Structural and Functional Characterization of Disulfide Isoforms of the Human IgG2 Subclass

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Thomas M. Dillon, Margaret Speed Ricci, Chris Vezina, Gregory C. Flynn, Yaoqing Diana Liu, Douglas S. Rehder, Matthew Plant, Brad Henkle, Yu Li, Songpon Deechongkit, Brian Varnum, Jette Wypych, Alain Ballard, and Pavel V. Bondarenko

From the Departments of Pharmacetics, Inflammation Research, and Analytical Sciences, Amgen Inc., Thousand Oaks, California 91320

In the accompanying report (Wypych, J., Li, M., Guo, A., Zhang, Z., Martinez, T., Allen, M. J., Fodor, S., Kelner, D. N., Flynn, G. C., Liu, Y. D., Bondarenko, P. V., Ricci, M. S., Dillon, T. M., and Balland, A. (2008) J. Biol. Chem. 283, 16194–16205), we have identified that the human IgG2 subclass exists as an ensemble of distinct isoforms, designated IgG2-A, -B, and -A/B, which differ by the disulfide connectivity at the hinge region. In this report, we studied the structural and functional properties of the IgG2 disulfide isoforms and compared them to IgG1. Human monoclonal IgG1 and IgG2 antibodies were designed with identical antigen binding regions, specific to interleukin-1 cell surface receptor type 1. In vitro biological activity measurements showed an increased activity of the IgG1 relative to the IgG2 in blocking interleukin-1β binding from the receptor, suggesting that some of the IgG2 isoforms had lower activity. Under reduction-oxidation conditions, the IgG2 disulfide isoforms converted to IgG2-A when 1 m guanidine was used, whereas IgG2-B was enriched in the absence of guanidine. The relative potency of the antibodies in cell-based assays was: IgG1 > IgG2-A > IgG2-B. This difference correlated with an increased hydrodynamic radius of IgG2-A relative to IgG2-B, as shown by biophysical characterization. The enrichment of disulfide isoforms and activity studies were extended to additional IgG2 monoclonal antibodies with various antigen targets. All IgG2 antibodies displayed the same disulfide conversion, but only a subset showed activity differences between IgG2-A and IgG2-B. Additionally, the distribution of isoforms was influenced by the light chain type, with IgG2A composed mostly of IgG2-A. Based on crystal structure analysis, we propose that IgG2 disulfide exchange is caused by the close proximity of several cysteine residues at the hinge and the reactivity of tandem cysteines within the hinge. Furthermore, the IgG2 isoforms were shown to interconvert in whole blood or a “blood-like” environment, thereby suggesting that the in vivo activity of human IgG2 may be dependent on the distribution of isoforms.

Recombinant monoclonal antibodies, typically human or humanized, are used as protein-based therapeutic agents because of their high degree of specificity and the ability to alter their functional properties when desired. In vivo, therapeutic antibodies can function via several mechanisms, including as antagonists that compete with ligand binding and interfere with receptor response, as signaling molecules that elicit a response in the target cells (e.g. apoptosis), or as agents that target specific cells populations (1). The latter mechanism may involve attaching an effector moiety (e.g. enzymes, toxins, and radionuclides) to the antibody or using the antibody’s natural effector functions, which are mediated through the immunoglobulin Fc domain. These natural functions include antibody-dependent cellular cytotoxicity and activation of the complement cascade, leading to complement-dependent cytotoxicity. Effector functions have been shown to be dependent on the immunoglobulin γ (IgG) subclass affinity for Fc receptors (IgG1 > IgG3 > IgG4 > IgG2) (2, 3), and this feature serves as a common determinant for therapeutic use. The human IgG2 subclass in particular has emerged as an attractive framework for therapeutic antibodies in clinical applications for which effector functions are undesirable or unnecessary for therapeutic activity (4, 5).

