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Assessment of naturally occurring covalent and total dimer levels in human IgG1 and IgG2

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ABSTRACT

Antibody dimers, two self-associated monomers, have been detected on both recombinantly expressed and endogenous human IgG proteins. Nearly 10 years ago, Yoo et al. (2003) described low levels of IgG2 covalent dimer, in human serum, but did not quantify the levels. Here we quantify the total and covalent dimer levels of IgG2 and IgG1 in human blood, and study the origin of covalent dimer formation. Low levels (<1%) of total IgG1 and IgG2 dimers were measured in freshly prepared human plasma. Both IgG1 and IgG2 covalent dimers were also found in plasma. Whereas IgG1 covalent dimer levels were significantly reduced by steps intended to eliminate artifacts during sample preparation, IgG2 covalent dimer levels remain stable in such conditions. About 0.4% of IgG2 in plasma was in a covalent dimer form, yet very little (<0.03%) of IgG1 covalent dimer could be considered naturally occurring. IgG2 dimer also formed in vitro under conditions designed to mimic those in blood, suggesting that formation occurs in vivo during circulation. Thus, small amounts of covalent IgG2 dimer do appear to form naturally.

1. Introduction

Antibodies of the IgG isotype are comprised of two identical heavy chains (HCs) and two identical light chains (LCs), forming the H_2L_2 molecule. Beyond non-covalent forces, the individual polypeptide chains are bound together through disulfide bonds. Each LC is linked to an HC by a single disulfide bond, and the two HCs are linked together by a number of disulfide bonds, which differ between IgG subclasses. IgG1, the most abundant IgG subclass, has two inter HC disulfide bonds, whereas the IgG2 subclass is considered to have four. Although the disulfide connectivities were mapped some time ago for all the human IgG subclasses, recent work has shown that alternate linkage patterns can exist due to disulfide exchange reactions. In the case of IgG4, inter HC disulfide bonds break, resulting in the exchange of half molecules (HL) and antibodies in which the two Fab domains react with different antigens (Aalberse and Schuurman, 2002; Schuurman et al., 1999; van der Neut Kolfschoten et al., 2007). Some interchain disulfide bonds on IgG2 molecules are labile, generating an ensemble of structural isomers with different hinge and Fab connectivities (Wypych et al., 2008; Zhang et al., 2009). While the IgG2 disulfide heterogeneity was originally observed on antibodies expressed in artificial cell culture systems, the same phenomena have been confirmed to occur naturally in humans (Dillon et al., 2008; Liu et al., 2008; Wypych et al., 2008).

Prior to the discovery of IgG2 disulfide isoforms, Yoo et al. (2003) reported the existence of naturally occurring IgG2 covalent dimers. The authors detected IgG2 dimers under non-reducing but denaturing conditions in serum from individual subjects and in IgG obtained from pooled serum. No IgG1 covalent dimers were detected. Cyanogen bromide cleavage experiments indicated that the hinge region was involved in the cross-linking between monomers. Because the hinge disulfides in IgG2 also participate in the IgG2 disulfide exchange of isoform conversion, the mechanisms of dimer formation and disulfide isomer conversion could be related. Multiple reviews (Nezlin, 2010; Swann et al., 2008) have cited this work as proof that IgG2 antibodies can form covalent dimers in vivo, while the original authors suggest that IgG2 is secreted in vivo as a dimer (Yoo et al., 2003).

Since the Yoo et al. paper was published in 2003, no additional observations or characterization work have been published, despite several outstanding questions. For example, IgG2 dimer was detected in this paper, but no attempt was described to quantify its levels. In addition, some controls to determine whether dimer formed in vitro were not performed. Finally, although IgG1 dimer was not detected, the results could not distinguish between the absence of its existence and the poor sensitivity of the method.

Abbreviations: DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; GFB, glufibrinopeptide B; HC, heavy chain; HL, half molecule; LC, light chain; LC/MS, liquid chromatography/mass spectrometry; NEM, N-ethylmaleimide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography; TFA, trifluoroacetic acid; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; V_o, void volume.

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IgG2 and IgG1 dimers, both covalent and non-covalent, are present in low amounts in recombinant therapeutic antibody drug products (Chen and Flynn, 2009; Remmele et al., 2006). While the immunogenicity risk for antibody dimers is generally considered low (Rosenberg, 2006), much effort is made to monitor and reduce their levels through purification. Information about whether dimers are naturally occurring and, if so, at what levels, can aid in a safety risk assessment. Due to these outstanding questions about the natural occurrence of IgG2 dimers and their impact on process development, further work was performed to characterize IgG2 dimer formation and to quantify their levels in blood.

2. Materials and methods

2.1. Materials

Glufibrinopeptide B (GFB) was purchased from American Peptide Company (Sunnyvale, CA). L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated, mass spectrometry (MS)-grade trypsin was from Thermo Scientific (Rockford, IL). N-ethylmaleimide (NEM) was from Sigma–Aldrich (St. Louis, MO). Ethylene diamine tetraacetic acid (EDTA)-coated blood collection tubes were from Becton, Dickinson and Company (Franklin Lakes, NJ). Amersham ECL Plex Fluorescent Rainbow Markers were purchased from GE Healthcare Life Sciences. All other chemicals were reagent grade or better quality. IgG2 (mAbA and mAbB) and IgG1 (mAbC and mAbD) monoclonal antibodies were expressed in CHO cells and purified using conventional chromatography methods at Amgen. These four antibodies all have kappa light chains.

