Production of non-fucosylated antibodies by co-expression of heterologous GDP-6-deoxy-d-lyxo-4-hexulose reductase

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All IgG-type antibodies are N-glycosylated in their Fc part at Asn-297. Typically, a fucose residue is attached to the first N-acetylgalactosamine of these complex-type N-glycans. Antibodies lacking core fucosylation show a significantly enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) and an increased efficacy of antitumor activity. In cases where the clinical efficacy of an antibody is to some extent mediated by its ADCC effector function, afucosylated N-glycans could help to reduce dose requirement and save manufacturing costs. Using Chinese hamster ovary (CHO) cells as a model, we demonstrate here that heterologous expression of the prokaryotic enzyme GDP-6-deoxy-d-lyxo-4-hexulose reductase within the cytosol can efficiently deflect the fucose de novo pathway. Antibody-producing CHO cells that were modified in this way secrete antibodies lacking core fucose as demonstrated by MALDI-TOF mass spectrometry and HPAEC-PAD monosaccharide analysis. Engineering of the fucose de novo pathway has led to the construction of IgGs with a strongly enhanced ADCC effector function. The method described here should have broad practical applicability for the development of next-generation therapeutic antibodies.

Keywords: antibody/cell culture/core fucose/glycoengineering/sugar nucleotide metabolism

Introduction

The pharmaceutical activity of therapeutic antibodies is often mediated by their effector functions. Antibody-dependent cell-mediated cytotoxicity (ADCC) is one of the major effector functions by which IgG and IgE can limit and contain infection and kill tumor cells (Santoli and Koprowski 1979; Clark 1997). Once an antibody has bound to a specific target cell, it recruits natural killer cells (NK cells), monocytes, or eosinophils to the site where it has bound to its target (Clark 1997). The classic ADCC response is mediated by NK cells upon the binding of the Fcγ receptor IIIA (CD16a, FcγRIIIa) to the Fc region of IgG molecules (Perussia and Loza 2000). Upon binding to the antibody-Fc-region, NK cells release cytokines and cytolytic agents that enter the target cell and trigger apoptosis. An NK cell’s Fc receptor recognizes residues located within the hinge region, the B/C loop, C′/E loop, and the F/G loop of the IgG1-Fc (Sondermann et al. 2000; Radaev et al. 2001; Houde et al. 2010). IgG1 Fc domains bind to their cognate Fc receptors CD16 (FcγRIII), CD32 (FcγRI-B1 and -B2), and CD64 (FcγRI) (Gessner et al. 1998), while IgE domains bind to their cognate Fc receptors FceRI and CD23 (Holgate 1998). Most human therapeutic antibodies are IgG1 isotypes containing two diantennary complex-type glycans that are N-linked to asparagine 297 in the constant region (Rademacher et al. 1986). Antibodies lacking the (α1,6)-linked fucose (core fucose) show enhanced in vitro target cell lysis compared to fucosylated antibodies (Shields et al. 2002; Shinkawa et al. 2003; Niwa, Hatanaka, et al. 2004; Niwa, Shoji-Hosaka, et al. 2004; Niwa, Natsume, et al. 2005; Niwa, Sakurada, et al. 2005; Kanda, Yamada, et al. 2006; Kanda, Yamane-Ohnuki, et al. 2006; Suzuki et al. 2007). The ability of these antibodies to support the ADCC response in vivo can be deduced from the superior clinical response of breast cancer patients carrying the high-affinity FcγRIIa allotype (FcγRIIa-158Val) for Trastuzumab in contrast to that of patients carrying the low-affinity allotype (FcγRIIa-158Phe) (Musolino et al. 2008). Although it has been suggested that the impact of Fc glycosylation on ADCC is critically dependent on the recruited effector cell type and that the lack of fucose may be detrimental to the ADCC activity mediated by polymorphonuclear cells (Peipp et al. 2008), more recent work on the in vivo ADCC activity of non-fucosylated antibodies showed that such glyco-engineered antibodies were effective in eliciting a proper ADCC response by evading the inhibitory effects of both plasma IgG on FcγRIIIa binding and of fucosylated antibodies on antigen binding (Iida et al. 2009). Recent data suggest that the presence or absence of core fucose has only a minor to negligible impact on the overall IgG1 conformation (Houde et al. 2010), suggesting that removal of fucose does not alter monoclonal antibody (mAb) stability. Because of the strong enhancement of ADCC response, several groups have explored routes to block core fucosylation of antibodies intended for therapeutic application. However, many of the currently applied methods for providing
therapeutic antibodies with reduced or absent fucose content have significant drawbacks. For example, treatment of manufactured IgG with recombinant fucosidases is inefficient due to steric hindrance caused by the large N-terminal (β/α)8-TIM barrel catalytic domain typical of many glycosyl hydrolases (Coutinho and Henrissat 1999; Intra et al. 2007). In addition, such post-manufacturing enzymatic treatment would entail additional manufacturing steps bearing significant drug consistency risks. In order to achieve the production of afucosylated antibodies from vertebrate cells, several key enzymes such as (α1,6)-fucosyltransferase 8, GDP-mannose 4,6-dehydratase and transporters such as the GDP-fucose transporter have already been identified as targets for knock-out, or suppression by RNAi (Yamane-Ohnuki et al. 2004; Kanda, Yamada, et al. 2006; Kanda, Yamane-Ohnuki, et al. 2006; Kanda et al. 2007). In addition, it was also discovered that overexpression of b(1,4)-N-acetylglucosaminyltransferase III (GnTIII) could modify Chinese hamster ovary (CHO) cells in such a way that they secrete predominantly afucosylated glycoproteins with bisecting N-acetylglucosamine (GlcNAc) (Umaña et al. 1999). Since non-homologous recombination events occur several orders of magnitude more frequently than homologous recombination in somatic cells (Sedivy and Sharp 1989), knockout strategies have their limitations and do not allow for a fast and reliable conversion of existing producer cell lines. A comparison of a given fucosylated and non-fucosylated biotherapeutic still requires parallel cell line development from both the knockout and unmodified host cell lines. Other approaches suggest the use of cell lines treated with RNAi or antisense molecules to knock down the expression level of key enzymes (Mori et al. 2004; Imai-Nishiya et al. 2007). These approaches, however, may suffer from unpredictable off-target effects and are impractical to implement at manufacturing scale. Also, in light of the fact that more details about the link between antibody N-glycosylation and ADCC are still emerging, a comparison between an unmodified product and a fucose-deficient product may still be required by regulatory authorities. Thus, a method that allows the fast and reliable generation of fucose-deficient producer cells showing the equivalent average specific productivity (qP) of the original unmodified producer cell line—without introducing major changes in cell character beyond the lack of fucosylation—are still industrially desirable (Imai-Nishiya et al. 2007).