The increased prevalence of therapeutic IgGs has led to a renewed interest in understanding antibody structure and its relationship to biological function. Structural heterogeneity in proteins can result from genetic differences or from many common post-translational modifications, such as glycosylation, protein folding, disulfide bond formation, and chemical modifications to amino acid side chains or the peptide backbone (6). For example, structural changes caused by glycan variants have been shown to impact antigen binding and antibody effector functions (7–10). Other examples demonstrate how cysteinylation of cysteines and incomplete disulfide bond formation in antibodies can interfere with antigen recognition and ultimately lead to reduced binding or inactivity (11, 12). Disulfide heterogeneity of human IgG4 molecules represents a clear example of how unstable disulfide bonds can disrupt the structural integrity of an antibody, generating half-molecule forms. In this case, the half-molecule IgG4 is still capable of specific binding, although in a diminished capacity due to the loss of multivalent binding.

Disulfide bond formation is a post-translational process that can affect the structure and function of proteins. Incorrectly completed disulfide bonds have the potential to generate improperly folded proteins. Although disulfide heterogeneity is
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less common in mammalian expression systems possessing the proper intra-cellular redox environment and post-translational machinery for protein folding, incomplete or improper disulfide bond formation of bacterially expressed mammalian proteins is commonly observed. Restoring native disulfide bonds in these proteins (often produced as inclusion bodies or soluble aggregates) has typically been accomplished by solubilization in the presence of a high concentration chaotropic agent, typically 6 M guanidine hydrochloride (GuHCl), followed by exposure to redox agents while slowly decreasing the concentration of the chaotrope (13). Additionally, redox procedures without chaotropic agents have been used for Fc fusion proteins and antibodies produced in mammalian cells (12, 14), primarily to modify the disulfide structure without denaturing the proteins and improve binding to their targets.

In the companion report (15), we describe the existence of multiple disulfide isoforms of human IgG2 antibodies that can be partially resolved by cation exchange, capillary electrophoresis, and reversed-phase chromatography. Three discrete isoforms were identified, each having different disulfide linkages between the light chain (LC) and heavy chain (HC), as detailed by nonreduced peptide mapping. In the first part, thorough covalent characterization of the IgG2 disulfide isoforms has been presented. In this study, we describe a redox procedure for enrichment of the IgG2 disulfide isoforms utilizing a relatively low concentration of GuHCl. We investigated the individual biophysical properties of the human IgG2 isoforms, their potency, and the mechanism of disulfide conversion.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human monoclonal IgG antibodies were produced from Chinese hamster ovary cells and purified using well established purification protocols (16) at Amgen. The purified proteins were stored in a formulation buffer at 4 °C. Unless otherwise noted, the same representative mAb expressed as an IgG1κ versus IgG2κ construct was used throughout this study. Antibodies purified from human plasma were purchased from Sigma and ICN biochemicals. The antibodies were purified from human plasma using well established purification protocols, which generally include acid precipitation of non-IgG proteins, ammonium sulfate precipitation, and affinity chromatography.

Reversed-phase Chromatography—An Agilent 1100 HPLC system with a binary pump was equipped with a UV detector and an autosampler. The proteins were injected onto a Zorbax 300SB C8 column (150 × 2.1 mm, 5 μm, 300 Å) operated at 75 °C. The flow rate was 0.5 ml/min. Mobile-phase A was water containing 0.1% trifluoroacetic acid. Mobile-phase B was 70% isopropanol alcohol, 20% acetonitrile, and aqueous 0.1% trifluoroacetic acid. Samples were injected at a loading condition of 10% B and increased to 19% B over 2 min. A linear elution gradient of 1.1% B/min started at 2 min and ended at 24 min. The column was then flushed for 5 min with 95% B. The column was reequilibrated with the loading condition for 5 min.

Size-exclusion Chromatography—An Agilent 1100 HPLC system with a binary pump was equipped with a UV detector and an autosampler. The proteins were injected onto two Tosoh Bioscience TSK-Gel G3000 SWxl columns in series (7.8 mm × 300 mm, 5-μm particles) operated at 25 °C. The flow rate was 0.5 ml/min. The mobile phase contained 100 mM sodium phosphate, 500 mM sodium chloride, 5% ethanol, pH 7.0.

