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IgG2 disulfide isoform conversion kinetics

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ABSTRACT

Human IgG2 antibodies contain three types of disulfide isoforms, classified by the number of Fab arms having disulfide links to the heavy chain hinge region. In the IgG2-B form, both Fab arms have interchain disulfide bonds to the hinge region, and in IgG2-A, neither Fab arm are disulfide linked to the hinge. The IgG2-A/B is a hybrid between these two forms, with only one Fab arm disulfide linked to the hinge. Changes in the relative levels of these forms over time are observed while IgG2 circulates in humans, suggesting IgG2-A → IgG2-A/B → IgG2-B conversion. Using a flow-through dialysis system, we studied the conversion kinetics of these forms *in vitro* under physiological conditions. For two IgG2κ antibodies, *in vivo* results closely matched the kinetics observed *in vitro*, indicating that the changes observed *in vivo* were solely conversions between isoforms, not differential clearance of specific forms. Moreover, the combined results validate the accuracy of the physiological model for the study of blood redox reactions. Further exploration of the conversion kinetics using material enriched in the IgG2-A forms revealed that the IgG2-A → IgG2-A/B rate was similar between IgG2κ and IgG2λ antibodies. In IgG2κ antibodies, conversion of IgG2-A/B → IgG2-B was slower than the IgG2-A → IgG2-A/B reaction. However, in IgG2λ antibodies, little IgG2-A/B → IgG2-B conversion was detected under physiological conditions. Thus, small differences in the C-terminus of the light chain sequences affect the disulfide conversion kinetics and impact the IgG2 disulfide isoforms produced *in vivo*.

1. Introduction

The human IgG antibody class is comprised of four sub- or isotypes: IgG1, IgG2, IgG3 and IgG4, based on their heavy chain (HC) identity. Each subtype molecule contains two identical HCs and two identical light chains (LC) of either the kappa (κ) or lambda (λ) subclass, linked together by disulfide bonds. All the IgG isotypes possess a single interchain disulfide bond between the light chain (LC) and the heavy chain (HC) but differ in the number of HC to HC disulfide links in the hinge region: IgG1 and IgG4 each has 2, IgG2 has 4, and IgG3 has 11 (Alarazi et al., 1988). While the disulfide linkage pattern in the IgG subtypes was published some time ago, more recent studies have discovered alternative linkages in some isotypes. For example, in IgG4 molecules, hinge disulfides can form intrachain linkages, which allow half molecules (1HC + 1LC) to exchange between antibodies *in vivo* (Schuurman et al., 1999; van der Neut Kolfschoten et al., 2007). IgG2 isotypes also have alternate disulfide linkages. Like IgG4, the IgG2 hinge cysteines can form intrachain as well as interchain linkages (Zhang et al., 2010), though no half molecule exchange has been reported. More strikingly, alternative linkages have been observed between the Fab arms and the hinge region (Wypych et al., 2008).

These alternate Fab arm linkages have been classified by whether the LC:HC interchain links are restricted to the Fab arm or are linked to the HC hinge region. In the IgG2-A form (A), the cysteine near (λ) or at (κ) the C-terminus of each LC is linked to the Fab arms of the HC (Wypych et al., 2008). This is the originally described structure (Milstein and Frangione, 1971). In the IgG2-B structure (B) both Fab arms are disulfide linked to the hinge, and in the hybrid IgG2-A/B structure (A/B), only one Fab arm is disulfide linked to the hinge. Models of the IgG2 alternative inter chain linkages are shown in Fig. 1, with intrachain disulfide linkages omitted for simplicity. Single examples are given for A, A/B and B, though multiple linkage patterns may exist for each structural class (Zhang et al., 2010).

Mixtures of the IgG2 disulfide isoforms have been found in endogenous antibodies purified from human blood, as well as in recombinant therapeutic antibodies produced using animal cells (Dillon et al., 2008; Wypych et al., 2008). The function of disulfide isoform heterogeneity *in vivo*, if any, is not clear. For some

Abbreviations: HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; PBS, phosphate buffered saline; NEM, N-ethyl maleimide; UV, ultraviolet; Redox, oxidation–reduction.

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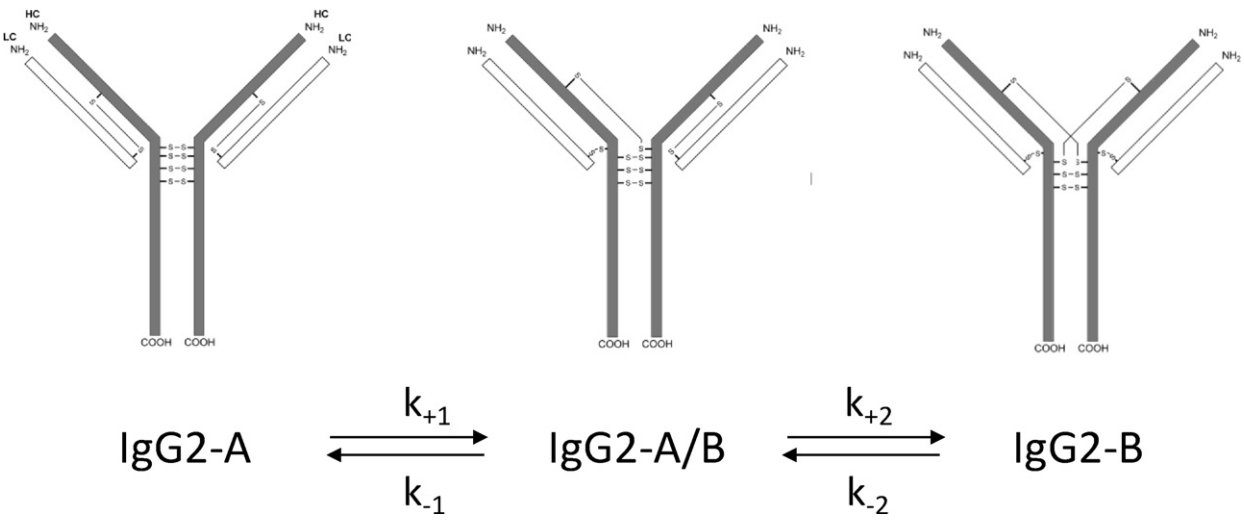

