parent CHO/DG44 and higher than that of *FUT8* knockout CHO/DG44 Ms704, which was similar to the LCA-staining pattern of a CHO cell line Lec13 partially deficient in *GMD* (Fig. 3C). Among all the clones, one LCA-resistant clone, designated as CHO SM, showed a very low reactivity to LCA similar to that shown by Ms704 (Fig. 3B). However, CHO SM was distinguished from Ms704 by its unique ability to restore LCA reactivity when cultured in the medium containing 10 mM L-fucose (Fig. 3B). The intracellular GDP-fucose concentration of CHO SM was measured and compared with those of other CHO cell lines, including the parent CHO/DG44, Lec13, and Ms704 in the presence and absence of L-fucose (Fig. 3E). In the case of culturing in the medium without L-fucose, CHO/DG44 and Ms704 contained 3.5 and 3.3 nmol $10^8$ cells$^{-1}$ of intracellular GDP-fucose, respectively. These amounts were comparable with the intracellular GDP-fucose content in HeLa S3 cells previously reported (Yurchenco and Atkinson, 1975). Lec13 had one tenth of the amount of intracellular GDP-fucose present in CHO/DG44, and there was no detectable intracellular GDP-fucose in CHO SM. On the other hand, the intracellular GDP-fucose content was increased to 35–40 nmol $10^8$ cells$^{-1}$ irrespective of the cell lines when cultured in the presence of L-fucose.

Southern blot analysis of CHO SM using the 193-nt sequence of the CHO *GMD* genome region from A346 to G538 of CHO *GMD* cDNA (GenBank accession no. **AF525364**) as a probe revealed that the band corresponding to the *GMD* allele-specific genome fragment had disappeared, though the band corresponding to the *FUT8* allele-specific genome fragment was detected when employed the 156-nt sequence of CHO *FUT8* exon 2 (Yamane-Ohnuki et al., 2004) as a probe (data not shown). RT-PCR analyses also confirmed that CHO SM expressed a short mutant *GMD* mRNA instead of the full-length wild-type (Fig. 4A and B). RT-PCR was performed three times independently to clone and sequence the mutant PCR-amplified products. Sequence analysis revealed that the mutant product consisted of a 693 bp *GMD* cDNA sequence lacking a 426 bp fragment from A346 to G771, which corresponded to the mouse/human *GMD* exon 5, 6, and 7 regions, encoding three functionally critical domains