Differential Scanning Calorimetry—Samples were analyzed using a MicroCal VP-Capillary differential scanning calorimeter system. All samples were diluted to 1 mg/ml in acetate buffer at pH 5. A scan rate of 1 °C/min was used.

Redox Treatment—To enrich for IgG2-B and IgG2-A, the antibodies were incubated at 3 mg/ml in two buffers: 1) 200 mM Tris buffer at pH 8.0; 2) 200 mM Tris buffer at pH 8.0 with 0.9 M GuHCl. A combination of cysteine and cystamine was added at concentrations of 6 mM to 1 mM. The ratios of redox reagents were determined by testing multiple conditions and monitoring for optimal IgG2 isoform conversion by reversed-phase chromatography. Several redox reagents listed in a previous study (14) and their ratios were evaluated before choosing the above conditions. The samples were protected from light and placed at 2–8 °C for 24–48 h.

Chondrocyte Bioassay—The anti-IL-1RI IgG samples were serially diluted from 400 nM to 1.5 pm in assay media. The diluted test antibodies (50 μl) were added to the wells of 96-well plates seeded with human chondrocytes at a density of 10,000 cells/well in a 100-μl volume. The final antibody concentration ranged from 100 nM to 0.38 pm. After a 30-min incubation, 50 μl of recombinant human IL-1β was added to a final concentration of 10 pm. After incubation overnight, the antibody activities were analyzed using an IL-6 immunoassay with electrochemiluminescence detection (Meso Scale Discov-

3 The abbreviations used are: LC, light chain; HC, heavy chain; mAb, monoclonal antibody; RP-HPLC, reversed-phase high performance liquid chromatography; ESI, electrospray ionization; IL-1RI, interleukin-1 cell surface receptor type 1; IL-1α, interleukin-1β; GuHCl, guanidine hydrochloride; CEX, cation exchange; CH1, constant region one of heavy chain; SEC, size-exclusion chromatography; Fc, fragment crystallizable; PBS, phosphate-buffered saline.
ery, Gaithersburg, MD). The inhibition of IL-6 production was calculated as a percentage of maximum IL-1/H9252 activity. The inhibition-response curve for each test antibody was established, and the corresponding IC50 values (the concentration of antibody which reduces the signal by 50%) were derived using GraphPad Prism software.

**IL-1β-induced IL-6 Human Whole Blood Bioassay**—The anti-IL-1RI IgG samples were evaluated in 50% human whole blood (final concentration) from 10 nM to 0.30 pM in half-log increments for a 10-point IC50 curve. After a 45-min preincubation with anti-IL-1RI IgG samples, the blood was stimulated with recombinant IL-1/H9252 for a final concentration of 30 pM (IC50). After incubation overnight, the antibody activities were analyzed using an IL-6 immunoassay with electrochemiluminescence detection (Meso Scale Discovery). The inhibition of IL-6 production was calculated as a percentage of maximum IL-1β activity. IC50 values were calculated using six separate donors (three donors on two different days).

**RESULTS**

**Structural Heterogeneity of Human IgG2 and Homogeneity of IgG1 by RP-HPLC**—RP-HPLC coupled with mass spectrometry has emerged as an effective analytical tool for characterizing and monitoring structural heterogeneity of antibodies (17, 18). In this study, several human IgG1 (Fig. 1, k–q) and IgG2 (Fig. 1, a–j) antibodies were analyzed generating the RP-HPLC profiles shown in Fig. 1. The IgG2 molecules consistently produced heterogeneous profiles for both monoclonal (Fig. 1, a–j) and endogenous antibodies purified from human serum (Fig. 1, g–j), whereas those of IgG1 were homogeneous. Furthermore, IgG2 antibodies containing kappa (κ) and lambda (λ) LC differed in their RP-HPLC profile. Both antibodies displayed a characteristic four-peak profile but displayed a different abundance of the earliest eluting peak. These results clearly show the inherent differences between the IgG1 and IgG2 subclass in addition to subtle variations among the LC type of IgG2.

