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Rapid LC–MS screening for IgG Fc modifications and allelic variants in blood

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ABSTRACT

A new method for simultaneously screening allelic variants and certain Fc modifications on endogenous human IgG1 and IgG2 directly from blood samples is described. The IdeS endoproteinase was used to cleave IgG in serum to generate Fc, which, after denaturation, was analyzed directly as monomeric Fc (Fc/2) by LC–MS to identify the haplotype(s) present in each individual. The relative levels of IgG isotype and haplotype ratios were generated along with the profile of the major Fc glycans and several other modifications associated with each IgG1 or IgG2 haplotype. Since only minute quantities (5 μL) of blood are required and analysis can be highly automated, this approach lends itself to screening large populations. We demonstrate its utility in examining possible correlations between Fc properties and allelic variants. IgG1 core fucosylation, which significantly impacts antibody dependent cellular cytotoxicity (ADCC), showed an asymmetric distribution, with a small number of individuals showing unexpectedly high core afucosylation levels. In these individuals, IgG2 afucosylation levels were normal. Finally, a new IgG1 allotype, previously not characterized, was identified using this analytical methodology.

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1. Introduction

Endogenous human antibodies are, at the molecular level, extraordinarily heterogeneous. This is due not only to the polyclonal nature of the immune response, but also the heterogeneity of the Fc consensus site glycan structure, as well as multiple post-translational modifications (PTMs) such as N-terminal pyroglutamic acid formation, heavy chain (HC) C-terminal lysine processing, deamidation and glycation occurring to varying degrees in vivo (Austin et al., 1987; Cai et al., 2011; Liu et al., 2011, 2009). Therapeutic monoclonal antibodies (mAbs) avoid the polyclonal diversity but still contain multiple product quality attributes (PQAs) related to glycan or PTM heterogeneities arising from enzymatic and chemical changes during manufacturing and storage. Characterizing, and understanding the impact, with respect to safety and efficacy, of these PQAs is a central requirement of the FDA's Quality by Design initiative for biotechnology products (FDA). Examination of endogenous antibodies for specific attributes can help clarify the potential impact of these attributes in therapeutic mAbs, on the premise that levels found endogenously are unlikely to represent safety concerns when present on therapeutics.

The glycan structures of endogenous human antibodies can be studied after enzymatic release (Chen and Flynn, 2007) but detailed characterization of other PTMs is generally limited to peptide mapping of the constant regions, due to the overwhelming variable region sequence diversity. Another approach for the characterization of PTMs is to limit study to the much more sequence-restricted Fc, generated by enzymatic fragmentation at, or near, the hinge. The Fc is responsible for most important biological functions of an antibody apart from antigen binding and contains the conserved glycosylation site; the glycan at this site can also modulate Fc activities (Arnold et al., 2007). However, the constant regions of immunoglobulin molecules, and especially the Fc, still contain multiple locations where amino acid sequence variants (alleles) occur naturally within subpopulations. Allotypes are defined as allelic variants recognized by unique serological reagents (Jefferis and Lefranc, 2009). Allotypes have been observed on the heavy chains (HCs) of human IgG1, IgG2, IgG3, IgA and on kappa light chains (LCs) (Grubb, 1994; Jefferis and Lefranc, 2009). The number of known allotypes per constant region ranges from one for IgG2 HC to 13 for IgG3 HC. Allotypes are inherited in fixed combinations, termed haplotypes, because the gene segments coding for the HC constant regions are closely linked within the IGH gene locus, with a low crossover frequency. Specific IgG HC haplotypes can be strongly linked to specific populations and, for this reason, have been extensively used in population genetics, forensics and

Abbreviations: ADCC, antibody dependent cellular cytotoxicity; CV, coefficient of variation; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Fuc, fucose; GlcNac, N-acetylglucosamine; HC, heavy chain; LC, light chain; mAb, monoclonal antibody; PQA, product quality attribute; PTM, post-translational modification; RP, reversed phase; TFA, trifluoroacetic acid; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

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paternity testing. (Cerutti et al., 2004; Dugoujon et al., 2004; Henke et al., 1989; Schanfield et al., 2008). The linkage disequilibrium demonstrated by the Gm haplotypes is extraordinarily high which hints strongly that selection during evolution has played a major role (Steinberg and Cook, 1981). This has led to suggestions that the haplotypes may influence the effectiveness of the immune response against various pathogens (viruses) or, since the majority of allotypes are located on the Fc, perhaps influence Fc effector function activities (Grubb, 1994; Steinberg and Cook, 1981). Viral Fc receptors have been shown to discriminate between IgG1 allotypes (Atherton et al., 2000; Pandey et al., 2008a) and Gm allotypes have shown associations with disease susceptibility (Dugoujon and Cambon-Thomsen, 1995; Jonsson et al., 2006; Pandey et al., 2008b; Skattum et al., 2008) as well as serum IgG concentrations (Oxelius, 1993; Sarvas et al., 1991).