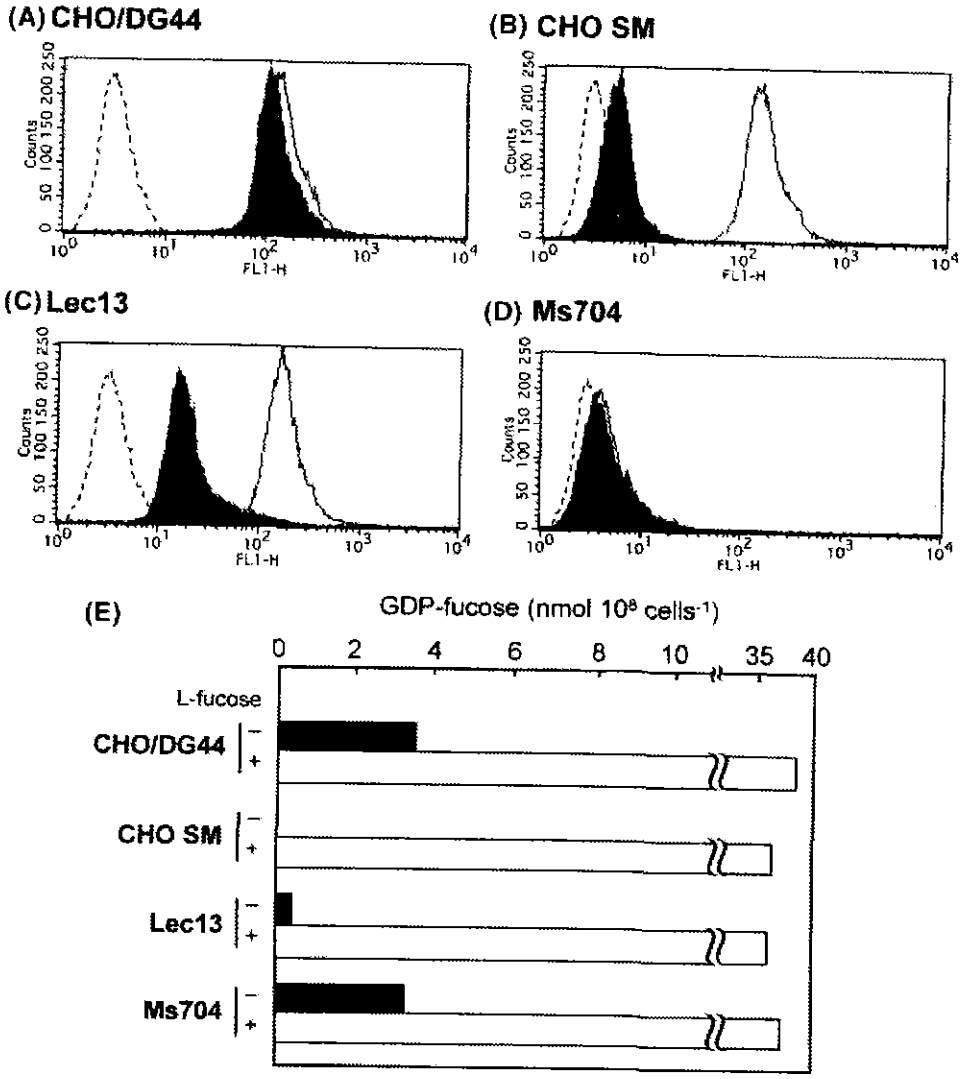


Fig. 3. LCA-binding of Chinese hamster ovary host cell lines. Cells, CHO/DG44 (A), CHO SM (B), Lec13 (C), and Ms704 (D), were harvested after 4-day culture in IMDM media containing dFBS and stained with FITC-labeled LCA (solid line) or FITC-labeled streptavidin (dashed line) as a negative control, and analyzed by flow cytometry. Cells were cultured in the presence (open pattern) or absence (filled pattern) of 10 mM L-fucose. The intracellular GDP-fucose concentrations (E) in the same cultures were measured using the HPLC system described in Section 2.

for GMD enzyme activity, i.e., the dimerization domain, reaction center, and substrate-binding domain (Fig. 4C), thus demonstrating that the CHO SM cells were *GMD* knockout cells.

### 3.3. Characterization of recombinant antibodies produced by GMD knockout CHO cells

To evaluate the characteristics of antibodies produced by *GMD* knockout cells, the recombinant anti-human CD20 mouse/human IgG1 rituximab was employed as a model and purified from the culture supernatant of 500 nM MTX-resistant CHO SM cells transformed by the expression vector for rituximab. The *N*-linked Fc oligosaccharides of the CHO SM-produced antibodies were of biantennary complex type, and the core fucosylation was strictly dependent on L-fucose feeding in the culture medium (Table 1). The oligosaccharide profile of the CHO SM-produced antibodies in the absence of L-fucose was equivalent to that of *FUT8* knockout CHO/DG44 Ms704, and the profile changed dramatically to that of parental CHO/DG44 in the presence of L-fucose (Table 1). Although there was no change in the antigen-binding activities among these purified anti-human CD20 IgG1s, the antibodies produced by culturing of Ms704 and CHO SM in the medium without L-fucose showed two orders of magnitude higher ADCC than the antibodies from CHO/DG44 (Fig. 5). The anti-human CD20 IgG1s purified from culturing of CHO/DG44 and CHO SM with L-fucose showed an ADCC equivalent to that of a control ritiximab currently on the market.

### 3.4. Serum-free fed-batch culture of antibody-producing GMD knockout CHO cells

Serum-free fed-batch cultures of *GMD* knockout and parent CHO/DG44 cells were performed using 250 ml Erlenmeyer flasks without pH and DO controls. CHO/DG44 32-05-12 (Mori

Table 1
Oligosaccharide analysis of anti-human CD20 mouse/human chimeric IgG1 produced by *GMD* knockout CHO/DG44 cells

| Cell line | Additive[b] | Relative composition of oligosaccharides[a] (%) | | | | | | Fucose[d] (−)% |
|---|---|---|---|---|---|---|---|---|
| | | F0G0[c] | F0G1 | F0G2 | F1G0 | F1G1 | F1G2 | |
| CHO/DG44 1H5 | – | n.d. | 3.6 | 2.6 | 44.9 | 42.3 | 6.6 | 6 |
| Ms704 1A7-15 | – | 63.7 | 32.7 | 3.6 | n.d. | n.d. | n.d. | 100 |
| CHO SM | – | 66.7 | 29.6 | 3.7 | n.d. | n.d. | n.d. | 100 |
| CHO SM | LF | n.d. | n.d. | n.d. | 63.7 | 33.3 | 3.0 | 0 |

n.d., not detected.
[a] Each value of composition is the relative amount to total oligosaccharide detected.
[b] LF, 10 mM L-fucose.
[c] Schematic oligosaccharides corresponding to each oligosaccharide are described. Galactose (open diamonds), GlcNAc (closed circles), mannose (open squares), and fucose (open triangles).
[d] Total percentage of fucose-negative oligosaccharides was measured by monosaccharide composition analysis.

