A method to detect the binding of hyper-glycosylated fragment crystallizable (Fc) region of human IgG1 to glycan receptors

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Summary

Engineering the fragment crystallizable (Fc) of human IgG1 can bring improved effector functions to monoclonal antibodies and Fc fusion-based medicines and vaccines. Such Fc-effector functions are largely controlled by post-translational modifications (PTMs) within the Fc, including the addition of glycans that introduce structural and functional heterogeneity to this class of therapeutic. Here we describe a detailed method to allow the detection of hyper-sialylated Fcs binding to glycan receptors that will facilitate the future development of new mAbs and Fc-fragment therapies and vaccines.

Key words: IgG1, glycans, glycosylation, Fc receptors, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), complement-dependent cytotoxicity (CDC), effector function, therapeutic antibodies.

Introduction

Immunoglobulin G antibodies are glycosylated at asparagine 297 (Asn297) of the unique N-linked sequon located within the Fc (Czajkowsky et al. 2012; Lund et al. 1996). The Fc glycans attached at Asn297 are typically biantennary complex types, exhibiting high levels of fucosylation of the core GlcNAc residue, partial galactosylation and bisecting GlcNAc, and of these structures less than 20% are sialylated (Dalziel et al. 2014). The low levels of branching and terminal structures, such as sialic acid, are a consequence of constraints imposed on Asn297 glycan processing by the Fc protein backbone (Frank et al. 2014; Subedi et al. 2014).

The composition of glycans attached at Asn297 significantly affects Fc-mediated interactions with different receptors (Dalziel et al. 2014), and multiple lines of evidence have shown that glycosylation is critical to driving either the anti- or pro-inflammatory capability of IgG1 (Schwab and Nimmerjahn 2013). Glycosylation of Asn297 in the Fc is thus essential for interactions with type 1 receptors (Fcγ) and type 2 receptors (glycan dependent) and is also necessary for driving interactions with the complement cascade (Czajkowsky et al. 2012). Although protocols essentially are well described for detecting interactions with type I Fcγ receptors, detailed protocols for the detection of such reagents to type 2 glycan receptors are less commonly described in the literature.

In humans, infusion of Fc fragments is sufficient to ameliorate idiopathic thrombocytopenic purpura (ITP) in children, demonstrating the therapeutic utility of the Fc *in vivo* (Debre et al. 1993). The anti-inflammatory property of the Fc is lost after deglycosylation of IgG1, and a small population of IgG1-bearing sialylated Fcs has been identified as making a significant contribution to the control of inflammation in animal models (Anthony et al. 2011; Anthony et al. 2008; Washburn et al. 2015). Higher levels of sialylation also lead to longer serum retention times (Li et al. 2006; Liu 2015), and studies in

humans and mice have shown that influx and efflux of IgG1 into the central nervous system (CNS) is glycan and sialic acid dependent (Finke et al. 2017; St-Amour et al. 2013; Zhang et al. 2004). Consequently, IgG1-Fc sialylation has emerged as an important but controversial concept for regulating anti-inflammatory activity of antibodies and Fc fragments (Schwab and Nimmerjahn 2013).

Methods to enhance the sialylation of the Fc have largely focused on modifications to Asn297 attached glycans (Fiebiger et al. 2015; Washburn et al. 2015). We have previously shown how Asn297-limited approaches can be overcome through the introduction of additional N-linked glycosylation sites into a limited number of exposed areas within the IgG1-Fc fragment (Blundell et al. 2017). For example, insertion of a N-terminal hinge-distal glycan site (N221) significantly increases sialylation, allowing engagement with glycan receptors not previously known to bind the Fc (Blundell et al. 2017). By adding a cysteine-silenced 18 amino-acid C-terminal extension containing an additional N-linked site (N563), further glycan complexity can also be brought to the IgG1 Fc (Fig. 1).

We provide a detailed protocol for the detection of glycan modified IgG1-Fc fragments containing one, two or three additional N-linked glycans to glycan receptors by enzyme-linked immunosorbent assays (ELISA).

Materials

Coating buffer: 0.05 M carbonate-bicarbonate solution (Sigma C3041), pH 9.6.

Incubation and wash buffer: TMS (20 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$).

Blocking buffer: TMS solution containing 5% bovine serum albumin (Sigma A7030).

Developing solution: Sigmafast p-Nitrophenyl phosphate dissolved in Tris-buffer (Sigma N2770).

Glycan receptors: lyophilized histidine-tagged recombinant Siglec-1 (R&D Systems, 5197-SL-050) or Siglec-4/MAG (Sinobiologicals, 13186-H08H) were reconstituted to 100 μg/ml with ddH_2O.

Detecting antibodies: Affinity-purified alkaline-phosphatase conjugated $F(ab')_2$ fragment goat anti-human IgG1 Fcγ-fragment specific (Jackson Immuno Research 109-056-008, or Invitrogen H10108).

Specialist equipment: Microplate reader capable of measuring absorbance at 405 nm wavelength, microplate washer, data analysis and graphing software.

Methods

Nunc microtiter plates are coated down with recombinant glycan receptors[1], typically 100 μl per well at 2-10 μg/ml in carbonate-bicarbonate coating buffer pH9.6, and incubated overnight at 4°C.

The following day plates are washed five times with excess TSM incubation buffer (1-5 min incubation between washes), prior to blocking for 2 hrs in 150 μl per well of TMS buffer containing 5% bovine serum albumin. Plates are then washed as before. 100 μl of varying concentrations of Fc fragments in TSM incubation buffer are added[2] in duplicate wells. We typically titrate the Fc fragments by doubling dilution down the plate from 50 to 0 μg/ml. Receptors are allowed to bind the Fc fragments overnight at 4°C.

