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A method for high-throughput, sensitive analysis of IgG Fc and Fab glycosylation by capillary electrophoresis

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ABSTRACT

The N-glycan of the IgG constant region (Fc) plays a central role in tuning and directing multiple antibody functions in vivo, including antibody-dependent cellular cytotoxicity, complement deposition, and the regulation of inflammation, among others. However, traditional methods of N-glycan analysis, including HPLC and mass spectrometry, are technically challenging and ill suited to handle the large numbers of low concentration samples analyzed in clinical or animal studies of the N-glycosylation of polyclonal IgG. Here we describe a capillary electrophoresis-based technique to analyze plasma-derived polyclonal IgG-glycosylation quickly and accurately in a cost-effective, sensitive manner that is well suited for high-throughput analyses. Additionally, because a significant fraction of polyclonal IgG is glycosylated on both Fc and Fab domains, we developed an approach to separate and analyze domain-specific glycosylation in polyclonal human, rhesus and mouse IgGs. Overall, this protocol allows for the rapid, accurate, and sensitive analysis of Fc-specific IgG glycosylation, which is critical for population-level studies of how antibody glycosylation may vary in response to vaccination or infection, and across disease states ranging from autoimmunity to cancer in both clinical and animal studies.

1. Introduction

Beyond their ability to neutralize pathogens, antibodies are able to mediate an array of effector functions through their interaction with Fc-receptors, complement molecules, and mammalian lectin-like molecules (Kapur et al., 2014). While the neutralizing activity of an antibody is mediated largely by its variable domain (Fab, antigen-binding fragment), its ability to perform extra-neutralizing functions is determined by the constant domain (Fc, crystallizable fragment) (Schroeder and Cavacini, 2010). Though the Fc is referred to as constant, it is in fact variable in two major aspects: a) protein sequence varies through subclass or isotype selection (Nimmerjahn and Ravetch, 2010) and b) glycosylation variation of N-linked glycans which change rapidly during an immunologic response (Xue et al., 2013). Together, these alterations in the antibody Fc significantly modify the effector function of antibodies, such as antibody-dependent cellular cytotoxicity (ADCC) (Davies et al., 2001; Shields et al., 2002; Shinkawa et al., 2003; Shoji-Hosaka et al., 2006) and complement-dependent cytotoxicity (CDC) (Karsten and Köhl, 2012). While high-throughput methods are

Abbreviations: APTS, 8-Aminopyrene-1,3,6-trisulfonic acid; CE, Capillary electrophoresis; Fab, Antigen binding fragment; Fc, Crystallizable fragment; HPLC, High performance liquid chromatography; MS, Mass spectrometry; THF, Tetrahydrofuran.

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available to profile the isotype/subclass selection profile of polyclonal antibody pools (Brown et al., 2012), comparable methods for efficient analysis of glycan profiles are not available.

Studies of therapeutic monoclonal antibodies have clearly demonstrated the critical nature of the antibody glycan; ADCC activity is significantly increased in monoclonal therapeutic antibodies that lack fucose (Shields et al., 2002; Shinkawa et al., 2003; Shoji-Hosaka et al., 2006) or contain a bisecting N-acetylglucosamine (GlcNAc) (Davies et al., 2001). In addition to their role in determining effector function, inflammatory responses are dramatically modulated by Fc glycosylation. In particular, the addition of terminal sialic acids to the Fc glycan results in the induction of a potent anti-inflammatory response (Anthony and Ravetch, 2010; Böhm et al., 2012). Moreover, population level studies have shown that IgG glycosylation varies significantly with age, pregnancy, and during autoimmune-disease flares (Chen et al., 2012; Keusch et al., 1996; Parekh et al., 1988; Van De Geijn et al., 2009). More recent analyses point to antigen-specific antibody glycan alterations suggesting IgG-glycosylation is specifically programmed during immune responses (Ackerman et al., 2013; Collin and Ehlers, 2013; Selman et al., 2012; Yamada et al., 2013).