Fig. 1. IgG2 disulfide isoform conversion. Interchain disulfides are shown on a simplified IgG2 model where intrachain disulfides have been removed. Heavy chains are shown in gray and light chains in white.

therapeutic IgG2 antibodies, activity differences have been observed (Dillon et al., 2008). Greater flexibility has been proposed for the A form, which may allow both Fab arms to more effectively reach two target antigens that have restricted mobility.

Relatively mild redox conditions can change the distribution of A, B and A/B isoforms *in vitro* (Dillon et al., 2008). Conditions similar to those found in the blood promoted IgG2 disulfide exchange, suggesting that disulfide shuffling could occur on endogenous IgG2's *in vivo* (Liu et al., 2008). Our previous study (Liu et al., 2008) followed disulfide isoform conversion *in vivo* for a therapeutic IgG2κ antibody using reversed phase HPLC (RP-HPLC) separation. The relative level of the A form decreased quickly, whereas the A/B form decreased slowly and the B form increased slowly. A later eluting peak in the chromatogram, which contained an IgG2 disulfide isoform indistinguishable from the A form, and which did not change in relative levels, was termed A* (for this work, the labile A form is termed A_1 and the non-convertible A form is termed A_2). The combination of relative level changes observed *in vivo* and the conversion reactions studied *in vitro*, suggested that the changes observed *in vivo* were disulfide isoform conversions, thus were kinetically modeled as such. However, the lack of a suitable *in vitro* system to model the blood redox conditions prevented an estimation of the degree of conversion *in vivo*. In previous redox experiments containing low concentrations of the free thiol compounds found in blood, rapid oxidization of the free thiol compounds interfered with the long-term monitoring of the disulfide shuffling reactions. Here we describe the use of a flow-through dialysis system to maintain blood-like redox conditions and allow the IgG2 disulfide isoform conversion kinetics to be studied under steady state physiological conditions. Studies with IgG2κ antibodies *in vitro* are compared to *in vivo* results and the *in vitro* studies were extended to IgG2λ antibodies.

2. Materials and methods

2.1. Materials and instrumentation

Recombinant human IgG2 monoclonal antibodies (mAbA [IgG2κ]; mAbB [IgG2κ]; mAbC [IgGλ]; and mAbD [IgG2λ]) were expressed in Chinese hamster ovary (CHO) cells and purified by standard manufacturing procedures (Shukla et al., 2007). Hu IL-1R (binds to mAbA) and hu IL-4R (binds to mAbB) were produced and purified at Amgen Inc. (Thousand Oaks, CA). Actigel ALD Superflow was from Sterogene Bioseparations (Carlsbad, CA). mAb–ligand resin was prepared using the Actigel ALD Superflow resin according to the manufacturer's protocol. The ZORBAX 300SB-C18 and 300SB-C8 (2.1 × 50 mm, 3.5 μm particles) columns were purchased from Agilent Technologies (Santa Clara, CA) and the Acquity UPLC BEH300 C4 Columns were purchased from Waters Corporation (Milford, MA). HyClone DPBS and Float-A-Lyzer dialysis devices were obtained from Thermo Fisher Scientific (Waltham, MA). L-Cystine, L-cysteine and sodium azide (NaN_3) were from MP Biomedical (Irvine, CA). DTNB (5,5′-dithio-bis 2-nitrobenzoic acid) was from Invitrogen (Carlsbad, CA). All organic solvents were of analytical or HPLC grade.

2.2. Affinity purification of mAbA and mAbB

Blood samples from human subjects with single intravenous doses of the antibodies were collected at specified time points. The mAb was purified from 0.17 to 0.5 mL serum samples using affinity purification procedures described elsewhere (Liu et al., 2008).

2.3. Disulfide isoform enrichment

The A_1 disulfide isoform was enriched from samples containing a mixture of isoforms either by cation exchange chromatography (CEX) or oxidation/reduction (redox) treatment. Redox enriched IgG2-A_1 material was prepared as described (Dillon et al., 2008), except that 1 M guanidine HCl was used. CEX enrichment was performed on an AKTA chromatography system as described (Wypych et al., 2008), with some modifications. A YMC Biopro SP-F (4.6 mm × 100 mm) column (YMC Co., Kyoto, Japan) was operated at ambient temperature. The mobile phase A consisted of 20 mM sodium acetate, pH 5.2 and mobile phase B consisted of 20 mM sodium acetate, 400 mM sodium chloride, pH 4.5. After injection (100 μg) onto the column, protein was eluted with a linear gradient of mobile phase A to mobile phase B, with some adjustments for each molecule. After purification or redox treatment, the IgG2-A_1 enriched samples were concentrated and buffer exchanged against 5 mM sodium acetate, 5% sorbitol, at pH 5.0, adjusted to the desired concentration of 30 mg/mL, and stored at −70 °C.

2.4. Non-reducing RP-HPLC analysis of mAb in vivo samples

Purified mAb samples were analyzed by non-reducing RP-HPLC as previously described (Dillon et al., 2006), except that column