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Technical note

The enzymatic removal of immunoglobulin variable domain glycans by different glycosidases

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ABSTRACT

About 15% of immunoglobulin G (IgG) molecules contain glycans linked to the antigen-binding fragments (Fab arms) in addition to the glycans linked to the crystallizable fragment (Fc tail) of all IgGs. Fab glycosylation appears to be an important feature of antibodies, for example by influencing antigen binding and antibody stability. The reliable generation of antibodies that either have or lack Fab glycans would be very helpful to study the role of Fab glycans in more detail. In this study, we set out to remove Fab glycans by treating polyclonal and monoclonal human IgG antibodies with two commonly used glycosidases and an improved version of one of the two (Endo F3, PNGase F, and Rapid™ PNGase F). Fc glycans can be removed using PNGase F and Rapid™ PNGase F, but not with Endo F3. For most antibody clones, Endo F3 partially cleaved off the Fab glycans. In contrast, PNGase F left the Fab glycans of most clones unaffected, but could remove glycans of some clones. Rapid™ PNGase F showed a higher glycosidase efficacy than PNGase F, and more clones could be deglycosylated using this enzyme. In summary, not all Fab glycans can be cleaved off by the tested glycosidases (under non-denaturing conditions), suggesting that Fab glycans are exposed to different degrees.

1. Introduction

Immunoglobulin G (IgG), the most abundant class of immunoglobulins in the circulation, contains glycans linked to the crystallizable fragment (Fc tail). In addition, about 15% of IgGs contain glycans linked to the antigen-binding fragments (Fab arms). These Fab glycans are mainly highly processed/sialylated complex biantennary glycans (Bondt et al., 2014). Fab glycans have not been studied as extensively as Fc glycans, but, like the latter, Fab glycosylation appears to be an important feature of antibodies (van de Bovenkamp et al., 2016). For example, we and others showed that Fab glycans can not only affect antibody stability (van de Bovenkamp et al., 2018a; Courtois et al., 2016), but also influence antigen binding (van de Bovenkamp et al., 2018b; Wright et al., 1991; Coloma et al., 1999; Leibiger et al., 1999; Schneider et al., 2015), which makes Fab glycosylation an additional layer of regulation of the humoral immune system.

So far, antibodies with Fab glycans were compared with antibodies without Fab glycans that were generated either by removing glycosylation sites through mutations or by expressing antibodies in the presence of tunicamycin, which blocks *N*-linked glycosylation. The removal of glycosylation sites through mutations not only prevents Fab glycosylation, but also changes the primary structure of the antibody. Expression of antibodies in the presence of tunicamycin blocks Fab glycosylation as well as Fc glycosylation, and tremendously reduces the antibody yield. Furthermore, neither method can be used to study effects of Fab glycosylation of serum-derived polyclonal IgG. Therefore, the establishment of an enzymatic method to generate antibodies that lack Fab glycans is much desired as an additional tool to study the role of Fab glycans in more detail.

In this study, we explored the efficacy of Fab glycan removal by treating antibodies with two relevant glycosidases. Endoglycosidase F3 (Endo F3) (Trimble and Tarentino, 1991; Plummer Jr and Tarentino, 1991; Tarentino et al., 1993) cleaves between the two *N*-acetylglucosamine residues, generating antibodies with only one monosaccharide (GlcNAc) attached. Peptide-*N*-glycosidase F (PNGase F) (Plummer Jr et al., 1984; Tarentino et al., 1985; Lemp et al., 1990) cleaves between the asparagine residue and the first *N*-acetylglucosamine residue, generating antibodies completely devoid of glycans. Rapid™ PNGase F cleaves at the same position as PNGase F, but was described to more effectively remove glycans than PNGase F. Our results show that not all Fab glycans can be cleaved off by the tested glycosidases (without denaturing conditions), and that Fab glycans at different positions are deglycosylated to different degrees, possibly caused by different degrees of Fab glycan exposure.

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2. Materials and methods

2.1. Polyclonal IgG

Intravenous immunoglobulin (IVIg) was obtained from Sanquin (Nanogam, Amsterdam, The Netherlands) and contains IgGs purified from thousands of voluntary plasma donors.