The following day plates are washed five times with excess TSM incubation buffer (1-5 min incubation between washes), prior to the addition of 100 μl per well of alkaline-phosphatase conjugated $F(ab')_2$ goat anti-human IgG1 Fcγ fragment-specific detection antibody diluted 1 in 500 in TMS buffer. Glycosylated Fc fragments that have bound to the glycan receptors are allowed to bind the conjugated antibody for 1 h at room temperature on a rocking platform.

Plates were washed as above and developed for 5-15 minutes with 100 μl/well of developing solution. The plates were read at 405nm wavelength using a LT-4500 microplate absorbance reader (Labtech), and the data plotted with GraphPad Prism (Fig. 1).

Notes

1. We have found that this protocol is amenable to the study of many other glycan receptors. However, from our experience we advise determining background binding of the detecting antibody to each glycan receptor first, as the Fab domains within the alkaline phosphatase-conjugated detecting antibody can themselves be glycosylated (van de Bovenkamp et al. 2016). For example, we have seen direct binding of both the Jackson and Invitrogen $F(ab')_2$ detecting reagents to Siglec-5, CLEC-1B and DC-SIGNR (Blundell et al. 2017). Preliminary removal of $F(ab')_2$ associated glycans with glycosidases e.g. PNGase F (New England Biologicals) may be required for the study of certain receptors.
2. This protocol works equally well for Fc fragments and Fab'_2 fragments (both available from Jackson Immuno Research) that have previously been digested with commercially available glycosidases e.g. PNGase F, Endo H or neuraminidase. For example, Siglec-5 binding above is lost after neuraminidase treatment.

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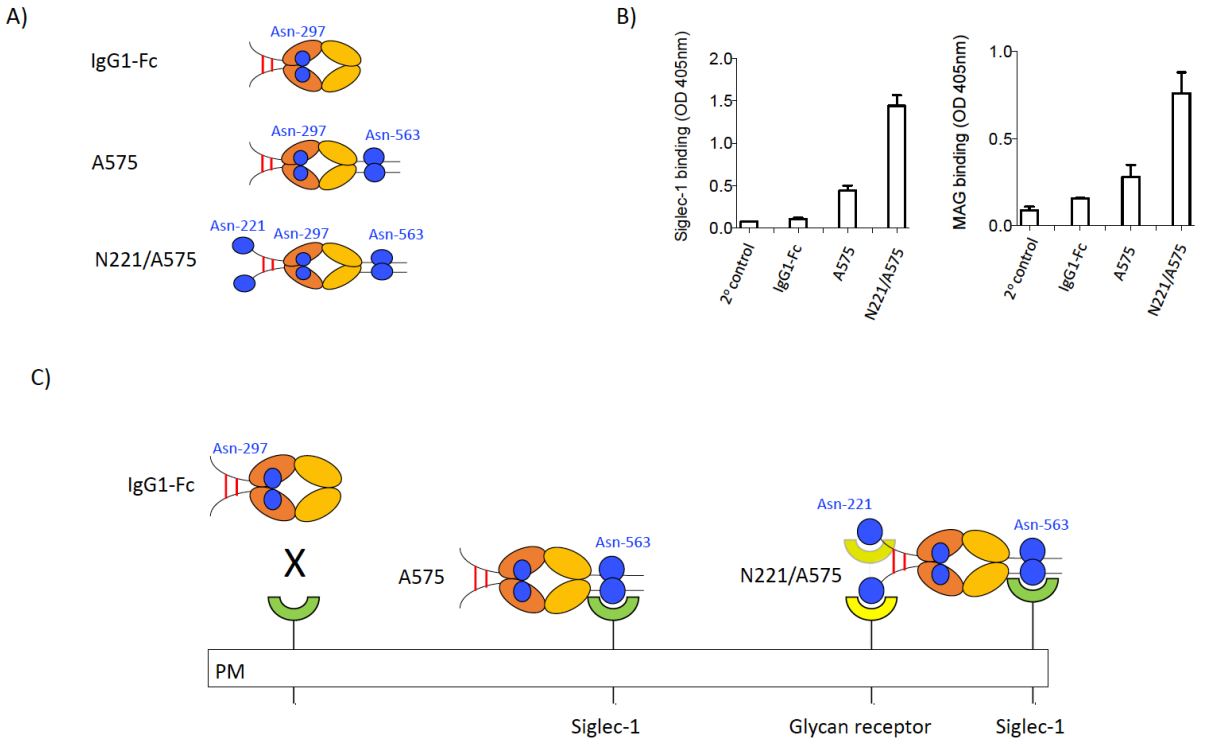

Fig. 1. Detection of hyper-sialylated Fc-fragment binding to glycan receptors by ELISA.

(A) Fc fragments containing one, two or three N-linked attachment sites together with methods for determining their glycan composition by HILIC-UPLC analysis have been described previously (Blundell et al. 2017).

(B) Fc fragments containing three N-linked glycans (N221/A575) bind more strongly to both Siglec-1 and Siglec-4 (myelin-associated glycoprotein), than Fc fragments only containing two (A575) or one N-linked sugar (IgG1 Fc control). Error bars represent standard deviations around the mean value, n = 3 independent experiments.

(C) In contrast with the Asn297 glycan, which is largely buried within the Fc cavity, Asn221 and Asn563 are located at the N- and C-terminal tips of the Fc respectively and, as our data show, would therefore be more accessible for post-translational modifications by glycan-modifying enzymes that permit A575 and N221/A575 to bind more strongly to sialic-acid dependent receptors.