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Stability of IgG isotypes in serum

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Abbreviations: FDA, Food and Drug Administration; IgG, immunoglobulin; MS, mass spectrometry; ER, endoplasmic reticulum; CDR, complementarity determining regions; PTM, post translational modification; cIEF, capillary isoelectric focusing; NA1F, asialo, monogalactosylated biantennary, core-substituted with fucose; NA2F, asialo, bigalactosylated biantennary, core-substituted with fucose; NGA2F, asialo, agalacto-, biantennary, core-substituted with fucose; NGA2F-GlcNAc, asialo, agalacto-, biantennary, core-substituted with fucose minus a bisecting N-acetylglucosamine; NA1F-GlcNAc, asialo, monogalactosylated biantennary, core-substituted with fucose minus a bisecting N-acetylglucosamine

Drug development from early discovery to late stage commercialization is a long arduous process where a number of factors are taken into consideration when deciding on a particular immunoglobulin isotype for a therapeutic purpose. There are no general rules for which isotype is selected; however, prior experiences, effector function and the specific therapy targeted, as well as extensive testing early in development help in paring the number of candidates. Over 20 monoclonal antibodies are FDA-approved, and most are IgG1 isotype, although a number of non-IgG1 molecules have been approved recently and the number in development is on the rise. Analytical techniques that examine the physicochemical properties of a molecule provide vital information on the stability and efficacy of candidate antibody therapeutics, but most of these studies are conducted using standard buffers and under well defined storage conditions.

It has recently become apparent that analysis of antibody therapeutics recovered after circulation in blood show altered physicochemical characteristics, and in many instances therapeutic molecules recovered from serum show lower potency. This review examines some of these studies, with a focus on the physicochemical changes observed in the molecules. Technologies that can facilitate rapid screening of candidate antibody therapeutics directly from blood are highlighted. The facts indicate that antibody therapeutic development programs must incorporate understanding of the basic biology of the isotype and its stability in serum, which is the intended environment of the therapeutic.

Introduction

Antibodies (immunoglobulins, Ig) have emerged as an important class of therapeutics in oncology, chronic inflammation, cardiovascular, transplantation and infectious diseases. To date, over 20 antibody therapeutics have been approved, with numerous others at various stages of development.[1-4] Antibodies are attractive as therapeutics due to their specificity and safety. This is reflected in their relatively high approval success rate (~25% for humanized antibodies) compared with the ~11% success rate for small molecule development.[4-6] Other advantages of antibodies, in general, are that they are well tolerated, and the knowledge and experience gained from one antibody during development, manufacturing and clinical use has the potential to be readily transferred to other therapeutics in the pipeline of an organization.[4,7] Two major disadvantages of antibody therapeutics are that targets are restricted to molecules in circulation or those expressed on the surface of cells and they are expensive, primarily due to high doses needed for treatments.[4,8]

The structure of antibodies, with complete amino acid content and disulfide bond pairing, was elucidated by Gerald Edelman and Rodney Porter in the early 1960s, and they were awarded the Nobel Prize in 1972 for this work.[9-11] The Ig monomer is composed of two identical heavy chains (HC) and two identical light chains (LC) that are linked by disulfide bonds (inter-chain disulfides). Each of the HC and LC contain structural domains (Ig domains) that resemble immunoglobulin folds composed of two beta sheets linked by cysteine residues (intra-chain disulfides); the Ig domains are further classified into variable (V) or constant (C) domains depending on structure and function.[12,13]

There are five classes of immunoglobulins that are classified on the basis of the constant region of the heavy chain; they are IgA, IgD, IgE, IgG and IgM. The constant heavy region of IgA, IgD and IgG has three Ig domains and a hinge region to provide flexibility; whereas, the constant regions of IgE and IgM has four Ig domains. The IgG and IgA classes are further classified into six isotypes (IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2). Despite the vast choice of immunoglobulin types to select for development of a therapeutic antibody, most antibody engineers have focused on the IgG class, although the IgG3 class is not used as a therapeutic candidate because it has a shorter half-life, a long hinge region that is easily accessible to proteolysis, and allotypic polymorphism.[1,4,5,14]

The intra-chain disulfides of the HC and LC immunoglobulin domains for the four IgG isotypes are similar; however, the inter-chain disulfide brides are different (see **Fig. 1**). Differences