Here, we demonstrate that a fast and simple heterologous overexpression of the bacterial oxidoreductase GDP-6-deoxy-d-lyxo-4-hexulose reductase (RMD) provides a fast and reliable method for conversion of an existing antibody producer clone into a cell line lacking the ability to efficiently attach core fucose to nascent N-glycan moieties of secreted therapeutic proteins.

Results
Heterologous expression of the RMD transgene
To evaluate the effects of RMD-transgene expression on the levels of fucosylation of secreted IgG, a vector equipped with a bicistronic expression cassette comprising the genes for RMD and green fluorescent protein (GFP) was generated and introduced into a CHO/DG44 clone that had previously been engineered for overexpression and secretion of a biosimilar version of the IgG1-type therapeutic antibody Trastuzumab (Herceptin®, Roche).

G418-resistant clones expressing the transgene were identified by their GFP-mediated fluorescence and appeared within 2 weeks of transfection. A transformation efficiency of approximately 80% was achieved by electroporation as assessed from GFP-fluorescence distribution (Figure 1A). Successful expression of the RMD transgene was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) using an RMD-specific set of primers (Figure 1B). The modified CHO cells expressing the RMD transgene are named RMD-CHO.
Serum-free fed-batch culture of CHO and RMD-CHO antibody-producing cells

Serum-free fed-batch culture of the unmodified parental CHO and the transfected RMD-CHO cells producing IgG was carried out in 50 mL bioreactor tubes containing a fucose-deficient growth medium supplemented with L-glutamine. Bioreactor tubes were inoculated at a starting cell density of 4 × 10⁵ cells/mL and then incubated at 180 rpm, 37°C, 7.5% pCO₂. Performance of the fed-batch cultures was monitored for 14 days and was compared side by side. Comparative analysis of parallel fed-batch cultures of CHO and RMD-CHO cells showed no significant deviations over a course of 14 days. Initial doubling rates, proliferation rates, and IgG titers at the respective sampling dates were congruent for both cell lines. Both clones retained the morphology typical of CHO cells. The pattern of declining viability over the duration of the fed-batch assay as well as the average specific productivity (qᵦ) between days 3 and 10 of the Fed batch shaker assay remained comparable for the two different clones (data not shown).

N-Glycan analysis of IgG produced from CHO and RMD-CHO cells

CHO and RMD-CHO cells were grown in a batch culture. Three different RMD-CHO clones producing IgG were used, namely H1, H2, and H3. Cells were inoculated at a starting cell density of 4 × 10⁵ cells/mL and grown for 7 days in biore-