2.2. SNA affinity chromatography

IVIg was enriched for Fab glycans using *Sambucus nigra* agglutinin (SNA) affinity chromatography as described previously (van de Bovenkamp et al., 2018b). SNA binds virtually exclusively to sialic acid residues in Fab glycans due to the relative inaccessibility of Fc glycans in natively folded IgG (Käsermann et al., 2012; Guhr et al., 2011; Stadlmann et al., 2009). In line with this, it was previously shown that SNA affinity chromatography does not enrich for Fc glycans, but does result in a 7-fold increase in Fab glycans (which is in agreement with ~15% Fab glycosylation of total IgG) (Guhr et al., 2011). Briefly, IVIg was applied to an SNA column and after washing away unbound proteins, bound proteins containing sialic acid were eluted with 0.5 M lactose in 0.2 M acetic acid. SNA-enriched IVIg was dialyzed against PBS.

2.3. Monoclonal IgG

Seven adalimumab (ADL) clones, five anti-ADL clones, four anti-infliximab (anti-IFX) clones, two anti-D clones, and two anti-TNP clones, all with Fab glycans ($n = 20$), and ADL wild-type, without Fab glycans, were used in this study. The ADL, anti-D, and anti-TNP clones were designed and produced as described previously (5, Koers et al., unpublished). The anti-ADL and anti-IFX clones were obtained, designed, and produced as described previously (van de Bovenkamp et al., 2018b; van Schouwenburg et al., 2014; van Schie et al., 2017). ADL wild-type, which was unmodified, i.e., had an identical amino acid sequence to Humira, was also produced as described previously (van de Bovenkamp et al., 2018b), and all antibodies were generated and purified as described previously (van de Bovenkamp et al., 2018b).

2.4. Endo F3 treatment

The glycosidase Endo F3 (Sigma-Aldrich) cleaves between the two *N*-acetylglucosamine residues. 50 µg glycoprotein was incubated for 24 h at 37 °C with 0.005 units (1 unit is defined as the amount of enzyme required to remove the carbohydrates from 1 µmole denatured porcine fibrinogen in 1 min at 37 °C) Endo F3 in reaction buffer (50 mM sodium acetate pH 4.5, Sigma-Aldrich) diluted in water in a total volume of 132 µL, and stored at −20 °C.

2.5. PNGase F treatment

The glycosidase PNGase F (New England BioLabs) cleaves between the asparagine residue and the first *N*-acetylglucosamine residue. 10 µg glycoprotein was incubated for 48 h at 37 °C with 25 units (1 unit is defined as the amount of enzyme required to remove > 95% of the carbohydrates from 10 µg denatured RNase B in 1 h at 37 °C in a total volume of 10 µL) PNGase F in PBS in a total volume of 30 µL, and stored at −20 °C. As a control, some samples were treated with PNGase F under denaturing conditions. To this end, samples were incubated for 10 min at 100 °C in glycoprotein denaturing buffer (0.5% SDS, 40 mM DTT, New England BioLabs), and treated with PNGase F in the presence of 1% NP-40 (New England BioLabs) and glycobuffer 2 (50 mM sodium phosphate pH 7.5, New England BioLabs) diluted in water for 1 h at 37 °C.

2.6. Rapid™ PNGase F treatment

The glycosidase Rapid™ PNGase F (New England BioLabs) cleaves between the asparagine residue and the first *N*-acetylglucosamine residue. 10 µg glycoprotein was incubated for 24 h at 37 °C with 0.33 µL Rapid™ PNGase F in 5× reaction buffer (New England BioLabs) diluted in water in a total volume of 10 µL, and stored at −20 °C.

2.7. Gel electrophoresis

Samples were analyzed by SDS-PAGE by loading 5 µg IgG on precast 4–12% Bis-Tris gels (NuPAGE), visualized with Coomassie Blue (InstantBlue). To examine heavy and light chains separately, samples were reduced with DTT. Band intensities were determined using ImageJ and the percentage of deglycosylated IgG was calculated by the following formula: $100\%-((I^+t/(I^+t + I^-t))/(I^+u/(I^+u + I^-u))*100\%)$, where I^+t = intensity band with Fab glycans treated, I^-t = intensity band without Fab glycans treated, I^+u = intensity band with Fab glycans untreated, and I^-u = intensity band without Fab glycans untreated.