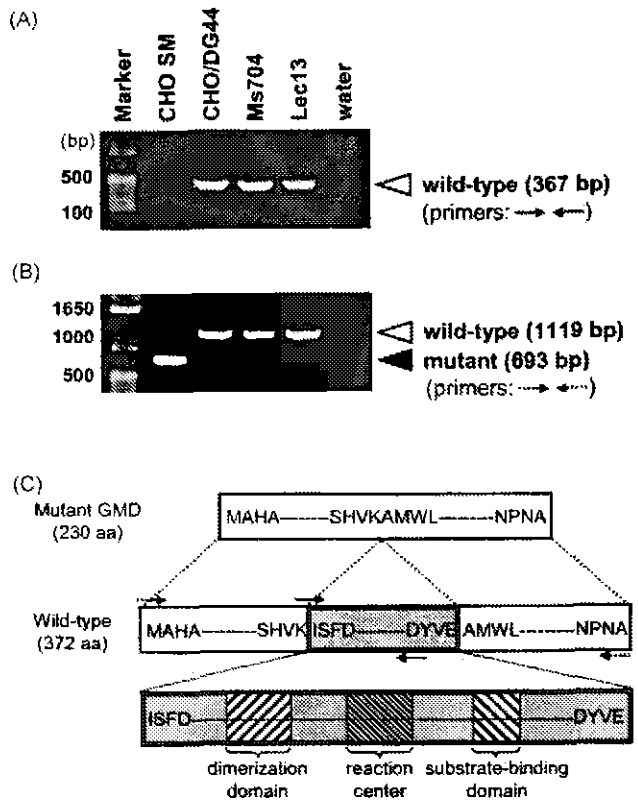


Fig. 4. Analyses of *GMD* cDNA. RT-PCR analyses were carried out using two sets of the indicated primers (arrows in C) for CHO *GMD* in order to amplify a partial sequence of the wild-type cDNA (A) and the full length sequence of GMD cDNA (B). The schematic polypeptide structure encoded by the mutant *GMD* cDNA is aligned with the wild-type polypeptide (C).

et al., 2004) was employed as a recombinant mouse/human chimeric IgG1 antibody-producing CHO/DG44 cell line, and CHO SM 3G1 was generated using the same antibody expression vector as used to generate CHO/DG44 32-05-12. The fed-batch cultures of CHO SM 3G1 in the presence and absence of 10 mM L-fucose were compared side-by-side, and the fed-batch culture of CHO SM 3G1 in the presence of 10 mM D-fucose was also performed as a control in order to adjust the osmotic pressure of the culture medium to the level in the culture with L-fucose. The cultures were carried out until the cell viability decreased to less than 50%, which occurred on day 13 post-inoculation. Culture aliquots were taken on days 3, 6, 9, 11, and 13 to analyze the cells and the produced antibodies. Cells grew logarithmically, reaching a maximum viable cell density within 9 days followed by declining viability in each culture (Fig. 6A and B). Cell growth, cell morphology, and antibody productivities of CHO SM 3G1 were comparable among the different culture conditions irrespective of whether or not D/L-fucose was added to the medium. The antibody productivities of CHO/DG44 32-05-12 and CHO SM 3G1 were almost equivalent, with the specific production rate being approximately 16.0 pg cell$^{-1}$ day$^{-1}$ for both cell lines. Monosaccharide composition analysis showed that CHO SM 3G1 stably produced non-fucosylated antibodies throughout the culture course (Fig. 6C). Oligosaccharide profile analysis of the produced antibodies purified from the final culture medium of CHO SM 3G1 cultured in the absence of L-fucose confirmed