Fig. 2. MALDI-TOF spectra of desialylated IgG N-glycans produced using (A) WT CHO cells; (B) RMD-CHO clone H1; (C) RMD-CHO clone H2; (D) RMD-CHO clone H3. All molecular ions are present in either sodiated [M+Na⁺] or potassiated [M+K⁺] form (black cross). Grey circle, Man; white circle, Gal; black square, GlcNAc; dark grey triangle, Fuc; white cross, does not contain any carbohydrate material.
actor tubes at 180 rpm, 37°C, 7.5% pCO2. Supernatants were harvested on day 7, and IgG samples were purified by protein A affinity chromatography. Purity and integrity of the eluted antibodies were confirmed by reducing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). N-glycans, released using peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase (PNGase F), were desialylated and subsequently analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Relative quantification of signal intensities of N-glycans was performed as it was demonstrated earlier to give reliable results when compared to chromatographic methods (Wada et al. 2007). High-mannose as well as complex-type diantennary N-glycan structures were detected in all the samples (Figure 2). Monofucosylated agalactosylated/monogalactosylated/digalactosylated diantennary N-glycans were the three most abundant N-glycan structures found in wild type (WT) IgG (Figure 2A). The presence of core fucose in those peaks, namely at m/z 1485.4, 1647.4, and 1809.4, was confirmed by MALDI-TOF/TOF. A diagnostic fragment ion dHex1HexNAc2 was observed in every spectrum at m/z 592.8. In the IgG samples that were produced from RMD-CHO cells, only trace amounts of fucose were observed (Figure 2B–D), representing a maximum of 2% of the total N-glycan pool (sample H2). Simultaneously, sample H2 contained a larger amount of high-mannose structures than WT IgG.

IgG1-Fc binding activity of IgG produced from CHO and RMD-CHO cells
Since the comparatively weak interaction ($K_d \sim 1 \mu M$) between IgG1 and its cognate Fc receptor FcyRIIIa is one of the major factors that contribute to the ADCC effector function (Sondemann et al. 2000), an FcyRIIIa binding assay is an indirect measure to predict ADCC activity of IgG1 monoclonal antibody samples. FcyRIIIa binding of afucosylated IgG secreted from RMD-CHO cells was greatly increased with equivalent binding at ~16-fold less protein to FcyRIIIa-158F when compared with fucosylated IgG secreted from the wild-type CHO cells (Figure 3A and B). The data are reported in Figure 3B as relative fold increase in binding of afucosylated IgG using the fucosylated IgG as a reference. Our result corroborates other previously published work on the differential FcyRIIIa-receptor binding activity of fucosylated and afucosylated mAbs (Shields et al. 2002; Shinkawa et al. 2003; Niwa, Natsume, et al. 2005; Niwa, Sakurada, et al. 2005; Kanda, Yamada, et al. 2006; Kanda, Yamane-Ohnuki, et al. 2006, Suzuki et al. 2007; Iida et al. 2009).

ADCC activity of afucosylated IgG produced using RMD-CHO cells
To analyze ADCC activity, isolated NK cells and HER2-expressing target cells were co-incubated with serial dilutions of afucosylated and fucosylated IgG. As a prerequisite for the assay, NK cells were isolated from whole blood samples at a purity level of 73% (Figure 4). The technical NK cellular cytotoxicity control showed a specific lysis activity of 77% for the donor material used for the ADCC assay (Figure 4). The HER2-expressing target cell line BT-474 (isolated from a human invasive ductal carcinoma of the breast) was used in the ADCC assay. The BT-474 target cell line is also attacked by NK cells by mechanisms other than ADCC. BT-474 cells show a mean value of 16% antibody-independent cell lysis (data not

Fig. 3. (A) Binding curves of FcgRIIIa-His (Phe158) to WT IgG and afucosylated IgG in the absence of plasma. FcyRIIIa-binding was detected by an ELISA using FcyRIIIa-His as a capture reagent, anti-human IgG peroxidase-conjugated antibody as a detection reagent and Tetramethylbenzidine (TMB) as a chromogenic substrate. Points indicate the median absorption of peroxidase reacted TMB at 450 nm, n = 2. Open circles, wild type fucosylated IgG (WT); closed circles, afucosylated IgG derived from clone H1 (H1); closed squares, afucosylated IgG derived from clone H2 (H2); upward pointing closed triangles, afucosylated IgG derived from clone H3 (H3). (B) Relative fold increase in FcyRIII-binding of afucosylated IgG over WT IgG. The relative fold increase in FcyRIII binding was calculated from EC_{50} values. WT, wild type fucosylated IgG; H1, afucosylated IgG derived from clone H1; H2, afucosylated IgG derived from clone H2; H3, afucosylated IgG derived from clone H3.
shown). Data for specific cell lysis induced by IgG samples are displayed in Figure 5. All three afucosylated IgG samples (H1–H3) induced an increased ADCC response compared to the WT antibody (Figure 5). The afucosylated IgG sample H2 induced the highest ADCC response (Figure 5). Similar results were obtained when SK-BR-3 cells (HER2 positive

Fig. 4. Preparation and analysis of NK cells for ADCC-activity assays. (A) Dot Plot showing the level of purity of the isolated primary NK cells used in the ADCC assays. Primary NK cells were isolated from whole blood from a healthy human donor by magnetic bead separation. The isolated NK cells were then analyzed for purity by flow cytometry using antibodies against the NK-cell markers CD16 and CD56. (B) Performance of the isolated NK cells. In order to determine the cytolytic activity of the isolated NK cells, the calcein Calcein acetoxyethyl ester (AM) stained K562 target cells were incubated either alone, with NK cells, or with the cell lysing agent saponin for 4 h. Mean fluorescence intensity (MFI) released from the incubated target cells indirectly indicates the extent of cell lysis. The MFI observed for NK-mediated cell lysis subtracted from the MFI obtained for spontaneous cell lysis indicates the maximum possible specific MFI to be observed in the ADCC assay. Note that NK-mediated lysis does not completely reach the MFI level obtained from total lysis.