2.2. Preparative size-exclusion chromatography (SEC) of fresh plasma sample

Blood from a healthy volunteer was collected into an EDTA-coated tube. The blood was immediately centrifuged (5 min at 2700 × *g*) to collect cell-free plasma and then centrifuged again (5 min at 20,800 × *g*) to clarify. Twenty microliters clarified plasma were injected within 30 min of collection onto two TSKgel SuperSW3000, 300 × 4.6 mm, 4-μm columns (Tosoh Bioscience LLC, Montgomeryville, PA) connected in tandem and flowing at ambient temperature at 0.16 mL/min with a mobile phase of 100 mM sodium phosphate, 250 mM NaCl, 1 mM EDTA, pH 6.8. Fractions (0.3 min, 48 μL) were collected between 23.0 and 36.8 min.

2.3. Analysis of size-exclusion fractions for IgG1 and IgG2 content by peptide mapping

To each SEC fraction were added 132 μL denaturing buffer (7.5 M guanidine–HCl, 0.25 M Tris–Cl, 2.5 mM EDTA, pH 7.87) and 20 μL GFB in denaturing buffer. One microliter 1 M dithiothreitol (DTT) was added and samples were reduced for 45 min at 45 °C. Samples were carboxymethylated by the addition of 7.6 μL iodoacetic acid and incubated for 40 min at room temperature in the dark. Samples were then buffer-exchanged into 50 mM Tris–Cl, pH 7.87 using NAP-5 columns (GE Healthcare, Piscataway, NJ); a final volume of 300 μL for each sample was obtained. Then, 10.5 μL 0.25 mg/mL trypsin were added and digestion allowed to proceed for 6 h at 37 °C. Digestion was quenched by the addition of 9 μL 10% trifluoroacetic acid (TFA), and 100-μL aliquots (in some cases, also 5-μL aliquots) were injected for liquid chromatography–mass spectrometry (LC–MS) analysis.

The resultant digests were analyzed by LC/MS/MS. The LC/MS/MS system consisted of an Agilent HP1100 HPLC system (Santa Clara, CA) connected in-line to a Thermo Scientific LTQ electrospray ion trap mass spectrometer. A reversed-phase HPLC column (Acquity BEH130 C18 1.7 μ, 2.1 × 50 mm, Waters Corp., Milford, MA) 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 0–29.2% B in 105 min) was performed with a flow rate of 0.2 mL/min. The chromatogram was monitored by both UV light absorbance (set at 280 nm) and MS. MS detection included full scan in positive mode, as well as data-dependent ultra zoom scan and MS/MS of the most intense ion of the scan in centroid mode. Monoisotopic masses and charges were determined from ultra zoom scans. Data analysis, including peptide identification and quantification, was performed using a custom written program, MassAnalyzer (Zhang, 2009; Zhang and Shah, 2010).

Three pairs of homologous Fc tryptic peptides (Table 1), each with a single amino difference between the corresponding IgG1 and IgG2 peptide, were used to estimate relative amounts of IgG1 and IgG2 in the SEC fractions covering the regions of IgG dimers, higher multimers, and monomers. None of these six peptides contain any known allelic sites; therefore, only a single amino acid sequence is expected for any donor. The mass spectral ion intensity of the dominant charge state of each of these peptides was used. Integration of the ion intensities corresponding to all dimer and all monomer fractions was used to calculate the dimer contents of IgG1 and IgG2.

2.4. Sample preparation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Plasma samples were analyzed by SDS-PAGE under non-reducing conditions on NUPAGE® 3–8% Tris–acetate, 10-well gels (Life Technologies, Grand Island, NY) using NUPAGE® LDS Sample Buffer 4× (Life Technologies). All samples were heated at 75 °C for 12 min.

Two alternate protocols were employed—one to create initially minimally diluted plasma and the other to create immediately diluted plasma sample. Each protocol included several sub-protocols, as NEM was optionally added immediately after blood collection, or prior to sample preparation for SDS-PAGE, or not at all.

To prepare “undiluted” human plasma, approximately 5 mL blood from a healthy volunteer was collected into each of two pre-chilled EDTA-coated tubes. Within 10 s of blood draw, 0.8 mL PBS or, optionally, NEM stock solution in PBS, was added to each tube. The final NEM concentration was about 57 mM. The blood-containing tubes were centrifuged at 4000 rpm for 10 min to collect plasma. This plasma was diluted 8.6-fold with PBS, then mixed 3:1 (v/v) with 4× sample buffer. In one sub-protocol, NEM from a 400 mM stock solution was added, prior to mixing with sample buffer, to samples that had not previously seen NEM. The final NEM concentration in all samples that contained NEM at the time of heating was 5 mM, irrespective of whether the NEM was added to the blood or prior to preparation for SDS-PAGE.

To prepare 10-fold diluted human plasma, two tubes containing 4.5 mL ice-cold dilution buffer were prepared beforehand, with one tube containing 4.5 mL 1.1 mM EDTA in PBS and the other tube containing, in addition, 7.4 mM NEM. Within 10 s of blood draw, 0.5 mL blood was pipetted into each of the two tubes. The blood-containing tubes were centrifuged at 4000 rpm for 10 min to collect plasma. The plasma samples were mixed 3:1 (v/v) with 4× sample buffer. In one sub-protocol, NEM from a 400 mM stock solution was added, prior to mixing with sample buffer, to samples that had not previously seen NEM. The final NEM concentration in all samples that contained NEM at the time of heating was 5 mM, irrespective of whether the NEM was added to the blood or prior to preparation for SDS-PAGE.

After preparation for SDS-PAGE, all samples contained the equivalent of 7.5% plasma (v/v). A typical sample load was 30 μL,