To further study the effects of this structural phenomenon, monoclonal IgG1 and IgG2 antibodies were designed with identical antigen binding regions, specific to interleukin-1 cell surface receptor type 1 (IL-1RI) (supplemental Fig. S1). The most significant differences between the human IgG1 and IgG2 subclasses are the primary structure of the hinge and two serine to cysteine substitutions (Ser → Cys) in IgG2 (Table 1). The IgG2 upper hinge sequence is three amino acids shorter than that of IgG1 and contains a cysteine substitution at position 219. Additionally, the CH1 loop of the IgG2 HC has a cysteine substitution at position 131 that is available for disulfide bonding to the LC. Although the overall IgG1 and IgG2 amino acid sequences are 95% identical, the proteins exhibited significant structural differences, as determined by the RP-HPLC analysis.
(Fig. 2). As shown in the accompanying report, the heterogeneity in the RP-HPLC profile was a result of disulfide linkage differences in the IgG2s (15). Therefore, as expected, high resolution mass spectral analysis in-line with RP-HPLC found the calculated molecular weight values of the four chromatographic peaks to be identical (data not shown) (18). Interestingly, the individual RP-HPLC peaks displayed unique mass spectral features, suggesting underlying structural differences. We measured the number of protons on the surface of each of the RP-HPLC peaks to be different (supplemental Fig. S2), according to the method initially proposed by Chait and coworkers (19). The results indicated that peak 1 had decreased solvent exposure and a more compact structure while peaks 2–4 showed an increased solvent exposure with a larger number of protons on the antibody surface and a more open structure.

**Biological Activity of IgG1 and IgG2 Anti-IL1-R1—IgG1 and IgG2 constructs were designed to compete with the IL-1β ligand, thereby inhibiting IL-1-mediated cellular events, including the production of IL-6 (20). IL-1RI is present on the surface of many cell types, including endothelial cells, fibroblasts, T cells, and chondrocytes. In response to IL-1, chondrocytes switch from synthesizing cartilage matrix molecules such as collagens and proteoglycans to releasing matrix degrading molecules like matrix metalloproteinases, leading to cartilage degradation (21). Biological activity of the anti-IL-1RI IgG1 and IgG2 mAbs were assessed by monitoring the inhibition of IL-1β-induced IL-6 production by primary human chondrocytes. The results of the bioassays showed statistically significant differences in IL-6 inhibition (Fig. 3) with the IgG2 having approximately one-third of the IgG1 potency. This raised the question of whether the lower IgG2 mAb potency was due to the disulfide heterogeneity, as observed by RP-HPLC. Ideally, the RP-HPLC peaks would be collected and tested for potency, but because the RP-HPLC assay utilizes denaturing conditions (high temperature, organic solvent, and low pH), the collected fractions would not retain native structure and function. Therefore, a different assay using non-denaturing conditions was needed. Fractionation of the disulfide heterogeneity was attempted by cation exchange (CEX) chromatography, which has been shown to partially resolve IgG2 disulfide isoforms (15). When the collected CEX fractions were analyzed by RP-HPLC, they showed low purity (<50%) of the isoforms. However, differences in activity were observed that correlated with differences in the disulfide isoforms. The normalized potencies (IC50) of CEX fractions ranged from 70 ± 13% to 125 ± 12% (data not shown). Although not definitive, these results indicated that this naturally occurring structural heterogeneity affects the bioactivity of the antibody. To confirm these results, a more efficient enrichment technique was sought.