Fig. 5. In vitro ADCC activity of afucosylated and fucosylated IgG derived from CHO and RMD-CHO. Points indicate mean specific cell lysis (%) at a given IgG concentration (%), n = 4; bars indicate ±SD, wild-type IgG (open squares), afucosylated IgG derived from clone H1 (closed circles), afucosylated IgG derived from clone H2 (downward pointing closed triangle), and afucosylated IgG derived from clone H3 (upward pointing closed triangle). Comparable data were obtained when SK-BR-3 cells were used as target cells (data not shown).
adenocarcinoma of the breast) were used as target cells in the assay. The calculated half maximal effective concentration (EC50) values for afucosylated and fucosylated IgG samples incubated with BT-474 and SK-BR-3 cells are summarized in Table I. The observed shift in EC50 between wild type IgG and the variants H1 to H3 remained comparable regardless of the target cell line used in the ADCC assay (Figure 5).

In the presence of the HER2-positive BT474 and SK-BR-3 target cell lines and purified NK cells, afucosylated IgG showed an average 16-fold antibody-mediated target cell depletion activity with average EC50 values of 0.443 and 0.00817 μg/mL, which indicated a much higher efficacy compared to fucosylated IgG.

In line with the findings of previous reports, the afucosylated IgG samples that had shown an increased FcγRIIIa-binding activity also induced a higher ADCC response compared to the WT IgG. However, despite this overall trend, the slight outperformance of the afucosylated H2-sample in the ADCC assay cannot be directly deduced from the superimposed FcRIIIa-binding assay data (Figures 4 and 5).

### Table I. Summarized results of the ADCC effector function of afucosylated (H1–3) and fucosylated (WT) IgG incubated with different antigen presenting target cells (BT-474, SK-BR-3). The calculated ratio EC50 (fucosylated)/EC50 (afucosylated) indicates the enhanced ADCC effector function.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>BT-474 EC50 [ng/mL]</th>
<th>BT-474 ratio (WT/H)</th>
<th>SK-BR-3 EC50 [ng/mL]</th>
<th>SK-BR-3 ratio (WT/H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>6.03</td>
<td></td>
<td>1.31</td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>0.454</td>
<td>13</td>
<td>0.0932</td>
<td>14</td>
</tr>
<tr>
<td>H2</td>
<td>0.227</td>
<td>27</td>
<td>0.0602</td>
<td>22</td>
</tr>
<tr>
<td>H3</td>
<td>0.647</td>
<td>9</td>
<td>0.0917</td>
<td>14</td>
</tr>
</tbody>
</table>

Similarly, N-glycans, released from purified antibodies produced using RMD-CHO cells, were hydrolyzed with trifluoroacetic acid (TFA), and the resulting monosaccharides were analyzed by HPAEC-PAD. The TFA-hydrolyzed N-glycans did not yield any rhamnose peak that exceeded the LOD (Figure 7). To conclude, neither RMD-CHO cells nor the secreted antibodies contain detectable amounts of rhamnose.

### Discussion

In recent years, it has emerged that fucose-deficient antibodies show a dramatically enhanced ADCC activity and increased clinical efficacy, particularly for oncology indications. In this study, we evaluated a glycoengineering approach to achieve secretion of fucose-deficient mAbs from cell lines that were modified for continuous removal of a key metabolic intermediate from the cytosolic fucose de novo synthesis pathway (Figure 8). Our data suggest that a transgenic expression of a heterologous bacterial enzyme leads to the desired block in the

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**Fig. 6.** HPAEC-PAD profile of monosaccharides hydrolized from the cytosolic fraction of (A) unmodified CHO cells and (C) RMD-CHO clone H2. (B), (A) spiked with 10 pmol/μL l-rhamnose and (D), (C) spiked with 10 pmol/μL l-rhamnose. As the monosaccharides were not re-N-acetylated after the TFA hydrolysis, GlcNAc was measured as GlcNH2. Under the selected conditions, the l-rhamnose peak elutes at a retention time of approximately 15.5 min. Note the absence of the fucose peak from the HPAEC-PAD chromatograms of the cell lysate from the modified clone H2.
synthesis of fucose on nascent glycoprotein N-glycans. The extent of fucose depletion by this method also indicates that omission of fucose from the culture medium was sufficient to completely block the salvage pathway, which may otherwise have served as an alternate source of cytosolic GDP-fucose. Culturing the cells in the presence of L-fucose is likely to replenish cytosolic GDP-L-fucose pools via salvage pathway activity and thus rescue the core fucosylation of antibodies secreted from RMD-CHO cells.