2.8. SNA ELISA

The SNA ELISA was performed as described previously (van de Bovenkamp et al., 2018b). Briefly, samples were coated on plates and detected with biotinylated SNA (Vector Laboratories), streptavidin labeled with horseradish peroxidase, and tetramethylbenzidine. Alternatively, to check for binding of IgG to the plates, samples were detected with anti-IgG labeled with horseradish peroxidase (MH16–1 HRP, Sanquin Reagents, Amsterdam, The Netherlands) and tetramethylbenzidine. The reaction was stopped with 2 M H_2SO_4, the absorbance was measured at 450 and 540 nm using a BioTek microtiter plate reader, and relative SNA binding was calculated relative to a calibration curve of IVIg. In parallel, IgG adsorption was measured using anti-IgG detection. The percentage of deglycosylated IgG was calculated by the following formula: (1-((SNA signal/IgG signal)_treated/(SNA signal/IgG signal)_untreated))*100%.

3. Results

3.1. The removal of Fab glycans on polyclonal IgG

To investigate the glycosidase efficacy of endoglycosidase F3 (Endo F3) and peptide-*N*-glycosidase F (PNGase F), polyclonal IgG was treated with these glycosidases. Intravenous immunoglobulin (IVIg) contains a fraction of IgG with Fab glycans (~15%). We enriched for this Fab-glycosylated fraction using SNA affinity chromatography, and glycosidase-treated samples were compared with untreated samples using gel electrophoresis and SNA ELISA. Since Fc glycans are not recognized by SNA (because they are poorly sialylated and partially buried), the SNA ELISA is specific for Fab glycans (Fig. 1). In contrast, the gel electrophoresis data provide information about both Fc and Fab glycans (Fig. 2A). Whereas Endo F3 does not cleave off Fc glycans (Fig. 2B), PNGase F does (Fig. 2C).

When SNA-enriched IVIg was first denatured and then treated with PNGase F, it was completely deglycosylated (Fig. 2D, 1st gel), showing that this enzyme is able to remove all glycans when accessible. Endo F3 treatment of SNA-enriched IVIg resulted in a partial size shift (Fig. 2D, 2nd gel), but the SNA ELISA did not show a difference between Endo F3-treated and untreated IVIg (Table 1). PNGase F treatment of SNA-enriched IVIg resulted in a size shift representing the removal of Fc glycans, but the ratio between the Fab-glycosylated and non Fab-glycosylated fractions did not change (Fig. 2D, 2nd gel). In line with this, the SNA ELISA did not show a difference between PNGase F-treated and untreated IVIg (Table 1). Using Rapid™ PNGase F (an improved version of PNGase F), treatment of SNA-enriched IVIg did result in a size shift

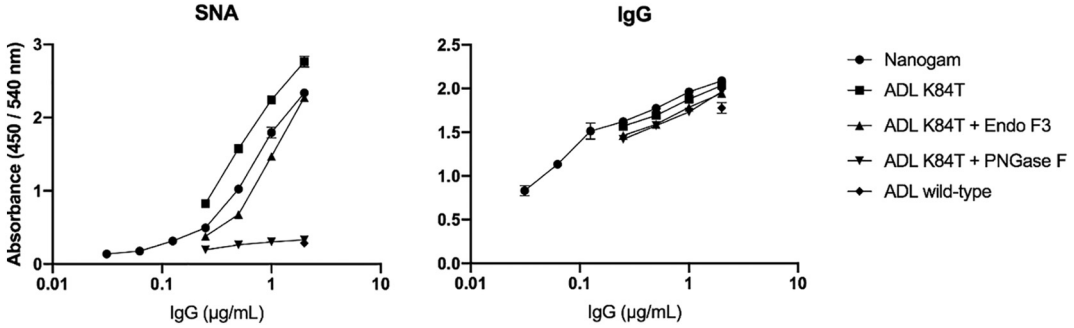

Fig. 1. SNA ELISA to estimate relative levels of Fab glycans. The absorbance was measured at 450 and 540 nm, and relative SNA binding was calculated relative to a calibration curve of IVIg (Nanogam). In parallel, IgG adsorption was measured using anti-IgG detection. In this example, ADL wild-type (without Fab glycans) does give a signal for IgG but not for SNA, showing that the SNA ELISA does not recognize Fc glycans, i.e., is specific for Fab glycans. The percentage of deglycosylated IgG was calculated by the following formula: (1-((SNA signal/IgG signal)_treated/(SNA signal/IgG signal)_untreated))∗100%.

representing the removal of Fc glycans as well as a change in the ratio between the Fab-glycosylated and non Fab-glycosylated fractions (Fig. 2D, 2nd gel), and the SNA ELISA confirmed that Fab glycans were partially removed (~15%) by this glycosidase (Table 1).