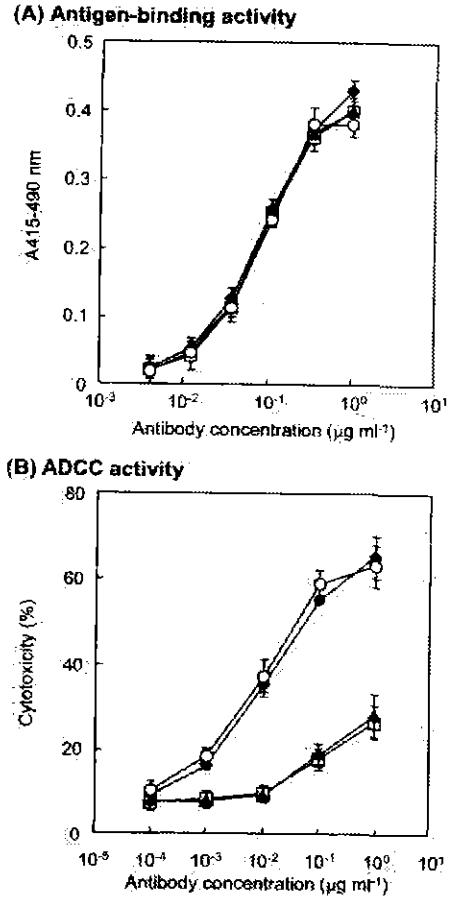


Fig. 5. Biological activities of anti-human CD20 IgG1s. Both antigen-binding (A) and ADCC (B) were measured in the following four different antibodies as the samples: the antibodies from CHO SM cultured in the absence (closed diamonds) or presence (closed triangles) of L-fucose, Ms704 1A7-15 (open circles), and Rituxan® (open squares) as a control. The mean values ± S.D. of triplicates are shown.

that the *N*-linked Fc oligosaccharides of the product were of the biantennary complex type completely lacking core fucosylation, consisting of F0G0 (61.0%), F0G1 (33.1%), and F0G2 (3.4%) (Fig. 6D). There was no significant change of the ratios during the culture (data not shown).

## 4. Discussion

L-fucose has the two distinctive structural features of the lack of a hydroxyl group on the carbon at the 6-position and the L-configuration compared to other six-carbon sugars present in mammals, and confers unique functional properties to *N*- and *O*-linked glycoproteins, which are related to a variety of physiological and pathological phenomena, including cancers, blood transfusion reactions, selectin-mediated cell adhesion, host–microbe interactions, ontogenic events, antibody functions, and signal transductions by cytokines (Becker and Lowe, 2003; Satoh et al., 2006). In the biopharmaceutical industry, control of fucosylation of the therapeutics is a matter of concern and still a challenging issue, especially in therapeutic antibodies (Satoh et al., 2006; Presta, 2006). In this study, we have searched among the genes involved in the oligosaccharide fucosylation pathway in mammalian cells in order to identify target genes that would allow us to manufacture completely non-fucosylated therapeutics.

Loss-of-function analyses using siRNAs against *FUT8*, *GMD*, and *GFT* in antibody-producing cells revealed that there was a positive correlation between the Fc oligosaccharide fucosylation and the mRNA expression through the origin (0, 0) in both *GMD* and *FUT8* (Fig. 2), suggesting that there is no functional redundancy in *GMD* and *FUT8*. On the other hand, the suppression of *GFT* mRNA was not as efficient for reduction of fucosylation of the products as the suppression of *GMD* and *FUT8*. To yield LCA-resistant clones, it was necessary to suppress over 90% of the *GFT* mRNA in the clones, resulting in only two clones from the many *GFT* siRNA-introduced transformants, and a very low LCA-resistant transformation frequency of 0.2%. The fact that the fucosylation capability was retained even in the case in which almost all (98%) of the endogenous *GFT* mRNA was suppressed suggested that there could be another mechanism responsible for intracellular GDP-fucose transport into the Golgi-lumen. Any of the sugar-nucleotide transporters other than GFT might work, since *GFT* is the only gene of the acting as a GDP-fucose transporter thus far reported (Handford et al., 2006). LCA-resistant clones transformed by *GMD* siRNA showed relatively high-level suppression of the target gene compared to those transformed by *FUT8* siRNA (Fig. 2). Indeed, all of the clones reduced over 50% of target *GMD* mRNA to be LCA-resistant. This phenomenon was well reflected by the fact that mammalian cells keep an excess endogenous intracellular GDP-fucose pool, i.e., GMD is not the rate-limiting step in the oligosaccharide fucosylation pathway in the cells. It seemed to be necessary to delete the excess amount of GMD to extinguish the intracellular GDP-fucose pool at first. In the LCA-resistant clones with a more than 50% reduction of *GMD* mRNA, the fucosylation level of the products was linearly proportional to the amount of *GMD* mRNA through the origin as observed in the clones transformed by *FUT8* siRNA. Therefore, we have chosen *GMD* as a candidate gene to generate a new host cell line to manufacture non-fucosylated therapeutics.