**Reduction-oxidation (Redox) Treatment of IgG2—Redox treatment of proteins from the inclusion body state is a common practice in the microbial production of recombinant proteins but is not typically implemented in mammalian cell production. However, because the IgG2 isoforms were discovered to be disulfide-mediated, the reactivity of the isoforms to redox treatment was tested. In the example shown, the anti-IL-1RI IgG2 mAb was subjected to a variety of redox conditions in the presence and absence of GuHCl. By changing the redox treatment conditions, either RP-HPLC peak 1 or peak 3 could preferentially be enriched (Fig. 4). Peak 1 was redox-enriched without using GuHCl, and peak 3 was enriched using ~1 m GuHCl, a concentration well below that known to affect overall secondary or tertiary structure of the antibody (11, 22). The major RP-HPLC peaks in the redox-treated materials eluted at approximately the same retention times as peaks in the untreated (no redox) IgG2 control material. Full characterization of IgG2 heterogeneity by RP-HPLC has been previously completed by performing nonreduced peptide mapping on collected RP-HPLC fractions (15). That characterization showed that the RP-HPLC method was able to resolve three unique disulfide structures in the elution order of IgG2-B (peak 1), IgG2-A/B (peak 2), and IgG2-A (peaks 3–4). The RP-HPLC
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**Far-UV CD analysis** was used to assess the overall secondary structure of the antibody. Comparison of the individual CD spectra for each of the enriched IgG2 isoforms and IgG2 control material showed no significant difference in secondary structure (data not shown). SEC analysis, which separates based on hydrodynamic radius, was used to assess the apparent molecular sizes of the IgG2 isoforms. The enriched isoforms and the control material generated similar peak shapes but eluted at slightly different retention times (Fig. 6a). The enriched IgG2-A eluted earlier than IgG2-B, with the untreated sample, containing a mixture of the isoforms, eluting at an intermediate time. These results suggested that IgG2-A had a larger apparent molecular size than IgG2-B. An alternate explanation of the retention time shift is that the isoforms were differentially interacting with the column matrix due to differences in solvent exposed surface area or hydrophobicity. Sedimentation velocity analytical ultracentrifugation was therefore used as an orthogonal technique to investigate the hydrodynamic properties of the disulfide isoforms. Similar to the SEC data, the sedimentation coefficient distribution was unique for each of the IgG2 isoforms analyzed in various buffers (Fig. 6b). The enriched IgG2-B isoform consistently showed a greater sedimentation coefficient (s) value than IgG2-A, supporting the SEC assessment of a more compact conformation for IgG2-B.

In addition, the thermal stability of IgG2-A and IgG2-B was tested using differential scanning calorimetry. This technique was used to assess whether changes in antibody structure impacted stability as a function of temperature. The IgG2-A and IgG2 control samples generated similar thermograms (Fig. 7), whereas the IgG2-B sample displayed a significantly increased enthalpy at a high temperature transition (~85 °C). Together, the biophysical analyses suggested that the IgG2 disulfide isoforms contain native-like secondary structure but display altered tertiary/quaternary structure and distinct thermal stability properties.

**Biological Activity of the IgG2 Disulfide Isoforms**—To determine whether the binding properties of the disulfide isoforms differ, the binding ability and biological activity of the redox-enriched IgG2-A and IgG2-B isoforms and IgG2 control were measured in three different cell-based assays (Fig. 8, a–c, supplemental Table S1, and Fig. S5).

The effect of redox treatment on biological activity of the anti-IL-1R1 IgG2 mAb samples was assessed by monitoring the inhibition of IL-1β-induced IL-6 production by primary human chondrocytes and human whole blood. The results of the bioassays showed differences in IL-6 inhibition between the enriched IgG2-A and IgG2-B isoforms (Fig. 8a). On average, there was a statistically significant difference in IC_{50} values between the enriched isoforms, with IgG2-B activity at about one-third of IgG2-A (Fig. 8, b and c). In addition, the binding properties were assessed from global nonlinear regression analysis of IL-1β dose shift experiments in human whole blood. Although all three antibody samples bound with high affinity to IL-1R1, measurable differences in dissociation constants were obtained (supplemental Table S1 and Fig. S5). The IgG2 control sample and IgG2-A sample bound with comparable affinities (~0.06 nM), whereas the affinity measurement of the IgG2-B sample was 3-fold weaker (~0.19 nM). The apparent correlation
between disulfide structure and changes in affinity and biological activity of the anti-IL-1RI mAb may be caused by limited conformational angles and a loss of flexibility between the Fab domains of the IgG2-B. The reduced Fab mobility may restrict the ability of IgG2-B to form multivalent interactions with IL-1RI on cell surface, thus reducing the measured affinity and biological activity of this isoform (see next section for more discussions).