3.2. The removal of Fab glycans on monoclonal IgG

We next treated monoclonal IgG with Endo F3 and (Rapid™) PNGase F to study the glycosidase efficacy of these glycosidases in more detail. Seven ADL clones, five anti-ADL clones, four anti-IFX clones, two anti-D clones, and two anti-TNP clones, all with Fab glycans (n = 20), that together span a variety of glycosylation sites across the VH and VL

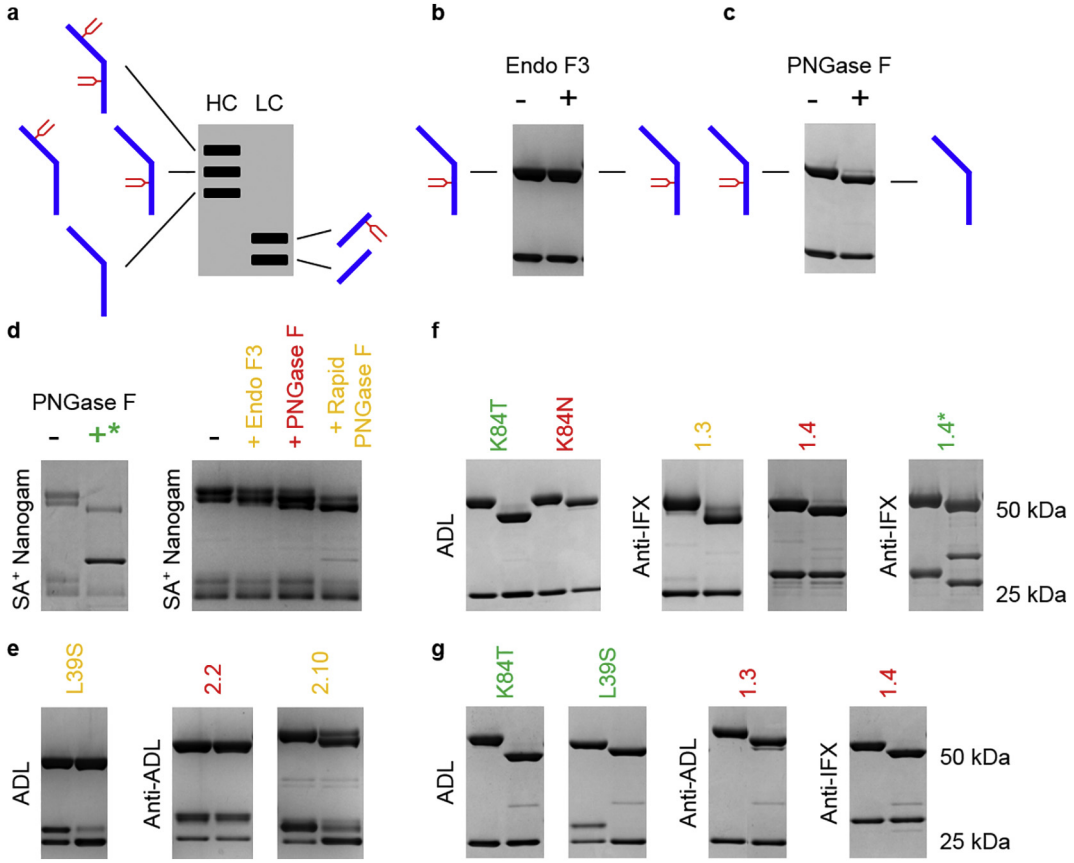

Fig. 2. Gels of antibody clones treated with different glycosidases. (A) Schematic representation of possible size shifts on gel upon glycosidase treatment. For both the heavy and light chains, no size shift indicates no deglycosylation. For the heavy chain (HC, ~50 kDa), a smaller size shift indicates either the removal of Fc or Fab glycans, and a bigger size shift indicates the removal of both Fc and Fab glycans. For the light chain (LC, ~25 kDa), a size shift indicates the removal of Fab glycans. Based on enzyme specificities, Endo F3 treatment would result in a slightly smaller size shift than PNGase F treatment. (B) Endo F3 treatment of ADL wild-type (with heavy chains with only Fc glycans) does not show a size shift, indicating that Endo F3 does not cleave off Fc glycans. (C) In contrast, PNGase F treatment of ADL wild-type does show a size shift, indicating that PNGase F does cleave off Fc glycans. (D) SNA-enriched (SA^+) Nanogam was treated with Endo F3, PNGase F, or Rapid™ PNGase F. As a control, SA^+ Nanogam was also treated with PNGase F under denaturing conditions (*). Representative examples of antibody clones for which Fab glycans are largely (green), partially (orange), or not (red) deglycosylated by (E) Endo F3, (F) PNGase F, or (G) Rapid™ PNGase F. As a control, anti-ADL 1.3 (not shown) and anti-IFX 1.4 were also treated with PNGase F under denaturing conditions (*). The molecular weight of Endo F3 and (Rapid™) PNGase F is 38.8 kDa and 36 kDa, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Overview of antibody clones and glycosidase efficacies.