First, we planned a gene targeting of *GMD* using a homologous recombination technique as *FUT8* knockout had been generated previously (Yamane-Ohnuki et al., 2004); Since we had experienced and known that the appearance of spontaneous LCA-resistant cells from the parent cell line CHO/DG44 hampered the efficient identification of the desired targeted cells, the characteristics of the spontaneous LCA-resistant cells were extensively investigated. The rate at which colonies of LCA-resistant cells appeared even in the absence of mutagen was quite high, i.e., $10^{-6}$, which was consistent with the previous reports (Stanley et al., 1975). The majority of spontaneous LCA-resistant cells from the parent cell line CHO/DG44 showed an LCA-staining pattern similar to that of a CHO cell line Lec13 partially deficient in the function of GMD (Kanda et al., 2006a), suggesting that there were some problems in the *de novo* pathway for GDP-fucose synthesis in these cells. Even though we used a CHO/DG44 clone prepared by two rounds of single cell cloning, spontaneous LCA-resistant clones appeared with the

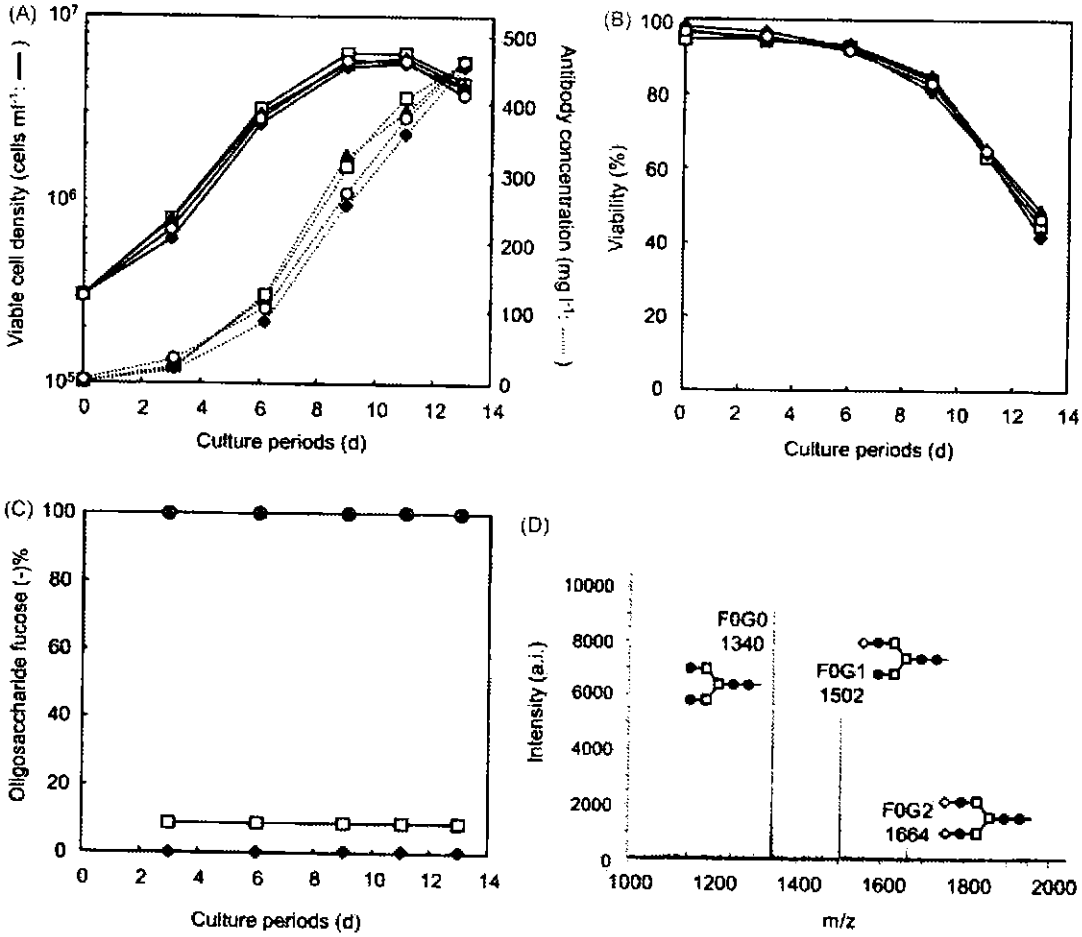


Fig. 6. Serum-free fed-batch culture of *GMD* knockout antibody-producing cells. Serum-free fed-batch culture using a 250 ml Erlenmeyer flask was performed using the recombinant IgG1-producing clone CHO SM 3G1. The clone was cultured in the absence of D/L-fucose (open circles), in the presence of L-fucose (closed diamonds), or in the presence of D-fucose (closed triangles), and four different parameters were analyzed: viable cell density (A; solid lines), antibody concentration in culture supernatants (A; dotted lines), cell viability (B), and total percentage of non-fucosylated oligosaccharides from recombinant products (C) analyzed by monosaccharide composition analysis. CHO/DG44 32-05-12 (open squares) was cultured as a control. The oligosaccharide structure of the final product from CHO SM 3G1 in the absence of D/L-fucose was analyzed using MALDI-TOF MS (D).

high frequency (data not shown). The genes involved in the *de novo* pathway might be instable, but the reasons for the high frequency of LCA-resistant cell occurrence in CHO/DG44 remains to be solved.

Among the spontaneous LCA-resistant clones, consequently, we have succeeded in finding a new cell line of *GMD* knockout cells from CHO/DG44, which we designated CHO SM. CHO SM has a genomic deletion that corresponds to the mouse/human *GMD* exon 5, 6, and 7 regions, in its *GMD* allele, and expresses a mutant GMD lacking three functionally critical domains: the dimerization domain, reaction center, and substrate-binding domain (Somoza et al., 2000; Webb et al., 2004). CHO SM is the first established mammalian cell line completely deficient in GMD function, though a few cell lines partially lacking GMD function, such as mouse $PL^R1.3$ and CHO Lec13, have been reported (Reitman et al., 1980; Ripka