The key metabolic target in our approach was GDP-4-keto-6-deoxy-D-mannose, which is an unstable intermediate in the synthesis pathways of a variety of monosaccharides (including fucose) in eukaryotes and bacteria (Bulet et al. 1984; Bonin and Reiter 2000). In plants and bacteria, the precursor GDP-4-keto-6-deoxy-D-mannose is a common intermediate for the synthesis of several different GDP-monodeoxyhexoses including GDP-L-fucose, GDP-4-deoxy-D-talose, as well as the GDP-dideoxyhexose GDP-colitose and the GDP-dideoxy amino hexose GDP-D-perosamine (Andrianopoulos et al. 1998; Kneidinger et al. 2001; Mäki et al. 2002, 2003; Beyer et al. 2003; Mäki 2003; Albermann and Beuttler 2008; King et al. 2009). Moreover, an active site Cys109Ser mutant of GDP-fucose synthase produces GDP-6-deoxy-D-altrose instead of GDP-fucose (Lau and Tanner 2008). The specific enzyme that we selected for our approach was GDP-4-keto-6-deoxy-D-mannose reductase (synonym with GDP-4-keto-6-deoxy-D-mannose reductase, abbreviated RMD) (Kneidinger et al. 2001; Mäki et al. 2002). This enzyme utilizes NADH and NADPH as hydrogen donors and catalyzes the targeted reduction of the 4-keto group of the fucose pathway intermediate GDP-4-keto-6-deoxy-D-mannose to yield GDP-D-rhamnose. The conversion of GDP-4-keto-6-deoxy-D-mannose to GDP-D-rhamnose by RMD appears to proceed quantitatively because no reverse reaction has been detected (Kneidinger et al. 2001). Our data also indicate that GDP-rhamnose, a 6-deoxyhexose found only in glycoconjugates of certain bacteria but not in animals (Webb et al. 2004), is a dead-end product within the context of the vertebrate cytosol. Vertebrate cells lack rhamnosyltransferases (Webb et al. 2004) and membrane transporters so that there is only a limited likelihood for GDP-D-rhamnose to be incorporated into nascent glycans within vertebrate cells. Moreover, GDP-D-rhamnose is a different enantiomer compared to the predominant vertebrate deoxyhexose nucleotide—GDP-L-fucose. Nevertheless, we needed to address possible concerns that the artificial D-rhamnose may be incorporated into the secreted IgG or elsewhere in the cell. In order to accomplish this, we conducted an in-depth comparative monosaccharide analysis based on HPAEC-PAD. Since HPAEC-PAD is not enantioselective (Horton 2004), the D-rhamnose peak co-elutes at the identical retention time of L-rhamnose. The L-rhamnose standard did elute at a retention time where no monosaccharides from either IgGs or cell lysate eluted. As expected, the D-rhamnose concentration remained well below the LOD. There is mounting evidence that in certain prokaryotic and eukaryotic cells, GDP-fucose biosynthesis is regulated by competitive feedback inhibition of GDP-mannose-4,6-dehydratase (GMD) by GDP-fucose—the final product in the pathway (Sturla et al. 1997; Sullivan et al. 1998; Bisso et al. 1999; Somoza et al. 2000). Besides GDP-L-fucose, other sugar nucleotides have also been shown to inhibit their own biosynthesis. In vitro reconstitution experiments of GDP-D-rhamnose biosynthesis suggest that the Pseudomonas aeruginosa GMD can be regulated by GDP-D-rhamnose mediated feedback inhibition (King et al. 2009). Crystal structure analysis of a GMD from the bacterium P. aeruginosa in the presence of the ligands NADPH and
GDP confirmed the structural homology with the GMDs from different prokaryotic and eukaryotic species (Webb et al. 2004). Since GMD is highly conserved throughout the domains archaea and bacteria and in higher eukaryotic organisms, such as plants and animals, it seems likely that the activity of the mammalian GMD enzyme may be inhibited by several different GDP-deoxyhexoses, including GDP-L-fucose and GDP-D-rhamnose.