Position	Group	Clone	Allele	Motif	Endo F3		PNGase F		Rapid™ PNGase F	
					SNA[a]	Gel	SNA[a]	Gel	SNA[a]	Gel[a]
–	Nanogam (SA^+)	–	–	–	–	+	–	–[b]	–[c]	+/++
N_H29	Anti-IFX	1.3	IGHV1–18	NFT	+	+	++++	+++		
N_H29	Anti-TNP	S29N	IGHV4–28[d]	NIT		+++	++	++++		
N_H59	ADL	G63S	IGHV3–9	NSS	+++	++	–	–		
N_H66	Anti-ADL	1.3	IGHV1–18	NYT	+++	+++	–	–[b]	–	–
N_H66	Anti-IFX	2.1	IGHV1–69	NYT	+++	+++	–[c]	++	++++	+++
N_H77	ADL	T77N	IGHV3–9	NIS	+++	++	–	–	++++	++++
N_H77	Anti-ADL	2.6	IGHV4–39	NIS	++	++	–	+	+++	++++
N_H77	Anti-ADL	2.10	IGHV1–03	NIT		+++		–		++++
N_H77	Anti-D	T77N	IGHV3–30-3	NIS	+++	+	–	–	–	–
N_H82	ADL	K84T	IGHV3–9	NAT	++	+	++++	++++	++++	++++
N_H84	ADL	K84N	IGHV3–9	NNS	++	+	–	–		
N_H84	Anti-IFX	2.3	IGHV3–30	NNT	++	++	–	–		
N_H90	Anti-ADL	2.7	IGHV4–34	NLT		–		–		
N_L26	Anti-ADL	2.10	IGKV3–20	NRS		+		–		+++
N_L37	ADL	L39S	IGKV1–27	NYS	++++	++	–	–	++++	++++
N_L79	ADL	S79N	IGKV1–27	NGS	+++	++	–	–	++	++++
N_L79	Anti-ADL	2.2	IGKV1–33	NGS	–	–	–	–	++	+
N_L79	Anti-D	S79N	IGKV1–39	NGS		++		–		
N_L86	ADL	D86N	IGKV1–27	NFT	+++	++	–	–		
N_L86	Anti-TNP	D86N	IGKV2D-29[d]	NFT		++	–	–		
N_L110	Anti-IFX	1.4	IGLV2–23	NIT	+++	++	–	–[b]	+	–

ADL = adalimumab, IFX = infliximab, TNP = trinitrophenyl.
– ≤ 20%, + = 20–40%, ++ = 40–60%, +++ = 60–80%, ++++ ≥ 80% deglycosylated.
[a] empty = not determined.
[b] completely deglycosylated when denatured.
[c] partially deglycosylated (~15–20%).
[d] concerns mouse variable domains.

domains, were first treated with Endo F3 and analyzed on gel and in SNA ELISA (Fig. 2E and Table 1). The treatment of ADL wild-type, without Fab glycans, showed that Endo F3 does not cleave off Fc glycans (Fig. 2B). For most clones with Fab glycans, Endo F3 partially cleaved off the glycans (Fig. 2E and Table 1). For two clones (anti-ADL 2.2 and anti-ADL 2.7), no deglycosylation was observed (Fig. 2E and Table 1).

Next, all monoclonal IgG clones were treated with PNGase F and analyzed on gel and in SNA ELISA (Fig. 2F and Table 1). The treatment of ADL wild-type, without Fab glycans, showed that PNGase F does cleave off Fc glycans (Fig. 2C). In contrast to Endo F3, PNGase F left the Fab glycans of most clones totally unaffected; deglycosylation was only observed for ADL K84 T, anti-IFX 1.3, and anti-TNP S29N, and partially for anti-IFX 2.1 (~20%) (Fig. 2F and Table 1). When two clones with Fab glycans that were totally unaffected by PNGase F (anti-ADL 1.3 and anti-IFX 1.4) were first denatured and then treated with PNGase F, they were completely deglycosylated, again showing that this enzyme is able to remove the glycans when accessible (Fig. 2F and Table 1).