Studies of IgG-glycosylation in vivo have been historically limited by the low-throughput of existing analytical techniques, which generally require prohibitively expensive instrumentation and large quantities of sample, thus limiting the scope of research into natural regulation of IgG-glycosylation. Traditional approaches to analyze IgG N-glycosylation have relied primarily on high performance liquid chromatography (HPLC) or mass spectrometry (MS), both of which require relatively large quantities of material/antibody for accurate analysis as well as significant time and expertise to acquire and analyze data (Huhn et al., 2009). While MS offers remarkable structural resolution of N-glycans, it is poorly quantitative. On the other hand, while HPLC is highly quantitative, it is expensive, and both methods are distinctly low throughput. As studies of IgG glycosylation begin to focus on in vivo modifications, both in human populations and in animal models, the volume of samples often decreases as the number of samples increases. Thus a clear need exists for the development of a simple technique that combines sensitive quantitation with high-throughput capacity.

Capillary electrophoresis (CE) offers a unique high-throughput, quantitative analytical tool for the analysis of antibody glycosylation. Specifically, the use of common DNA sequencing equipment to perform glycan structure analysis by capillary electrophoresis is an excellent alternative to the established methods, with advantages in simplicity, throughput, structural resolution, and sensitivity (Callewaert et al., 2001; Huhn et al., 2012; Laroy et al., 2006; Reusch et al., 2014). Previously described CE techniques for antibody glycan analysis have focused on the analysis of whole IgG, as the large majority of monoclonal antibodies lack Fab glycan-sites (Ritamo et al., 2014). However, as many as 30% of serum-derived Fab fragments contain an N-glycosylation motif, and Fab glycans differ significantly from those typically found on the Fc-domain, in particular, Fab N-glycans contain higher proportions of sialic acid and fewer fucosylated structures (Anumula, 2012; Holland et al., 2006; Mimura et al., 2007). Thus, studies interrogating polyclonal antibody glycosylation aimed at understanding the functional significance of these regulated post-translational modifications will depend on the ability to resolve Fc and Fab glycans separately.

Here we describe a high-throughput, inexpensive, sensitive, and accurate approach for IgG N-glycan analysis of polyclonal antibodies. This methodology allows for separate analysis of the N-glycans from whole IgG, Fc, or Fab domains using capillary electrophoresis performed on a DNA-sequencer, providing a fast, accurate, quantitative, and relatively inexpensive and simple tool to probe IgG glycosylation, even when sample quantities are limited. This technique will be useful for the analysis of changes in antibody glycosylation following vaccination, in natural infection, as well as in non-infectious pathological conditions both in humans and in animal models, facilitating our understanding of the immunological impact of a B cell's ability to tune antibody activity through variations in glycosylation.

2. Materials and methods

2.1. Samples

Optimization of digestion and separation conditions was performed on commercially available, pooled IgG from healthy donors (Sigma Aldrich, human IgG and mouse IgG) or a pool of IgGs purified from healthy rhesus monkeys (Non-Human Primate Reagent Resource). Healthy human subjects were recruited through Brigham and Women's Hospital PhenoGenetic Project. The Institutional Review Board of Partners Healthcare approved the study, and each subject gave written informed consent. Rhesus macaque plasma was obtained from healthy, non-immunized animals, provided by D. Barouch. The Harvard Medical School Institutional Animal Care and Use Committee (IACUC) approved all studies involving rhesus monkeys. Plasma from C57/Bl6 mice was purchased from the Jackson Laboratory (Bar Harbor, Maine).

2.2. Isolation of IgG

Human and rhesus plasma was collected from fresh blood drawn in ACD tubes by centrifugation and frozen at −80 °C. IgGs from human and rhesus were isolated using Melon Gel IgG purification resin (Thermo Fisher) according to the manufacturer's instructions. Mouse IgG was isolated using protein A/G columns (Thermo Fisher) and eluted in 0.1 M citrate buffer pH 2.9, and subsequently neutralized in potassium 0.1 M phosphate buffer pH 8.9. All IgGs were isolated and stored in buffers without primary amines, to avoid problems with downstream glycan labeling. IgG concentrations were determined by measuring A_{280} on a Nanodrop spectrophotometer.

2.3. Removal of N-glycan from protein

Once IgG was purified, glycans were released from protein using enzymatic digestion with Peptide-N-Glycosidase F (PNGaseF, New England Biolabs). Protein was denatured using 2 μl of the provided denaturation buffer and incubated at 95 °C for 10 min according to the manufacturer's instruction. Samples were cooled on ice before the addition of 4 μl of G7

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