et al., 1986). CHO SM completely lacks the ability to produce intracellular GDP-fucose and fucosylated glycoproteins in the absence of L-fucose. In the presence of L-fucose, however, CHO SM recovers this capability, since extracellular L-fucose is utilized through the salvage pathway. This unique characteristic enabled us to directly compare the effect of fucosylation on various cell characteristics using the same clone, and clearly demonstrate that loss of oligosaccharide fucosylation, probably including α1,2-, α1,3-, α1,4-, α1,6-, and *O*-fucosylation, has no impact on the cell phenotypes, including morphology, growth kinetics, and recombinant protein productivity even in serum-free fed-batch culture. Since a mutant knockout mouse of FX protein, which is another essential enzyme for GDP-fucose synthesis, has been reported to show postnatal failure to thrive (Smith et al., 2002), loss of oligosaccharide fucosylation is critical for the development of individual animals, but probably not for cell culture events. CHO SM proved capable of consistently producing completely non-fucosylated antibodies throughout the duration of a serum-free fed-batch culture with a constant ratio of F0G0, F0G1, and F0G2 carbohydrates until the end of the culture. The purified anti-human CD20 antibodies from CHO SM showed dramatically enhanced ADCC accompanied by no change in antigen-binding activity compared to a control rituximab on the market. Thus, CHO SM is considered to be a suitable host cell line for the manufacture of non-fucosylated therapeutic antibodies with enhanced ADCC.

In conclusion, by means of loss-of-function analyses, we have found that, in addition to *FUT8*, *GMD* is a key gene enabling us to control the fucosylation of oligosaccharides, and confirmed that this finding had practical application to industry by establishing a new host cell line deficient in the functionally critical regions of *GMD* from its genome. The *GMD* knockout CHO/DG44 cell line loses the ability for intracellular GDP-fucose synthesis, and produces completely non-fucosylated therapeutics without any changes of cellular phenotypes such as morphology, growth kinetics and recombinant protein productivity in serum-free fed-batch culture. Moreover, this cell line makes it possible to produce 0% and 100% fucosylated therapeutics at the same time using the same producing clone just by changing the amount of L-fucose in the medium. Thus, in chemically-defined medium culture, we contend that *GMD* knockout is a new strategy with great advantages for the manufacture of therapeutics with *N*- and *O*-linked oligosaccharide fucosylation. Application of this cell line to therapeutics, e.g., recombinant antibodies and tissue-plasminogen activators (t-PA), will be promising for manufacturing next-generation therapeutics (Hajjar and Reynolds (1994); Satoh et al., 2006).

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