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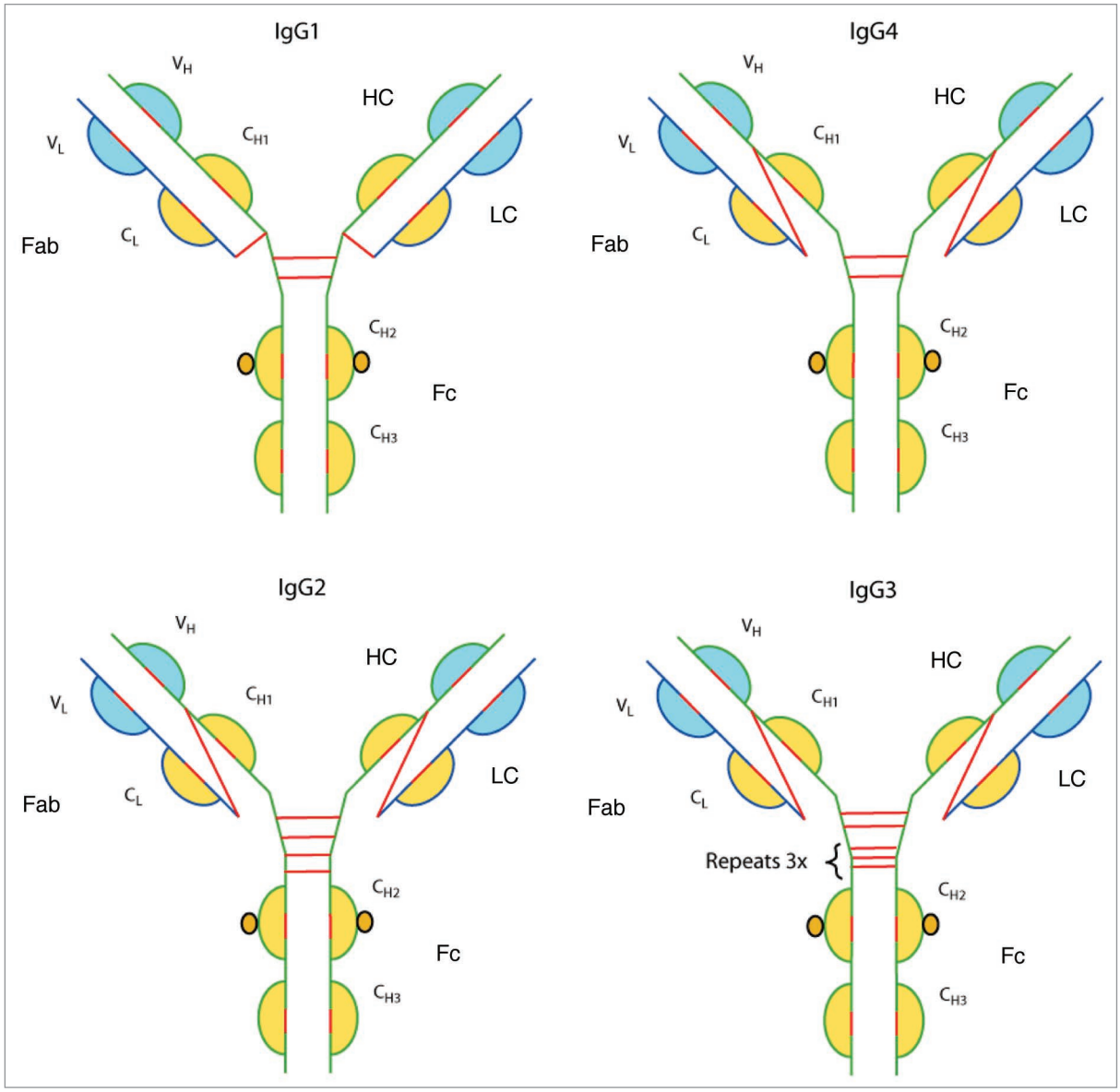

Figure 1. Schematic of the human IgG isotypes and their disulfide linkages.

Table 1. Amino acid sequence of hinge region for different IgG isotypes

IgG isotype	Core hinge region sequence (from refs. 96, 97)
IgG1	EPKS**C**DKTHT**C**PP**C**P
IgG2	ERK**CC**VE**C**PP**C**P
IgG3	ELKTPLGDTTHT**C**PR**C**P …… (EPKS**C**DTPPP**C**PR**C**P) **x 3**
IgG4	ESKYGPP**C**PS**C**P

in the inter-chain disulfide bridges between the HC is brought about by the amino acid composition of the hinge region and the number and position of the cysteine residues (**Table 1**). Both IgG1 and IgG4 have two disulfide linkages between their HC; whereas, IgG2 and IgG3 have four and eleven, respectively. The inter-chain linkage between the HC and LC of IgG1 is between the C-terminal cysteine residue of the LC and the first cysteine residue in the hinge region; whereas, the inter-chain linkage between the HC and LC of IgG2, IgG3 and IgG4 is between the terminal cysteine residue of the LC and the cysteine residue in Fab region (N-terminal of the C_{H1} domain).[15-22]

Protein therapeutics generally have a long half-life in blood and studies have revealed that the environment in blood can have a profound influence on the physicochemical properties of the molecule. Blood is composed of many types of cells, proteins and salts.[23] When the cells are removed without clotting, the liquid portion left behind is plasma; however, if cells are removed in the absence of anti-coagulants, the liquid portion is then called serum. Serum differs from plasma in that fibrin, as well as proteins that associate with fibrin, are removed; serum is estimated to have about 3–4% less protein than plasma.