Except for the lack of fucose, the afucosylated IgGs secreted from the genetically engineered clones showed a pattern of N-glycans that matched very closely with the pattern of N-glycans obtained from IgG secreted from unmodified CHO cells. In addition, certain RMD-modified cells secreted antibodies bearing a higher level of high-mannose structures. This phenomenon might well be explained by a putative feedback inhibition of GMD by GDP-D-rhamnose. The lack of GMD activity as an alternate metabolic sink for GDP-mannose may have contributed to an elevated pool of cytosolic GDP-D-mannose which in turn may explain the slight elevation of high-mannose structures occasionally observed in products secreted from RMD-modified host cells (Figure 2). In line with previously published reports, our results for the FcγRIII-binding assay as well as for in vitro ADCC assay also show that afucosylated IgGs show a significantly higher binding activity for FcγRIIIa and an enhanced ADCC response (Niwa, Hatanaka, et al. 2004; Niwa, Shoji-Hosaka, et al. 2004). The recent findings by Iida et al. (2009) suggest that the observed EC50 shift and fold increase in target cell depletion activity (Table I, Figure 5) would have been even higher if the assay had been conducted in the presence of whole blood, serum, or plasma. Interestingly, however, we observed that the afucosylated IgG sample H2 with the highest level of high-mannose structures also showed the highest ADCC activity, regardless of the target cell used in the assay (BT474 or SK-BR-3) (Table I).

Taken together, this study demonstrates that overexpression of RMD in vertebrate host cells causes a depletion of an important key intermediate for the synthesis of cytosolic GDP-fucose, GDP-4-keto-6-deoxy-D-mannose. This approach allows the generation of metabolically engineered cell lines but also offers a highly promising new strategy to convert existing antibody-expressing cell clones into producer cells for fucose-depleted therapeutics. The ability to reliably engineer fucose deficiency in already existing cell lines may help to accelerate drug development for next-generation monoclonal antibodies.

Materials and methods

Materials
PNGase F and Neuraminidase were obtained from Roche Diagnostics, Mannheim, Germany. Saponin, trypsin, and methyl...
iodide were obtained from Sigma-Aldrich, St. Louis, MO. NaOH, dimethylsulfoxide (DMSO) and ethanol used were from Merck, Hamburg, Germany. Acetonitrile (ACN) was from J.T. Baker, Deventer, the Netherlands. Sep-Pak C18-columns were from Waters, Milford, MA. Carbograph extract-clean columns were ordered from Alltech, Deerfield, IL. Protein A-Sepharose and CL-4B were obtained from Invitrogen, Carlsbad, CA. Alpha-modified Eagle’s medium (MEM) was purchased from Lonza, Belgium. G418 was from PAA, Pasching, Austria. C8862 cell culture medium was ordered from SAFC Bioscience, Lenexa, KA. (HIS)-tagged FcγRIIIA (F158) was purchased from R&D Systems, Minneapolis, MN. Anti-tetraHIS monoclonal antibody was obtained from Qiagen, Hilden, Germany.

**Gene optimization and synthesis**

The amino acid sequence for the oxidoreductase RMD (P. aeruginosa PAO1; 304 amino acids; GenBank Accession No. GenBank: AAG08839.1) was reverse translated, and the resulting nucleotide sequence was submitted to GeneArt (Würzburg, Germany) for optimization and gene synthesis. Gene optimization included knockout of cryptic splice sites and RNA destabilizing sequence elements, optimization for increased mRNA stability, and adaptation of codon usage to match the requirements of CHO cells (Cricetulus griseus).

**Construction of the RMD expression plasmid**

The synthesized cDNA construct encoding the deflacting enzyme was subcloned into a PBG-proprietary expression vector (ProBioGen AG, Berlin, Germany), which allows coordinated co-expression of RMD and green fluorescent protein from a bicistronic message (GFP). Integrity of the construct was confirmed by analytical restriction digest. The expression plasmid is equipped with a Neomycin resistance gene allowing for direct selection of cells that have stably integrated the expression cassette.

**Cell transfection, selection, and cloning**

The recombinant antibody producer cell line CHO was established earlier in our laboratory by stable transfection of the dihydrofolate reductase-deficient CHO cell line, CHO/DG44 (Urlaub et al. 1986) with a proprietary expression vector containing an antibody expression cassette (ProBioGen AG, Berlin, Germany) comprising nucleotide sequences encoding light and heavy chains of the therapeutic IgG antibody Trastuzumab (Herceptin®). Cells were grown in suspension cultures

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**Clone screening by fluorescence microscopy**

Single-cell clones were seeded into 96-well plates and screened for successful RMD integration by monitoring of GFP fluorescence with an Olympus IX-50 (Olympus Optical Co., Europe) fitted with a c-mount adapter. For GFP scan, a fluorescence-filter at 200-fold extension was used versus phase contrast. Images were edited using Viewfinder lite.

**RT-PCR**

mRNA expression of the RMD transgene was confirmed by RT-PCR analysis. mRNA was isolated from the cells and transcribed into cDNA using cloned avian myeloblastosis virus (AMV) Reverse Transcriptase (Cloned AMV First-Strand cDNA Synthesis Kit, Invitrogen, Carlsbad, CA). Reactions were carried out using oligonucleotide primers, 0.5 U Taq polymerase (Qiagen, Hilden, Germany) per reaction in a polymerase chain reaction (PCR) program with 25 cycles performed at an annealing temperature of 56°C. Amplicons were separated and visualized on 1% TRIS-acetate/EDTA agarose/ethidium bromide gels.

