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 receptor type 1. In vitro biological activity measurements showed an increased activity of the IgG1 relative to the IgG2 in blocking interleukin-1β ligand from binding to 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 >> 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. Incomplete or incorrect 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)\(^3\) 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 IgG1\(\kappa\) versus IgG2\(\kappa\) 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 re-equilibrated 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.

**Sedimentation Velocity Analytical Ultracentrifugation**—The solution properties of the IgG2 isoforms were evaluated by sedimentation velocity measurements conducted with a temperature-controlled Beckman XL-I analytical ultracentrifuge equipped with an An-60 Ti rotor and a photoelectric scanner (Beckman Instruments, Palo Alto, CA). Samples were diluted to 0.5 mg/ml in the desired buffer (see Fig. 6). Protein samples were loaded in a double sector cell equipped with a 12-mm Epon centerpiece and a sapphire optical window. The reference compartment was loaded with the matching buffer. The samples were monitored at 280 nm at a rotor speed of 40,000 rpm at 20 °C. Analyses of the raw data were completed using Sedfit v. 8.9 to obtain concentration distribution (c(s)) as a function of sedimentation coefficient. A confidence level of 0.7 (1 standard deviation) was used for the analysis. Meniscus and frictional ratio (f/f\(\infty\)) were fitted. Other fitting parameters used were default parameters suggested by Sedfit software. Replicate runs showed standard deviation of average sedimentation coefficients of <0.01 s. Partial specific volume for each antibody, buffer density, and buffer viscosity were calculated using Sednterp (v. 1.08) and used for hydrodynamic radii calculations.

**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 nm 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-

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\(^3\) 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-1β, interleukin-1β; GuHCl, guanidine hydrochloride; CEX, cation exchange; CH1, constant region of heavy chain; SEC, size-exclusion chromatography; Fc, fragment crystallizable; PBS, phosphate-buffered saline.
The inhibition of IL-6 production was calculated as a percentage of maximum IL-1β/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-1β/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).

**Statistical Analysis**—Bioassay data were analyzed using GraphPad Prism. IC50 values were derived by nonlinear regression (variable slope). Where dose-response curves were incomplete, the bottom was constrained to zero. Significance was calculated using a one-way analysis of variance with a Tukey multiple comparison post-test. A p value < 0.05 was considered significant.

**Crystal Structure Analysis**—Display and measurements were made using the DeepView/Swiss-PdbViewer version 3.7.

**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–f) 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.
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**FIGURE 3. IC₅₀ values for the inhibition of IL-1β-induced IL-6 in a chondrocytes assay for the IgG2 (●) and IgG1 (○) mAb constructs are shown (n = 3). The black bars represent the means. For statistical analysis the p value was <0.01.**

**TABLE 1**

Human IgG1 and IgG2 sequence alignments of the CH1 loop and upper hinge of the HC

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(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 peaks 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 ($\sim 85 \degree C$). 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-1RI IgG2 mAb samples was assessed by monitoring the inhibition of IL-1$\beta$-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-1$\beta$ dose shift experiments in human whole blood. Although all three antibody samples bound with high affinity to IL-1RI, 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 ($\sim 0.06$ nM), whereas the affinity measurement of the IgG2-B sample was 3-fold weaker ($\sim 0.19$ nM). The apparent correlation

elution profiles suggested that the two redox procedures preferentially enrich for the newly discovered IgG2 isoforms (IgG2-A and IgG2-B). Additionally, several other IgG2 mAbs with alternative antigen targets were redox-treated using similar conditions (supplemental Fig. S3). These results showed that all IgG2 mAbs tested were reactive to redox treatment. The redox-enriched isoforms showed no further conversion after being removed from the redox solution and stored in a PBS solution.

To confirm the identity of the redox-enriched IgG2 isoforms, nonreduced peptide mapping was performed on the redox-treated material and IgG2 control material (supplemental Fig. S4) using the earlier described protocols for nonreduced peptide mapping and mass spectrometry identification. The analysis confirmed that the redox procedures were able to enrich for two of the IgG2 isoforms (IgG2-A and IgG2-B) that were previously shown to exist in the IgG2 control material (15). Therefore, redox treatment of the IgG2 antibodies did not introduce new disulfide forms but effectively populated structural isoforms already present within the control material.

Based on the above results, the IgG2 disulfide isoforms were classified into three predominant structures, IgG2-A, -B, and -A/B (Fig. 5), defined by their disulfide connectivity. The structures differs by their interchain disulfide attachment of LC to the CH1 region or to the hinge. Two of the isoforms (IgG2-A and IgG2-B) contained symmetrical interchain disulfide connectivity in that the same light-to-heavy chain (LC-HC) attachment was present on both sides of the molecule (Fig. 5). In the third isoform (IgG2-A/B), one light chain was connected to CH1, whereas the other was connected to the hinge, which made it an asymmetrical disulfide structure. The IgG2-A/B disulfide form was an intermediate of the A and B forms.

Biophysical Characterization of Redox-enriched Isosforms—The structural and thermodynamic properties of the redox-enriched isoforms (IgG2-A and IgG2-B) were compared using a series of biophysical assays. These tests included far UV CD, size-exclusion chromatography (SEC), sedimentation velocity analytical ultracentrifugation, and differential scanning calorimetry.
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
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 Cys$^{220}$, the C-terminal Cys$^{214}$ of the LC, and the flexible loop of the conserved region of HC containing Ser$^{131}$ is evident in the IgG structure (Fig. 9b). Because only two cysteine residues, Cys$^{220}$ and Cys$^{214}$, 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 Ser$^{219}$ → Cys in the hinge, Ser$^{131}$ → Cys in the flexible loop of the HC of IgG2, and the truncation of three amino acids (Asp$^{221}$–Thr$^{223}$) in the hinge of IgG2. Assuming structural similarity of IgG1 and IgG2, these substitutions place several IgG2 residues of the HC Cys131, Cys219, and Cys214, the cysteine in the flexible loop of HC (Cys131), and the C-terminal Cys214 of the LC and Cys220 of the HC can be seen in a close proximity of the three-dimensional structure “with only a minor readjustment of a few key bond angles” (27). A flexible loop in the HC CH1 from amino acids Ser131 to Thr139 was disordered and lacked sufficient electron density and is not displayed.

FIGURE 9. A ribbon diagram of a human IgG1 antibody using atomic coordinates that were deposited in the Protein Data Bank (1HZH). The crystal structure of this full-length monoclonal human IgG1 antibody was solved to 2.7 Å. Although deposited 1HZH coordinates were numbered in Kabat and Wu format, the residue numbering in this figure is in Eu format, the same as in Table 1 and supplemental Fig. S1. The interchain disulfide bond between the C-terminal Cys131 of the LC and Cys220 of the HC can be seen in b. A flexible loop in the HC CH1 from amino acids Ser131 to Thr139 was disordered and lacked sufficient electron density and is not displayed.

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 (Ser$^{215}$) of the α-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

TABLE 1. Amino acid sequence comparison for human IgG1 and IgG2.
equal amounts for IgG2α and IgG2κ. Previous reports (4, 32, 33) have shown that the LC type can impact antigen/receptor binding and in vivo clearance. Some of these results were linked to specific differences in flexibility between the λ- 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 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 in vivo.

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