Plasma typically contains about 22 proteins that make up 99% of the total protein content—they include albumin, total IgG, transferrin, fibrinogen, total IgA, alpha-2-macroglobulin, total

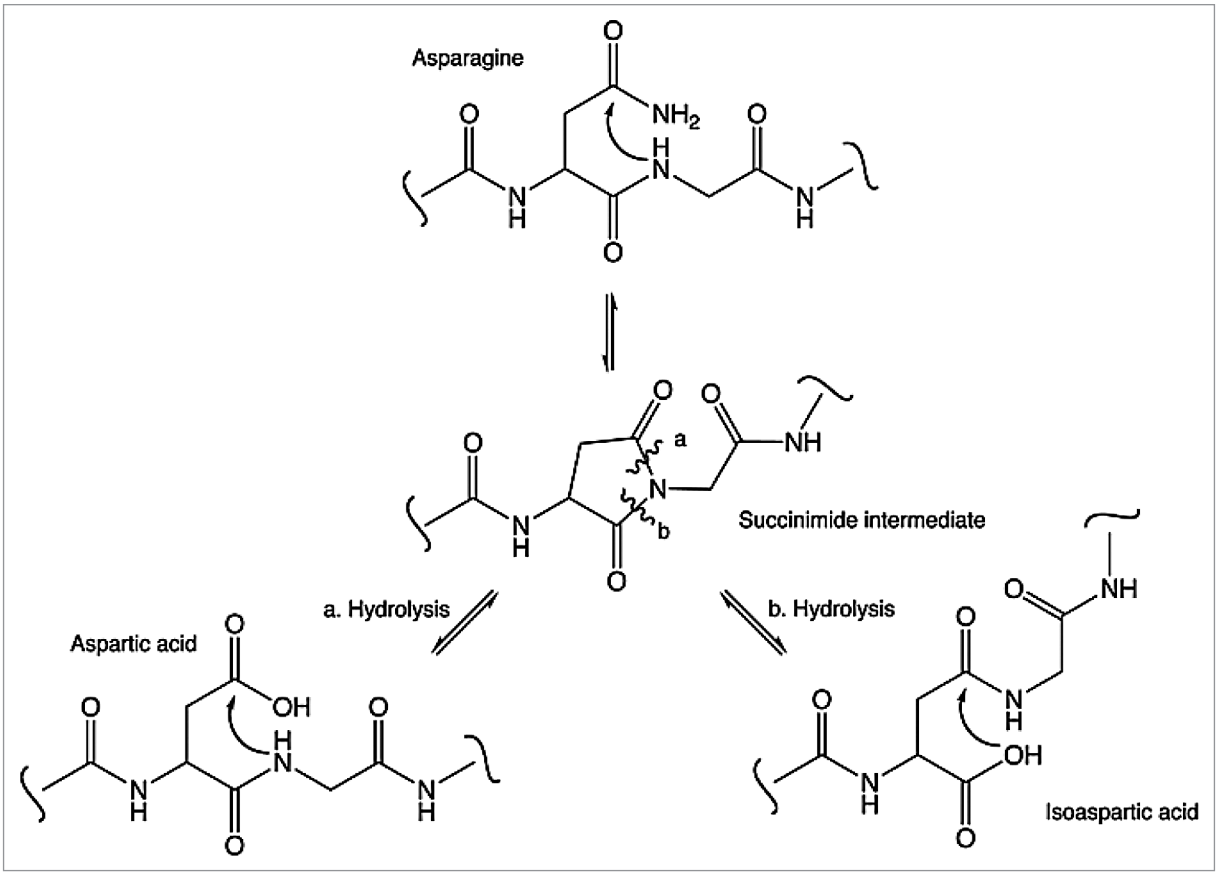

Figure 2. Deamidation of asparagine residue.

IgM, alpha-1-anti-trypsin, C3 complement, haptoglobulin, alpha-1-acid glycoprotein, apolipoprotein-B, apolipoprotein-A1, lipoprotein (a), factor H, ceruloplasmin, C4 complement, complement factor B, pre-albumin, C9 complement, C1q complement and C8 complement.[23-25] The remaining 1% of plasma is composed of hundreds of proteins that have been identified after removal of the most abundant proteins, using columns from Agilent, Sigma or GenWay Biotech, prior to MS analysis.[11,23,26-28] Serum proteins thus present an extremely "crowded" environment where the excluded volume results in antibody therapeutics adopting a compact structure, as well as showing enhanced association with antigen in serum.[29] High levels of albumin, cysteine, cystine, glutathione, homocysteine and other small thiols also present a "redox" environment, outside the endoplasmic reticulum (ER), that facilitates rearrangement of disulfide bonds in antibody therapeutics recovered from serum.[30-36] In circulation, the temperature of blood is at 37°C and antibody therapeutics are exposed for various lengths of time to receptors, as well as enzymes such as mannosidases. Due to these factors, serum has influences on the primary, secondary and higher order structures of antibody therapeutics.