**Fed-batch culture**

IgG were produced using both CHO and RMD-CHO cells (clones H1, H2, and H3) in order to compare their N-glycan structures. Cells were seeded at 4 × 10^5 cells/mL into 500 mL shake flasks in 100 mL of serum-free medium (custom for-
that the residuals for each calibration standard meet an acceptance limit of 20% relative error (RE).

**IgG purification using protein A affinity chromatography**

The cell culture supernatant was loaded on a 0.5 mL Protein A-Sepharose column, pre-equilibrated with 20 mM sodium phosphate, pH 7.0. After washing the column with two bed volumes of equilibration buffer, the antibody was eluted with four-column volumes 0.1 M glycine at pH 3.0. Fractions were collected and immediately neutralized with 1 M Tris-HCl, pH 9. Integrity and purity of each purified IgG was confirmed by reducing SDS-PAGE analysis. The protein concentration of the purified IgG was determined by Gyrolab sandwich immunoassay.

**Processing of IgG N-glycans**

IgGs (100 μg) were digested with trypsin for 16 h at 37°C. The reaction was terminated by heating the sample for 5 min at 95°C. Antibodies were further digested with 1 U PNGase F for 16 h at 37°C. Released N-glycans were isolated and desalted on reverse-phase Sep-Pak C18 cartridges followed by carboxypentaferrous (C18) extract-clean columns. Each N-glycan pool was digested with 10 μM Neuraminidase for 18 h at 37°C.

**Mass spectrometry**

N-Glycans were analyzed on an UltraFlex III TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with a smartbeam-II™ laser and a LIFT-MS/MS facility. Spectra were recorded in a reflector mode at an accelerating voltage of 25 kV and an extraction delay of 10 ns. Measurements were carried out in the positive-ion mode. External calibration was performed using a dextran ladder. Desialylated N-glycans were dissolved in H2O. Samples (0.5 μL) were mixed 1:1(v/v) with D-arabinosazone (5 mg/mL) dissolved in 70% aqueous ethanol on a steel target (Chen et al. 1997). Spectra were analyzed using Glyco-Peakfinder (Maas et al. 2007). Identified glycan structures were built with the GlycoWorkbench software (Ceroni et al. 2008).

**Fc-gamma receptor IIIA (FcγRIIIA) specific binding assay**

The FcγRIIIA-binding activity of the IgG samples was analyzed by an FcγRIIIA specific binding assay as described by Niwa, Hatanaka, et al. (2004) and Niwa, Shoji-Hosaka, et al. (2004) with slight modifications. A histidine (HIS)-tagged FcγRIIIA (F158) (22 kDa; 158F; R&D Systems, Minneapolis, MN) was used in combination with an anti-tetraHIS monoclonal antibody (Qiagen, Hilden, Germany) for receptor precipitation. Immunoplates (Maxisorp, Thermo, Waltham, MA) were coated with anti-tetraHIS antibody and blocked with blocking reagent (Roche Diagnostics, Penzberg, Germany). Subsequently, recombinant HIS-tagged FcγRIIIA was added to the immunoplates. Coated plates were then incubated with serial sample dilutions and controls so that they could bind the immobilized FcγRIIIA receptor. After a wash step, bound IgGs were detected by an anti-human IgG peroxidase-conjugated mAb (Dianova, Hamburg, Germany), and the amount of bound IgG was quantified via peroxidase activity. After each incubation step, the immunoplate was washed three times with phosphate buffer saline (PBS) containing 0.2% Tween-20. Tetramethylbenzidine (TMB; Seramun, Heidesee, Germany) was used as a chromogenic substrate; the reaction was terminated using 1 M sulfuric acid, and, finally, absorption was detected at 450 nm (Infinite F200 Reader, Tecan, Crailsheim, Germany). Based on the concentration-dependent absorption data, full curve fits were conducted using a four-parameter logistic curve model (Magellan Software 6.1, Tecan). The intraserial precision for this FcγRIIIA binding assay was determined to be within 15%CV.