To determine whether this structural isoform activity difference is a general property of the IgG2 subclass, studies were conducted with several other IgG2 mAbs differing in their therapeutic targets. For each antibody, IgG2-A and IgG2-B were enriched through redox treatment, and the resulting samples were tested with an appropriate activity assay. Some mAbs displayed activity difference between the disulfide isoforms, whereas others did not (data not shown). Approximately half of the IgG2 mAbs against cell surface receptors showed higher potency of IgG2-A compared with the IgG2-B. Only one of the IgG2 mAbs tested was directed against a non-membrane-bound antigen, and no measurable differences in potency were observed between IgG2-A and IgG2-B. The structure-function relationship is likely to depend on the nature and accessibility of the epitope for a particular IgG2 antibody (e.g., solution versus cell-surface receptor, receptor density, etc.) and the role avidity plays in the overall activity of the antibody.

**Whole Blood and PBS Redox Incubation**—Additional redox studies were conducted to determine whether the IgG2 disulfide isoforms were labile and prone to interconversion during in vivo circulation. The anti-IL-1RI IgG2 mAb was incubated in whole blood to determine if conversion between the isoforms

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**Figure 5.** A representation of the three different disulfide linkages of the human IgG2 subclass. The structures are defined by the interchain disulfide connectivity between the LC (gray), HC (black), and HC-HC. The broken lines represent the disulfide bonds that are different between the IgG2 isoforms. The yellow dots represent the individual cysteine residues (C) involved in forming the disulfide bonds. The numbering of the amino acids is in Eu format, as shown in Table 1 and supplemental Fig. S1. These disulfide linkages were used to identify the redox-enriched material through nonreduced peptide mapping (supplemental Fig. S4). The IgG2 isoforms each displayed a unique group of peptides (P1, P2, and P3), after enzymatic digestion, that were analyzed by RP chromatography and used to confirm the IgG2 structures (IgG2-A (top), IgG2-A/B (middle), and IgG2-B (bottom), respectively).

**Figure 6.** Size-exclusion chromatography and sedimentation velocity analysis of the IgG2 redox-enriched samples. **a**, size-exclusion chromatograms of IgG2 control material (black), IgG2-B (red), and IgG2-A (blue) samples. The enriched IgG2-A isoform eluted earlier suggesting an apparent larger molecular size as compared with the control material and enriched IgG2-B isoform. **b**, continuous sedimentation coefficient distribution analysis of enriched IgG2-B (red) and IgG2-A (blue) in acetate buffer pH 5 with 5% sorbitol (○), PBS solution, pH 7 (○), and size-exclusion buffer (100 mM sodium phosphate, 500 mM sodium chloride, 5% ethanol, pH 7.0) (x). The continuous sedimentation coefficient distribution analysis supported the size-exclusion analysis by consistently showing larger sedimentation coefficient (s) values for IgG2-B.
would occur or if the isoforms would remain stable in the redox environment of human blood. Samples were incubated in whole blood, at 0.1 mg/ml, from multiple donors \((n = 2)\). Incubation time in whole blood was limited to 48 h to maintain endogenous factor levels and limit cell lysis. Samples were taken at several time points and purified using a protein A column followed by an IL-1RI affinity column. RP-HPLC was used to analyze the purified IL-1RI IgG2 samples. The results showed conversion of the IgG2 isoforms with a decrease in IgG2-A and concomitant increase in IgG2-B and IgG2-A/B (data not shown). The limitations of the in vitro whole blood assay prevented conclusions on conversion between disulfide isoforms over a time course representative of in vivo circulation time.