Influence in Serum on Primary Structure of mAbs

Deamidation of asparagine residues in serum. Deamidation of asparagine, a non-enzymatic process, is a common post-translational modification (PTM) seen in protein therapeutics and is considered one of its major degradation pathways. Deamidation of asparagine is spontaneous and takes place during storage or sample handling. Due to steric exposure, asparagine residues, followed by glycines (NG sites) in flexible regions of a molecule, are found to be most susceptible to deamidation; the first step involves a nucleophilic attack of an α-amino group, from the peptide bond, on the side chain carbonyl group of the asparagine residue, resulting in a cyclic succinimide intermediate (**Fig. 2**). The succinimide intermediate is then hydrolyzed at either the alpha or beta carbonyl group to give either aspartate or isoaspartate (beta aspartate) respectively; hydrolysis of the beta carbonyl linkage is generally favored due to the asymmetry of the intermediate.[37,38] The Fc domain of the IgG1 and IgG2 isotypes share about 97% sequence homology.[39] Comparison of the rates of deamidation of 2 NG sites (Asn-315 and Asn-384) in the Fc domain revealed a slower rate of deamidation at Asn-315 (0.03% per day) compared to Asn-384 (0.63% per day).[40] An inspection of the two sites reveal the influence of secondary structure as hydrogen bonding of Gly-316 to Asp-312 reduced flexibility of the local region, thereby influencing the rate of deamidation of Asn-315.

A comprehensive analysis of sites of deamidation in murine mAbs (IgG1 and IgG2a) and recombinant human mAbs reveal that asparagine residues in the C_{H2} domain (WLN*GK) and

C_{H3} domain WESN*GQPEN*NY were consistently found to undergo deamidation.[41-43] Deamidation of asparagine residues in the CDR region was also observed in many of the molecules studied. The introduction of a net negative charge via deamidation is detrimental in many instances to protein function, as well as the pharmacokinetics (PK) of the molecule.[44] Loss of activity due to deamidation has been reported for two antibody therapeutics when it occurred in the CDR region.[44,45] Other evidence links isoaspartyl PTM to altered immunogenicity.[46,47] Consequently, long term stability studies at the recommended storage conditions of the protein therapeutic, as well as stressed stability studies done typically with different formulations, are extensively employed by the biotechnology industry to predict the molecule's capacity to undergo deamidation, monitor its loss of efficacy and demonstrate process consistency.[48-51] Deamidation is monitored by a variety of techniques since net negative charge as well as hydrophobicity of the molecule is altered and protein mass is changed (addition of 1 Da). Analytical techniques used to detect deamidation include capillary isoelectric focusing (cIEF) and ion exchange, which both measure change in charge, reverse phase chromatography measuring change in hydrophobicity and mass spectrometry measuring change in mass. An enzymatic assay that uses protein isoaspartyl methyltransferase (PIMT) is also frequently used for detecting isoaspartic residues.

Therapeutic antibodies tend to circulate in the blood for many days, or in some instances weeks, thus exposing the molecule to deamidation.[44] It is argued that the instability induced by deamidation of endogenous proteins in serum is a normal biological process that serves as a molecular clock.[37,52-55] However, deamidation of antibody therapeutics in vivo raise concerns about both loss in activity and the potential immunogenicity of the therapeutic. Thus, PK information for the antibody therapeutic and an estimate of the kinetics of deamidation for the different asparagine residues in serum are important for assessing the exposure of patients to the PTM and establishing criticality of this attribute, which has additional consequences concerning monitoring and controlling levels of the PTM.

For in vivo studies carried out with human therapeutic antibodies, the antibody is recovered from human serum using cross-liked antigen.[40] For studies carried out in monkey or other animals, serum recovery of the antibody was either recovered via cross-linked antigen or an anti-human Fcγ antibody.[44] The purified antibody is subsequently digested and peptides analyzed by LC/MS/MS. Extracted ion chromatograms for each asparagine-containing peptide are created to monitor the deamidation rates of Asn residues; the half-life for deamidation of different residues in the molecule can then be obtained. In vivo studies on antibody therapeutics have shown that rates of deamidation are independent of the administration route and deamidation in the Fc region of antibodies occurs naturally; both IgG1 and IgG2 are believed to undergo the same rates of deamidation.[40,44]