**Antibody-dependent cellular cytotoxicity assay**

Primary human NK cells were isolated from peripheral blood mononuclear cells (PBMCs). PBMCs were separated from whole blood of healthy human donors by density gradient centrifugation, and NK cells were subsequently isolated by negative magnetic bead separation (Miltenyi, Bergisch Gladbach, Germany). The purity of the isolated NK cells was confirmed by flow cytometry (PE-conjugated CD16 and Alexa488-conjugated CD56 antibodies, BD, San Jose, CA). The cell lines BT-474 (Lasfargues et al. 1978, invasive ductal carcinoma of the breast, human, CLS, Eppelheim, Germany) and SK-BR-3 (Zabrecky et al. 1991, adenocarcinoma of the breast, human, ATCC, Manassas, VA) were used as target cells. Both cell lines were confirmed positive for the Her2/neu marker by flow cytometry (data not shown). The target cell lines were revitalized from a research cell bank 3 days prior to inoculation. Antibody-dependent NK cell induced target cell lysis was quantified by release of a vital stain (Calcein AM, Life Technologies, Carlsbad, CA). Target cells were stained according to the manufacturer’s protocol and seeded at 2 × 10^5 viable cells/well in 50 μL RPMI1640 (Life Technologies) + 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany) in 96-well micro titer plates. Serial 1:3 dilutions of antibodies in RPMI1640 + 10% FCS were prepared. Fifty microliters per well of each dilution was pipetted with n = 3 and pre-incubated with the target cells for 30 min at 37°C prior to NK cell inoculation. At the end of the antibody pre-incubation, effector cells were seeded at an effector to target cell ratio (E:T) of 5:1. The plates were subsequently centrifuged at 200 × g for 3 min and incubated for 4 h at 37°C and 5% CO2. For each cell line, a spontaneous target cell lysis control (with NK cells), and a total target cell lysis control (induced by saponin) were induced. The total lysis in the control wells was induced by adding 15 μL/well 0.1 mg/mL saponin in RPMI1640 + 10% FCS 15 min before the end of the incubation period. In all other wells, 15 μL RPMI1640 + 10% FCS were added. The release of calcine AM was quantified by fluorescence detection in the culture supernatant. The plates were centrifuged (150 × g; 3 min), and 100 μL supernatant from each well was transferred into a 96-well black fluorescence plate (Thermo). The mean fluorescence intensity (MFI) was detected using an Infinite F200 reader (Tecan, 485/535 nm excitation/emission filter). Curve fitting was done using a four-parameter logistic dose–response model (Magellan software version 6.1). The specific cell lysis was calculated as follows: Specific cell lysis [%] = [MFI(sample) – MFI(spontaneous)]/[MFI(total) – MFI(spontaneous)] × 100.

Whereas MFI(sample) is the mean fluorescence intensity released by specific target cell lysis, MFI(spontaneous) is the gradual release of the fluorescent dye by the target cells, and
MFI(total) is mean fluorescence intensity obtained after detergent induced total target cell lysis.

**Monosaccharide analysis and rhamnose determination**

Cells (3 × 10⁷) were separated from the supernatant by centrifugation at 100 × g for 5 min. They were subsequently lysed by three freeze–thaw cycles. Cell membranes were then separated from cytosolic fractions at 21,000 × g for 30 min at 4°C. Cell culture supernatants, cell membranes, cytosolic fractions, as well as IgG N-glycans were hydrolyzed in 2 N TFA for 4 h at 100°C. After evaporation under reduced atmosphere, samples were analyzed by HPAEC-PAD on a PA-1 column using a Dionex ICS-3000, and 2-deoxyribose was used as the internal standard. Neutral monosaccharides were separated by isocratic elution with 2.25 mM NaOH. Postcolumn addition of 200 mM NaOH enabled amperometric detection. Since HPAEC-PAD does not allow assignment of enantiomeric identity (Horton, D. 2004), L-rhamnose was used as a standard to determine the retention time expected for D-rhamnose. The LOD for rhamnose was determined as described in Chapter 6.3 of the ICH harmonized tripartite guideline for validation of analytical procedures (ICHQ2 (R1)).

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**Conflict of interest statement**

H.H.v.H, C.O. and V.S. have applied for patent protection for the described procedure to modify pharmaceutical producer cell lines.

**Abbreviations**

ACN, acetonitrile; ADCC, antibody-dependent cell-mediated cytotoxicity; AMV, avian myeloblastosis virus; CHO, Chinese hamster ovary; CV, coefficient of variation; dHex, deoxyhexose; DMSO, dimethylsulfoxide; EC₅₀, Half maximal effective concentration (i.e. the concentration of agonist that provokes a response halfway between the baseline and maximum response); FCS, fetal calf serum; Fuc, fucose; Gal, galactose; GFP, green fluorescent protein; GlcNAc, N-acetylgalcosamine; GMD, GDP-mannose-4,6-dehydratase; GlcTIII, b(1,4)-N-acetylglucosaminyltransferase III; HPAEC-PAD, high-pH anion-exchange chromatography with pulsed amperometric detection; LOD, limit of detection; mAb, monoclonal antibody; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MEM, modified Eagle’s medium; MFI, mean fluorescence intensity; NK, natural killer cells; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffer saline; PCR, polymerase chain reaction; PNGase F, peptide-N4-(N-acetyl-β-glucosaminy) asparagine amidase F; Rha, rhamnose; RMD, GDP-6-deoxy-D-lyxo-4-hexulose reductase; SD, standard deviation; SDS, sodium dodecyl sulfate; TFA, Trifluoroacetic acid; TMB, tetramethylbenzidine; WT, wild type.

**References**