Because the IL-1RI IgG2 mAb could not be incubated in whole blood for longer than 48 h, a “blood-like” surrogate system was developed for a long term incubation that mimicked in vivo redox conditions. In the surrogate system, an IgG2 was incubated in a PBS solution (pH 7.2) containing the redox reagents levels of 250 \(\mu M\) cystine and 15 \(\mu M\) cysteine, near those found in human serum (23–25). Analysis in the PBS system was simpler than in whole blood, because the material could be analyzed directly by RP-HPLC without prior time consuming affinity purification. Incubations were performed at 37 °C and monitored up to 4.5 days (supplemental Figs. S6–S11). A consistent trend was observed for all samples showing an increase in IgG2-B and a decrease in IgG2-A. In addition, the samples incubated in PBS only (no redox) showed no significant change in the levels of IgG2 isoforms (supplemental Figs. S7 and S10). These results demonstrate that the disulfide isoforms of the human IgG2 were labile in the presence of blood-like redox conditions and generally showed conversion toward the IgG2-B disulfide structure. It should be noted that the free cysteine is gradually oxidized and, therefore, does not represent a true physiological steady-state redox environment. Therefore, the conversion rates for the isoforms determined in this study may be used as a lower estimate of in vivo events. Nevertheless, based on this trend we concluded that the endogenous heterogeneity of the IgG2 subclass could occur in circulation in vivo.

**DISCUSSION**

The biological mechanism enabling the interconversion of IgG2 isoforms can only be speculated upon because of the lack of detailed structural data for human IgG2 unlike IgG1 (26).
early as 1969, it was suggested that, although the point of attachment for the LC and HC in all IgGs may appear distant in the primary structure, the disulfide connectivity should occur in a close proximity of the three-dimensional structure “with only a minor readjustment of a few key bond angles” (27). A more recent publication provided support for the earlier hypothesis as it showed that a mutation in the point of attachment, at residue 131 (from Cys to Ser) of the HC of an IgG3 antibody can force the LC to form an interchain disulfide bond with the hinge (28). Although there is no solved crystal structure for a human IgG2, the overall orientations of the Fc and Fab regions of the IgG are considered to be similar for all subclasses and mainly differ in the sequence of the hinge (28).

By utilizing the atomic coordinates from a full-length human IgG1 (29) (PDB, ID 1HZH), we prepared a ribbon diagram (Fig. 9) to illustrate how the amino acid sequence homology of the IgG1 and IgG2 and structural similarities of the subclasses could be used to describe a plausible mechanism for IgG2 disulfide exchange. The close proximity of the hinge Cys220, the C-terminal Cys234 of the LC, and the flexible loop of the conserved region of HC containing Ser131 is evident in the IgG structure (Fig. 9b). Because only two cysteine residues, Cys220 and Cys234, are in close proximity to each other, the disulfide linkage between them is stable and unambiguous in IgG1. A comparison of the amino acid sequences for human IgG1 and IgG2 (Table 1 and supplemental Fig. S1) shows that the most significant differences between the subclasses are substitutions Ser131 → Cys in the hinge, Ser131 → Cys in the flexible loop of the HC of IgG2, and the truncation of three amino acids (Asp221–Thr223) in the hinge of IgG2. Assuming structural similarity of IgG1 and IgG2, these substitutions place several IgG2 cysteines in close proximity: the C-terminal cysteine of the LC (Cys234), the cysteine in the flexible loop of HC (Cys131), and the two cysteines of the upper hinge (Cys219 and Cys220). It is interesting that, by linear sequence, the HC Cys131, Cys219, and Cys220 from the hinge are separated by ~100 amino acids; however, the distance between the cysteines within the three-dimensional structure is only ~10 Å. This spatially situates the hinge and the flexible loop containing the disulfide LC–HC linkage in a range compatible with disulfide exchange (30, 31). This proximity, in combination with the unique disulfide-rich (Cys219 and Cys220) hinge of the IgG2, provides a structural framework by which the IgG2 subclass can interconvert among an ensemble of naturally occurring disulfide isoforms.