Influence of methionine oxidation on serum half-life. Oxidation of therapeutic proteins can take place on a number of different amino acid residues in the molecule, although methionine residues exposed to solvent tend to be the most susceptible.[39,56-58] The end product of methionine oxidation is frequently methionine sulfoxide, which increases mass by 16 Da and makes the molecule more polar and less hydrophobic. Technologies that allow for detection of oxidation include hydrophobic interaction chromatography (HIC), where oxidized species are found to elute earlier that the non-oxidized species; weak cation exchange chromatography (WCX-10), where oxidized species elute later than non-oxidized species; and peptide mapping, where peptides containing oxidized methionine residues elute earlier than non-oxidized methonine residues when separated on a reverse phase (RP) column.[42,57,59,60]

Oxidation of therapeutic proteins can occur during the cell-culture process, purification, formulation and storage of the molecules, and studies reveal that oxidation of methionine residues not only results in loss of activity, but also reduced stability of the molecule, potential immunogenicity and decreased in vivo half-life.[56,59-62] IgG1 and IgG2 molecules share considerable sequence homology (97%) in the Fc potion of the molecule and a number of studies using chemical oxidation, ultraviolet light, or elevated temperatures have shown that two solvent exposed methionine residues in the C_{H2} domain (Met-252) and C_{H3} domain (Met-428) are highly susceptible to oxidation compared to other methionine residues in the molecule.[39,42,56,58] Further, NMR analysis revealed that oxidation of Met-252 and Met-428 introduced structural changes in regions of the IgG molecule involved in binding to the neonatal receptor (FcRn), e.g., Met-428 interacts with residues on FcRn that are important for their pH dependent binding.[58,63,64] In fact, two studies confirmed that oxidation of methionine residues in the Fc region decreased its affinity for the FcRn receptor.[39,58] The FcRn receptor has an established role in maintenance of antibody half-life, and decreased binding of an IgG molecule to FcRn translates into a shorter half-life in serum.[58,65-67] Oxidation of methionine residues in the Fc region of an IgG molecule in serum can potentially have a significant impact on its half-life.

Influence of Fc glycan forms on clearance in serum. Glycosylation of recombinant protein drugs is closely monitored by the biotechnology industry because receptors for specific glycan structures are known to bind and rapidly clear these glycans from circulation. For example, studies have shown clearance of glycoproteins via two well-known receptors, the asialoglycoprotein receptor, which binds terminal galactose residues, and the mannose receptor, which binds terminal mannose and N-acetylglucosamine.[68-73] The Fc glycans present on human antibodies comprise an N-linked oligosaccharide (Asn-297) that shows considerable heterogeneity attributable to 32 oligosaccharides that can give rise to over 400 glycoform species as a result of random pairing of the heavy chains.[74] Antibodies manufactured by recombinant DNA technology and expressed in CHO cells show similar oligosaccharide profiles compared with those found in normal human IgG molecules. The major species typically reported e.g., the biantennary fucosylated species with 0, 1 or 2 terminal galactose residues (NA1F, NA2F, NGA2F, NGA2F-GlcNAc, NA1F-GlcNAc) and the high mannose non-fucosylated species (Mannose 5, 6, 7) are shown in **Figure 3**.

Fc glycans are partially buried within the C_{H2} domain and are believed to be not easily accessible to asialoglycoprotein and

mannose receptors.[75] A number of studies have attempted to study the impact of Fc glycans on clearance. In these studies, Fc glycans are either enriched by a variety of techniques, e.g., affinity purification, use of inhibitors, genetic mutation or enzymatic manipulation, or the entire glycan profile of the therapeutic antibody is studied after administration.[76-82] The major advantage of the latter method is that the glycan and antibody are typically very well-characterized prior to administration. Further, these studies are not limited to animals, and in many instances clinical PK studies facilitate analysis of human antibodies in human serum. The therapeutic antibody is recovered from serum via cross-linked antigen or using anti-Fc gamma antibodies. Various analytical techniques can then be used to evaluate the different glycans; the most common are fluorescent or LC/MS methods.[75,82]

Table 2 summarizes some of the major studies performed and the conclusions on the impact of Fc glycans on clearance, which are conflicting. An important study by Chen et al. conducted on a recombinant human antibody in human serum, uncover circulating mannosidases in serum that trim high mannose species (M6-M9).[75] The major conclusion of this study is that Fc glycan have no impact on clearance. In contrast, in studies where mannosidases in serum were carefully evaluated, oligomannoses in a human IgG1 antibody are preferentially cleared from human serum and the fucosylated biantennary oligosaccharides were unaffected (Correia IR, Alessandri L, Ouellette D, Piparia P, Aikhoje A, Radziejewski C, et al; unpublished results). In summary, the effects of Fc glycan structure on clearance for different therapeutic antibodies should be evaluated on a case by case basis, preferably during clinical PK studies. These studies are relevant not only for clinical justification for setting glycan specifications, but they also establish bioequivalence for the

