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Microscale purification of antigen-specific antibodies

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ABSTRACT

Glycosylation of the Fc domain is an important driver of antibody effector function. While assessment of antibody glycoform compositions observed across total plasma IgG has identified differences associated with a variety of clinical conditions, in many cases it is the glycosylation state of only antibodies against a specific antigen or set of antigens that may be of interest, for example, in defining the potential effector function of antibodies produced during disease or after vaccination. Historically, glycoprofiling such antigen-specific antibodies in clinical samples has been challenging due to their low prevalence, the high sample requirement for most methods of glycan determination, and the lack of high-throughput purification methods. New methods of glycoprofiling with lower sample requirements and higher throughput have motivated the development of microscale and automatable methods for purification of antigen-specific antibodies from polyclonal sources such as clinical serum samples. In this work, we present a robot-compatible 96-well plate-based method for purification of antigen-specific antibodies, suitable for such population level glycosylation screening. We demonstrate the utility of this method across multiple antibody sources, using both purified plasma IgG and plasma, and across multiple different antigen types, with enrichment factors greater than 1000-fold observed. Using an on-column IdeS protease treatment, we further describe staged release of Fc and Fab domains, allowing for glycoprofiling of each domain.

1. Introduction

Research on antibody (Ab) responses has generally focused on assessment of Ab titer. However, Ab quantity provides an incomplete assessment of in vivo activity, which depends upon both Fv and Fc activities that do not necessarily scale linearly with antibody quantity. Such qualitative functional aspects include Fv activities such as neutralization or agglutination, as well as Fc-dependent effector functions. These effector functions are in part post-translationally encoded via variant glycosylation on the conserved (Fc) portion of the antibody (Raju, 2008), which can be recognized by innate immune cells that drive several types of productive responses including antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular viral inhibition (ADCVI) and complement-dependent cytotoxicity (CDC). Long recognized as important to the efficacy of recombinant monoclonal therapeutics in the setting of cancer, these Fc-dependent functions have been identified as potential contributors to the mechanisms of action of even immunomodulatory Abs (Furness et al., 2014), as well as important drivers of protection in a number of infectious disease settings, such as Ebola (Wilson et al., 2000; Zeitlin et al., 2011), smallpox (Benhnia et al., 2013), anthrax intoxication (Bournazos et al., 2014), and in some settings in which broad, antibody-mediated protection has proven challenging to achieve, such as influenza (DiLillo et al., 2014) and HIV (Halper-Stromberg et al., 2014; Pincetic et al., 2014). Among monoclonal therapeutics, a growing number of antibodies have been designed specifically to elicit optimized cell-based killing as a primary mechanism of action, often through engineering of the Fc N-glycan composition (Gasdaska et al., 2012; Jefferis, 2012; Junttila et al., 2010).

The importance of the conserved Fc N-glycan site (N297) to these functions has been shown in numerous settings (Arnold et al., 2007; Jefferis, 1993, 2009), as cleavage of this glycan ablates binding of IgGs to numerous Fc Receptors (FcR). However, fine level modulation of IgG activity based on specific glycoforms is also well-established; the most prominent example being the potentiated ability of afucosylated Abs to strongly bind the FcγR3a and drive ADCC (Ferrara et al., 2011). Variation in the glycosylation patterns of naturally induced antibodies has also been implicated in a variety of autoimmune and infectious diseases (Albert et al., 2008; Mehta et al., 2008; Moore et al., 2005; R. Parekh et al., 1989; R. B. Parekh et al., 1985). Accordingly, recent efforts have begun to focus on understanding the role of natural variation in

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the glycosylation of antigen-specific antibodies in the setting of infectious disease protection and autoimmune pathology (Ackerman et al., 2013; Espy et al., 2011; Mehta et al., 2008; Scherer et al., 2010; Winkler et al., 2013), as well as beginning to determine the potential role that adjuvants, T cell help, or specific immunization regimens may play in driving the production of Abs with specific glycan profiles via vaccination (Guo et al., 2005; Hess et al., 2013; Oefner et al., 2012; Selman et al., 2012; J. Wang et al., 2011). These studies point toward the potential utility of evaluating variation in IgG glycosylation of Ab specificities of interest at the population level to provide critical information about antibody activity or immune status in vivo; for instance, IgG glycomics may aid in evaluating and comparing candidate vaccines as well as identifying putative mechanistic correlates of protection, or in screening subjects with auto-antibody responses to identify those at risk for more severe disease.

Despite its potential utility, evaluation of IgG glycoforms has not been widely utilized as a clinical biomarker of antibody activity or of immune status. Traditional glycan analysis methods such as high performance liquid chromatography (HPLC) and peptide mass spectrometry (MS) require relatively high sample input, significant operator expertise, and generally operate at low throughput (Huhn et al., 2009). These limitations have made analyzing antigen-specific antibodies, which are often present at very low concentrations, challenging to implement. Furthermore, while MS analysis can greatly reduce sample demands, the isolation of antigen-specific antibodies has generally utilized considerable quantities of antigen (Ackerman et al., 2013). However, recent advances in sensitive and simple glycan analysis methods (Bakovic et al., 2013; Mahan et al., 2015) which allow evaluation with greatly reduced sample quantities and higher throughput, have made high-throughput antigen-specific IgG N-glycan profiling a realistic goal, motivating the development of improved means to affinity purify these rare antibodies more efficiently.