Because the three-dimensional structures of the human IgG subclasses are similar, it allowed us to develop hypothetical models for each of the IgG2 isoforms (Fig. 10). We postulated that the IgG1 (1HZH) crystal structure (Fig. 9) would closely resemble the IgG2-A/B isoform. The oblique orientation of the Fc with respect to the Fab domains allows for a single Fab region of the IgG to interact (through disulfide bonds in the case of IgG2-A/B) with the hinge, whereas the other Fab is spatially decoupled from this region (26). This asymmetrical Fab orientation relative to the Fc portion of the antibody shows how Fab-Fab flexibility can still be achieved while Fab-Fc interactions coexist. The IgG2-A isoform is depicted as a canonical Y-shaped structure and the IgG2-B isoform as a constrained T-shaped structure. In the IgG2-B isoform, both LC and Cys131 residues of the HC are disulfide-bonded to the hinge, likely limiting flexibility of the antigen-binding Fab regions.

Structural characterization by CD showed no difference in secondary structure of the IgG2 isoforms but SEC, sedimentation velocity analytical ultracentrifugation, RP–HPLC, and ESI-mass spectrometry analyses consistently showed the IgG2-B isoform to possess a more compact global structure than the IgG2-A isoform. These conformational differences between the disulfide forms were likely a result of variations in Fab-Fc orientation and flexibility.

The three-dimensional analysis helped to elucidate the structural basis for the predominance of IgG2-B in IgG2x, but not in IgG2A antibodies (Fig. 1, e, f, i, and j). In general, IgG2A showed relatively low levels of this disulfide isoform while IgG2x generally populated this isoform in the highest abundance. Based on the close examination of the proximity of the LC and hinge in Fig. 9b, we hypothesize that the additional C-terminal serine residue (Ser215) of the λ-LC sterically interferes with the LC forming a disulfide bond with the hinge cysteines. Although IgG2-A seems to be preferred for IgG2A, IgG2-A/B is detected in approximately...
equal amounts for IgG2α and IgG2κ. Previous reports (4, 32, 33) have shown that the LC type can impact antigen/receptor binding and \textit{in vivo} clearance. Some of these results were linked to specific differences in flexibility between the α- and κ-LC, and, as we have shown in this report, may also be attributed to different distribution of IgG2 isoforms.

The disulfide heterogeneity in IgG2, both recombinantly derived and isolated from human serum, may arise from disulfide conversion post expression. A single structure (IgG2-A) could be produced in the cellular endoplasmic reticulum and only later covert to other forms. If so, this suggests that the environment of the endoplasmic reticulum, where the original disulfides are formed, differs from that of the extracellular environment, which leads to structural changes in the antibody. The relatively low level of GuHCl used in our studies to populate IgG2-A provides a mild denaturing environment that mimics the folding environment in the secretory pathway. Such low levels of denaturant may be required to induce structural flexibility that allows repositioning of the cysteines at the hinge without affecting secondary structure. On the other hand, the extracellular surroundings \textit{in vivo} generally represent a nondenaturing environment that is preferred by IgG2-B. This suggests that IgG2-B would predominate in these conditions unless additional factors such as antigen binding, receptor binding, or other interactions are able to induce structural changes.

We have demonstrated for the first time that the disulfide-mediated heterogeneity of human IgG2 antibodies can impact structure and function. The impact of structural differences on biological activity for a specific IgG2 manifested itself in a number of cell-based assays and was dependent on binding affinity, cellular surface density of the receptor, and cooperative receptor binding through both Fab domains. By developing a unique redox treatment method we were able to enrich the disulfide isoforms of the IgG2 and subsequently characterize the structure and function of the isoforms. Moreover, in a blood-like redox environment the disulfide isoforms were shown to interconvert. The ability of human IgG2 to modulate structure by shuffling disulfide bonds may regulate the overall function of the antibody \textit{in vivo}. Disulfide bonds have been generally divided into three main types: structural, catalytic, and allosteric (34). The regulation of protein function through “allosteric disulfide bonds” is a relatively new area of research. These disulfide bonds have been shown to act as physiological switches by eliciting conformational changes in proteins (35). We speculate that this type of disulfide bonds may be closely related to the human IgG2 phenomenon presented herein.

By showing that human IgG2 antibodies exist as an ensemble of structures that are able to convert under physiological conditions and that differ in their biological activity, we have revealed a new structure-functional relationship for this class of immunoglobulins. Additional studies are required to characterize the physiological function for this conversion and its role in antibody response.

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