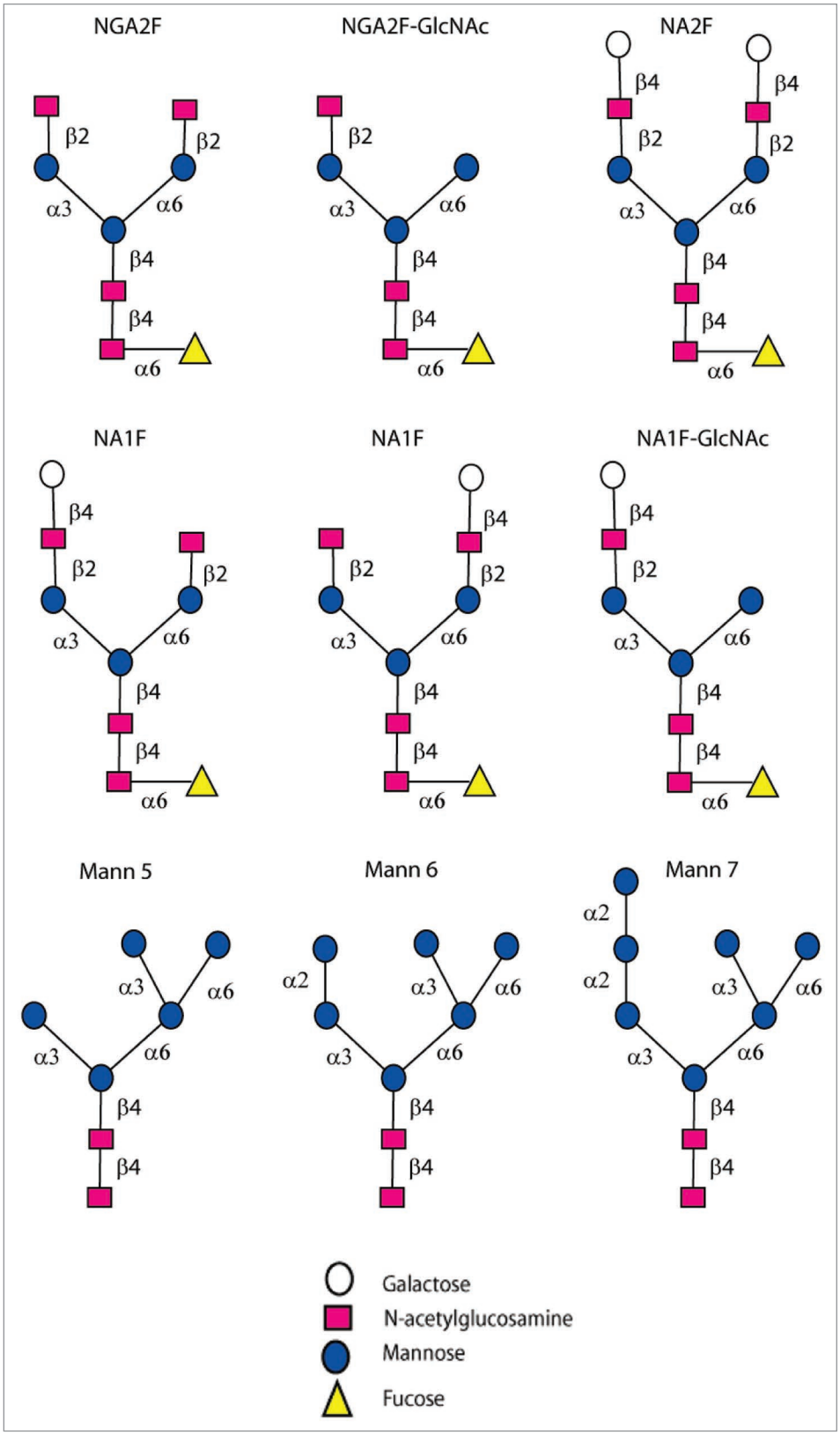

Figure 3. The different Fc glycans present on antibodies.

Table 2. Summary of studies to evaluate influence of the Fc glycan structure on serum clearance

Author(s)	Antibody	Fc glycan studied	Host	Effect on clearance
Wright and Morrison[77]	Chimeric mouse/ human	Oligomannoses	Mice	Oligomannoses rapidly cleared
Kanda et al.[81]	Chimeric mouse/ human	Fucosylated vs. non-fucosylated and oligomannoses	Mice	Oligomannoses rapidly cleared. No difference between fucosylation
Zhou et al.[76]	Human	Non-fucosylated oligomannoses	Mice	No impact on clearance
Millward et al.[79]	Human	Complex type or high mannose enrichment	Mice	No impact on clearance
Newkirk et al.[78]	Mouse (IgG1 and IgG2a)	G_0 (NGA2F) versus NA2F	Mice	G_0 has longer half-life for IgG2a
Chen et al.[75]	Human	All glycans	Human	No impact on clearance
Jones et al.[106]	Fusion Fc with TNF receptor	Terminal N-acetyl-glucosamine	Human/cyno monkey	Terminal N-acetyl glucosamine is rapidly cleared
Keck et al.[80]	Fusion Fc with TNF receptor	Terminal N-acetyl glucosamine	Human	Terminal N-acetyl glucosamine is rapidly cleared
Wawrzynczak et al.[107]	Mouse (IgG2b)	Aglycosylated vs. glycosylated	Rat	Aglycosylated is rapidly cleared

therapeutic, especially if a particular glycan is preferentially cleared from serum.