A confounding factor in glycan analysis of antibodies is the potential for N-glycan sites to be present in the Fab region, as N-linked glycosylation motifs are observed in up to 10–30% of Fab fragments among naturally produced polyclonal mixtures (Ritamo et al., 2014). These Fab glycans typically exhibit a strikingly different glycosylation pattern than the Fc, particularly with respect to the level of sialylation (Anumula, 2012; Mimura et al., 2007), making their presence potentially problematic, even at low levels. Intriguingly, Fab glycosylation has been found to impact antigen binding in multiple settings (Rombouts et al., 2015b; Song et al., 2013) and may be a general marker of Abs with relatively high levels of somatic hypermutation, however, it is thought to have a limited role in modulating traditional FcR-driven effector functions. Thus, in order to best determine the role of glycosylation on both antigen recognition as well as the effector potential of an antibody, it is ideal to separate the Fab and Fc domains prior to glycan analysis. Isolation of the Fc domain N-glycan is a challenge relatively unique to the analysis of natural IgG, as candidate therapeutic monoclonal antibodies with N-linked Fab glycosylation motifs are typically not selected for clinical development. While some downstream glycan analysis methods such as glycopeptide-based mass spectrometry can resolve Fab and Fc glycosylation, traditional glycan mass spectrometry, HPLC, and CE-based methods rely on enzymatically or chemically released glycan. Accordingly, these methods require additional upstream processing to isolate Fc glycans. This isolation is generally performed by enzymatic cleavage of the antibody with papain or similar enzymes followed by affinity chromatography-based separation of Fc from Fab prior to glycan release, adding additional manipulation steps likely to reduce overall yield.

Fortunately, the significance of the Fc domain in vivo has led to the evolution of microbial proteins whose functions are to restrict antibody effector function. The best known example of such a molecule is Protein A, which is utilized by *Staphylococcus aureus* to mis-orient IgG; however, microbes possessing a number of diverse alternative means of antibody-evasion exist (Collin and Killian, 2014). Enzymes such as IdeS and SpeB restrict the Fc domain by cleaving Abs in their hinge region, and a number of glycosidases with activity against IgG and IgA glycans have been identified. While one of these glycosidases, EndoS is relatively specific to the IgG Fc domain (M. Collin and Olsen, 2001) and is consequently of interest in therapy of antibody-mediated autoimmune diseases (Collin et al., 2008) Endo S cleaves the IgG glycan after the N-linked acetylchitobiose core, which is variably fucosylated. Thus, its use as an alternative to the pan N-glycosidase PNGaseF is limited by the resulting loss of resolution of core-fucosylation, which is known to dramatically modulate IgG Fc binding to FcγR3a and FcγR3b. Nonetheless, collectively these microbial defense mechanisms represent useful biotechnological tools for IgG glycan analysis. Here, IdeS (von Pawel-Rammingen et al., 2002), a hinge protease, was chosen as a means to separate antibody Fc for glycan profiling.

In this work, we present a 96 well plate-based method for microscale purification of antigen-specific antibodies in high throughput, suitable for profiling of large-scale, population-based studies, such as vaccine trials or clinical cohorts. In addition, the method can be used to separately elute the Fc domain alone without additional steps, via an on-resin digestion with the IdeS enzyme that cleaves the hinge portion of the antibody. With this method we demonstrate isolation of various antigen-specific antibodies from human and non-human primate (NHP) samples in sufficient yield to permit highly quantitative chromatography-based glycan analysis. This method has proved useful across a variety of types of antigens, including peptides, and for purification of even epitope-specific antibodies. We have been able to quantify routine antigen-specific Ab enrichment of several hundred fold over serum concentrations in clinically relevant settings, as well as the ability to obtain useful glycan data from relatively small sample volumes (200 μL of plasma).

2. Materials and methods

2.1. Sample processing

IgG from human or NHP plasma was either separated from other common serum proteins via Melon Gel purification according to the manufacturer's instructions (Thermo 45214) or simply diluted 10-fold in PBS and then filtered through a 0.22 μm syringe filter (Millipore SLGP033RB). Filtered or purified samples were then concentrated to approximately 10 mg/mL total antibody concentration via centrifugal concentration (Amicon UFC801024). Pooled polyclonal human IgG from healthy donors, IVIG, Sigma (#I2511-10 mg), and HIV-infected subjects, HIVIG (NIH AIDS Reagent Program #3957), were used as controls.

2.2. Preparation of affinity resin cartridges

HIV gp41, gp120, p24, and influenza HA antigens (Immune Technologies IT-001-005p, IT-001-0027p, IT-001-017p and IT-003-0011p), and SIVmac239 gp120 (IT-001-022p) were diluted to 0.1 mg/mL in 20 mM Tris pH 8.2 to which a 5-fold molar excess of 10 mM Sulfo-NHS-Biotin (Themo 21335) dissolved in dH_2O was added. Biotinylation was allowed to proceed for 1 h at RT with end-over-end mixing. To remove excess biotin, the biotinylated antigen was then buffer exchanged 3 times into Phosphate Buffered Saline (PBS) using Amicon spin concentrators with 10 min spins at 3000 ×*g* (Amicon UFC801024); final volumes were brought up with PBS to establish a biotinylated antigen concentration of 0.5 mg/mL. A synthetic N-terminally biotinylated cyclic SIVsmE543 V2 peptide (Barouch et al., 2012) (JPT Peptide Technologies GmbH); GenBank U72748) was likewise prepared in PBS. Agilent Bravo Streptavidin Cartridges (Agilent G5496-60010) were loaded into the provided 96-well cartridge racks and 170 μL of PBS was added to each tip. Loaded tip racks were placed in receiver plates and spun at 1000 ×*g* for 2 min. A 100 μL volume of biotinylated antigen in PBS (50 μg antigen) was then loaded on each tip and spun through at 50 ×*g* for 10 min. The PBS wash and antigen loading steps were then repeated to ensure maximum antigen binding. Finally, tips were washed