Since PNGase F was not able to cleave off the Fab glycans of all clones, we tested the improved version of PNGase F, Rapid™ PNGase F, for some of the clones (Fig. 2G and Table 1). Rapid™ PNGase F cleaved off the majority of Fab glycans for 7 out of 11 clones, and only two of the tested clones (anti-ADL 1.3 and anti-D T77N) were totally unaffected (Fig. 2G and Table 1). Compared to normal PNGase F, Rapid™ PNGase F showed a higher glycosidase efficacy for ADL T77N, ADL L39S, ADL S79N, anti-ADL 2.2, anti-ADL 2.6, anti-ADL 2.10, and anti-IFX 2.1 (Table 1).

Table 1 shows the Fab glycan position, VH/VL allele, and *N*-glycosylation motif for all antibody clones. We did not find a correlation between these variables and glycosidase efficacy. Together, our results show that not all Fab glycans can be cleaved off by the tested glycosidases (without denaturing conditions), and that Fab glycans at different positions are deglycosylated to different degrees.

4. Discussion

In this study, we analyzed the glycosidase efficacy on polyclonal IgG as well as monoclonal IgG of different glycosidases and found that PNGase F and Rapid™ PNGase F cleave off Fc glycans, whereas Endo F3 does not. For most clones, Endo F3 partially cleaved off the Fab glycans. In contrast, PNGase F left the Fab glycans of most clones totally unaffected. On the other hand, Rapid™ PNGase F showed a higher glycosidase efficacy, and could efficiently remove the Fab glycans for a subset of the tested monoclonal antibodies.

Our data suggest that Fab glycans are exposed to different degrees, and therefore the Fab glycans of some clones are easily cleaved off by both glycosidases (ADL K84T, anti-IFX 1.3, and anti-TNP S29 N), whereas the Fab glycans of other clones are probably less exposed and therefore resistant for Endo F3 and PNGase F (anti-ADL 2.2 and anti-ADL 2.7). For the antibody clones examined in this study, PNGase F does not cleave off Fab glycans of the light chain. We are not aware of other studies addressing deglycosylation of light chain variable domain glycans. Fab glycans at position 29 of the VH (anti-IFX 1.3 and anti-TNP S29 N) seem to be cleaved off by PNGase F. Overall, Fab glycans at different positions are deglycosylated to different degrees, possibly caused by different degrees of Fab glycan exposure.

Endo F3 is reported not to cleave off high-mannose and hybrid glycans, and non-fucosylated complex biantennary glycans at a slow rate. It does efficiently cleave off core fucosylated complex biantennary glycans. Nevertheless, we did not observe removal of Fc glycans using Endo F3, despite the fact that Fc glycans are highly (~95%) fucosylated complex biantennary glycans, as was shown for ex vivo purified (Bondt et al., 2014) as well as in vitro expressed (Dekkers et al., 2016) antibodies. Why Endo F3 does not cleave off Fc glycans remains elusive. Our finding that Endo F3 partially cleaved off the Fab glycans of most clones might indicate that these glycans are only partially fucosylated, leaving the non-fucosylated glycans unaffected by this enzyme.

It is noteworthy that PNGase F left the Fab glycans of most clones unaffected. Our results were unexpected, since Fc glycans have been

described to be partially buried and thus less exposed than Fab glycans (Lammerts van Bueren et al., 2011), whereas our findings indicate that PNGase F easily cleaves off all Fc glycans and only the Fab glycans of some clones, suggesting that Fc glycans actually are more exposed than Fab glycans. In contrast, certain lectins, in particular SNA, do not recognize Fc glycans of natively folded IgG (but do bind to Fc glycans of denatured IgG) (Dalziel et al., 1999), which is not in line with Fc glycans being more exposed than Fab glycans. However, PNGase F (36 kDa) is smaller than the fairly bulky lectin SNA (140 kDa), which might explain why PNGase F is able to reach the Fc glycans while SNA is not. In contrast, size does not explain why Endo F3 does not cleave off Fc glycans, since this glycosidase (38.8 kDa) has a similar size as PNGase F. Since the position of Fab glycans varies between different antibodies, the local structure around the Fab glycans probably influences the efficacy of PNGase F.

In summary, Endo F3 and Rapid™ PNGase F may be used to generate (natively folded) antibodies without Fab glycans for some clones, although they cannot be universally applied.

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