Influence in Serum on Secondary Structure of mAbs

Disulfide bond formation is an important post-translation event that occurs in the ER with the aid of many chaperones and enzymes such as protein disulfide isomerase (PDI). The primary function of the disulfide bonds is to ensure proper folding and activity of the protein; however, the dissociation energy of a disulfide bond is about 40% less than C-C and C-H bonds and is generally viewed as a "weak link" in a protein. Incorrect disulfide bond assembly and reduction of the bonds are both known to affect the antigen-binding capacity of an antibody, and lead to reduced activity.[83,84] The redox environment in serum is due to cysteine/cystine levels, albumin and thiols such as glutathione, and homocysteine.[30-36] Dynamic re-arrangement of disulfide bonds in serum, facilitated by the thiols, have been reported for both IgG2 and IgG4 antibodies and the general consensus is that the rearrangements serve as a mechanism for dampening the activity of these molecules; "allosteric" disulfide bonds may be another method by which protein function is regulated.[1,85,86]

IgG4 in serum (Fab exchange). Effector functions for the different IgG classes are directly correlated with their affinity for the Fc receptor, with IgG1>IgG3>IgG4>IgG2.[1,4,63] In addition, IgG4 molecules have a low affinity for C1q; thus, recombinant IgG4 molecules have emerged as an important therapeutic class of molecules when recruitment of the host effector function is not desirable. Natalizumab (Tysabri) and gemtuzumab ozogamicin (Mylotarg) are both IgG4 molecules that are currently marketed for multiple sclerosis (MS) and acute myeloid leukemia (AML), respectively.[1,4] TGN1412 another IgG4 molecule was discontinued after showing side-effects in a Phase 1 study of healthy volunteers.

IgG4 is a tetrameric molecule (H_2L_2) with two hinge region cysteines (**Fig. 1**). Although structurally similar to IgG1, significant levels of IgG4 half-antibodies, composed of HL dimers of IgG4, can be detected by a variety of techniques.[85,87,88] Evidence now links the wild type sequence (CPSC) in the hinge region of IgG4 molecules to unfavorable inter-chain disulfide bond formation. Mutations, e.g., from CPSC to CPPC (**Table 1**), greatly reduce formation of half-antibody molecules.[89-91] The C_{H3} domain of the IgG4 molecule is believed to also play a significant role in promoting half-antibody formation.[92]

Fab arm exchanges, where half-antibodies from one endogenous IgG4 molecule exchange with that of another endogenous molecule resulting in formation of bispecific antibodies, is postulated to be a normal biological characteristic of this class of anti-inflammatory molecules that may serve a protective role.[1,85,92] However, exchange of Fab arms between a therapeutic antibody and endogenous IgG4 molecules may be highly undesirable (**Fig. 4**). In an elegant study, Labrijn et al. found that natalizumab, which contains wild type sequence in the hinge region, exchanged its Fab arms with endogenous IgG4 molecules in individuals treated with the drug. In contrast, the stabilizing mutation is incorporated in gemtuzumab ozogamicin and the molecule did not exchange its Fab arms either in vitro or in studies conducted in mice. The authors also show Fab exchange between TGN1412, which contains wild type sequence, and endogenous IgG4 molecules.[93] The use of therapeutic IgG4 mAbs in which Fab exchange can take place has profound implications potentially relating not only to adverse events associated with the therapeutics, but also the pharmacokinetic and pharmacodynamic properties of the molecules. Stabilizing mutations in the hinge region or the C_{H3} domain will facilitate development of the IgG4 isotypes as therapeutics.

IgG2 in serum (disulfide shuffling). Several IgG2 molecules are either in late stage development (tanezumab, figitumumab, denosumab) or approved for commercial use (panitumumab). Like IgG4, this class of therapeutic antibodies is selected for development when neutralization of soluble antigens with reduced effector function is required. IgG2 molecules are unique in that fully functional covalent dimers can be detected both in recombinant antibodies secreted by myeloma cells, as well in sera