Choosing an optimal allotype may be an important consideration in the design of human therapeutic antibodies, as the therapeutic allotype represents a potential immunogen to homozygous populations lacking that allotype (Jefferis and Lefranc, 2009). Likewise, allotyping of clinical subjects may be warranted if anti-therapeutic antibodies are observed, in order to differentiate anti-allotype from other types of anti-therapeutic responses. To simplify discussion, we have expanded the traditional definition of haplotype in this study to include a fixed combination of allelic variants. Allele information is now possible through gene sequencing but gives no information regarding protein modifications whereas serological tests cannot generally identify the individual haplotypes present. Traditionally, detailed characterization of endogenous antibodies is further complicated by the need to purify IgG from individual donors. We have taken advantage of an endoproteinase highly specific for a single peptide bond in the hinge region of IgG that has just recently been made commercially available, to generate Fcs in serum from individual donors. LC–MS analysis was used to simultaneously determine both allotypic composition and major PTMs of isotype-specific Fc from individuals, without IgG purification. Fc glycan profiles, or, in an approach we prefer for screening purposes, core fucosylation levels, could be estimated for individuals along with Fc glycation levels. Core afucosylation levels influence an individual's IgG1's innate potential to engage ADCC, an important component of the cellular immune response. These PTMs were studied in the context of the specific haplotype(s) present for each individual and assessed for correlations among IgG isotype concentration, allotypes present and individual haplotype concentrations, thereby providing an endogenous context for better understanding these properties on therapeutic mAbs.

2. Materials and methods

2.1. Materials

L-1-Tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated (to inhibit chymotrypsin activity), MS grade bovine trypsin was obtained from Thermo Scientific and Asp-N protease from Roche. The glycosidase IgGZERO^{TM}, a histidine tagged recombinant EndoS glycosidase from *Streptococcus pyogenes* and the protease FabRICATOR®, the endoproteinase IdeS, were obtained from Genovis AB, Lund, Sweden. NAP-5 columns were from GE Healthcare.

2.2. Serum samples

Blood from healthy donors was collected into serum separator tubes. After allowing time for clotting (30 min at room temperature) and centrifugation (13 min at 1500 × *g*), the resulting serum was either used immediately or frozen at −70 °C until use. In several cases the blood samples were collected using a finger lancet into micro centrifuge tubes (250 μL) and processed similarly. Donor's ethnicity, age and other information were self-reported.

2.3. Enzymatic fragmentation of IgG

Five microliters of serum were diluted into 60 μL of Cleavage Buffer 1 (10 mM sodium phosphate, 150 mM NaCl, pH 7.4), 60 U EndoS was added, and deglycosylation was allowed to proceed for 30 min at 37 °C. Forty-five micoliters of Cleavage Buffer 2 (50 mM sodium phosphate, 150 mM NaCl, pH 6.6) was added followed by 67 U IdeS and digestion was allowed to proceed for an additional 30 min at 37 °C. Purified IgG was digested similarly but with 5 μL serum replaced with 5 μL IgG at 10 mg/mL. To each digested serum or IgG sample was added 12 μL 1 M Tris–Cl, pH 8 buffer, 145 mg guanidine-HCl and 5.2 μL 1 M dithiothreitol (DTT) followed by reduction for 30 min at 37 °C. Samples were analyzed immediately or stored frozen prior to DTT addition.

2.4. LC–MS analysis

Analysis was performed using an Agilent 1100 HPLC system directly coupled to a Waters LCT Premier electrospray time-of-flight mass spectrometer. A reversed phase HPLC column (Poros R1/10, 4.6 mm × 50 mm) flowing at 0.2 mL/min at 75 °C was employed with the following solvents: A = 0.1% TFA, B = 0.09% TFA in 90% acetonitrile. Either of two gradients, giving similar results, was employed. In the first, 10% B was held for 6 min, followed by a gradient from 10 to 55% B over 44 min, followed by a gradient from 55 to 90% B over 5 min. In the second method, 10% B was held for 6 min, followed by a gradient from 10 to 30% B over 4 min, followed by a gradient from 30 to 60% B over 25 min, followed by a gradient from 60 to 90% B over 5 min. A processed serum volume equivalent to 20 μg IgG was injected (assuming 10 mg/mL IgG in serum). The UV elution profile at 214 nm was also monitored.

Deconvolution of the MS spectra was performed using Waters MassLynx software. The spectra were summed across the chromatographic peaks and deconvoluted with MaxEnt1 algorithm for 12 iterations. An appropriate peak width was selected with an output resolution of 1.0 Da/channel; other parameters were default values for the software. Ion intensities of the deconvoluted peaks were used for quantification.

2.5. AspN peptide mapping

Purified IgG samples were denatured by diluting, to a final concentration of 0.5 mg/mL, into a buffer containing 6.25 M guanidine-HCl, 20 mM Tris–Cl, 2 mM EDTA, pH 7.87. Samples were reduced by adding DTT to 10 mM and incubating 45 min at 45 °C. Subsequently, samples were carboxymethylated by adding iodoacetic acid to 20 mM and incubating 40 min at room temperature. Samples were buffer exchanged into 50 mM potassium phosphate, pH 8 using NAP-5 columns. Zinc acetate was added to 0.5 mM and samples were digested with endoprotease AspN at a ratio of 1:33 for 4 h at 37 °C. Finally, digested samples were quenched by the addition of 10% formic acid to 0.3%.

The resultant digests were analyzed by LC/MS/MS. The LC/MS/MS system consisted of an Agilent HP1100 HPLC system connected in-line to a Thermo Scientific LTQ electrospray ion trap mass spectrometer. A reversed phase HPLC column (Acquity BEH130 C18 1.7 μm, 2.1 mm × 50 mm, Waters Corp.) was used to separate the peptides with the column temperature at 50 °C. Mobile phase A: 0.1% formic acid in water and mobile phase B: 0.09% formic acid in 10% water/90% acetonitrile. The gradient (hold at 0% B for 5 min, then 0–36% B in 130 min) was performed with a flow rate of 0.2 mL/min. About 30 μg of sample was injected. The

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