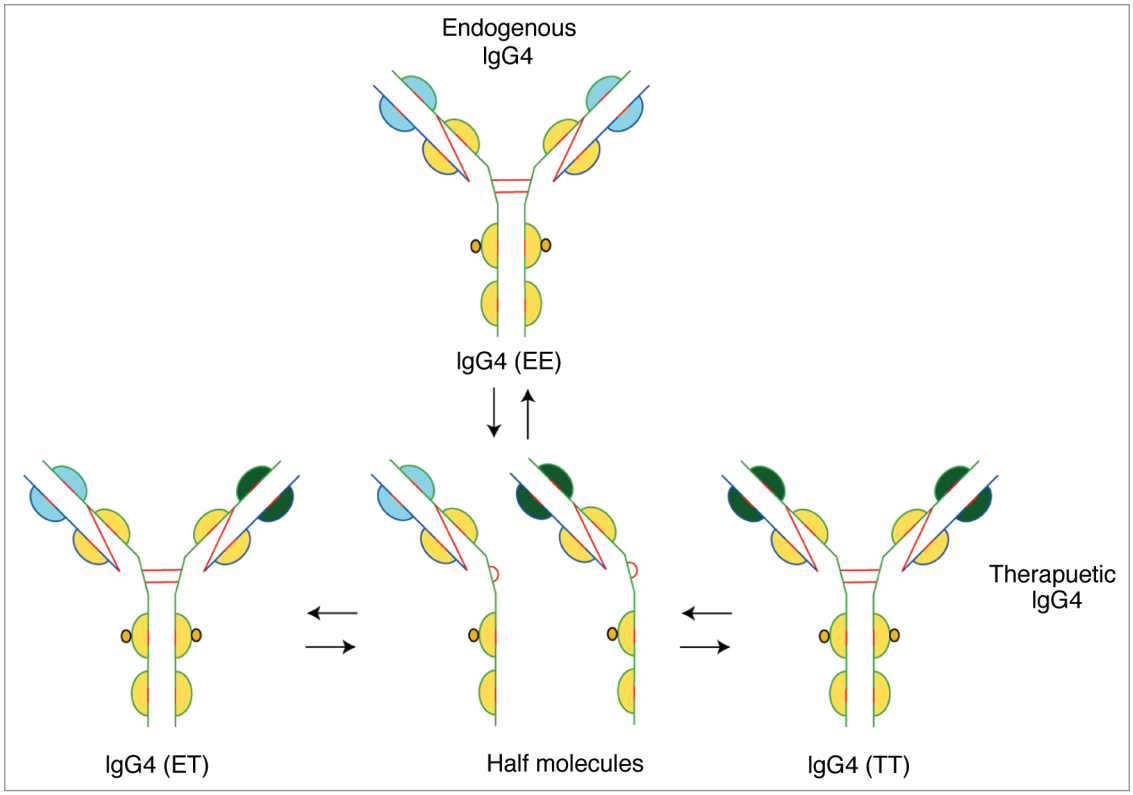

Figure 4. Exchange of Fab arms between endogenous IgG4 molecules and a therapeutic IgG4 molecule.

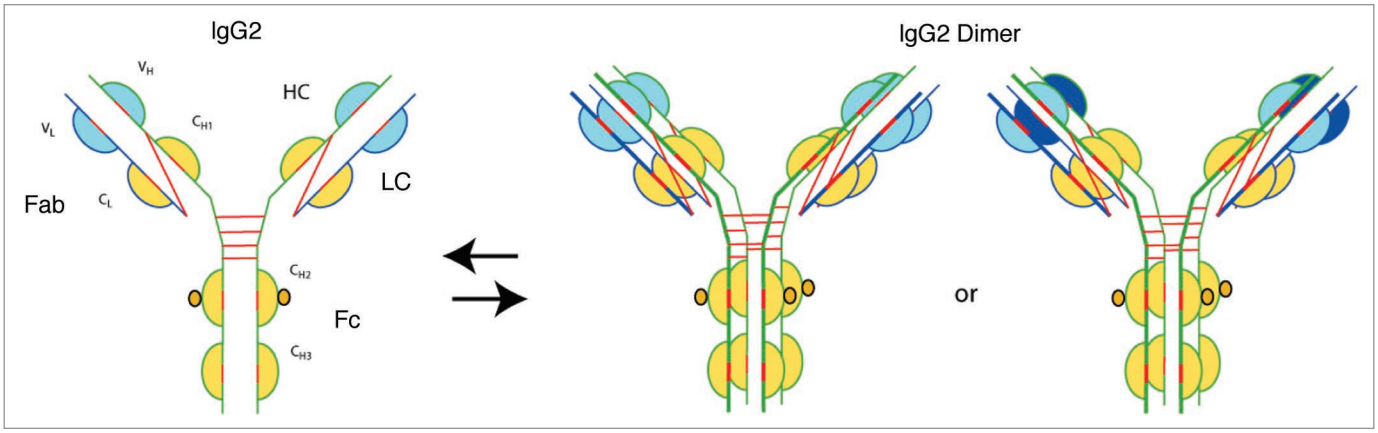

Figure 5. Formation of functional covalent dimers between identical as well as different IgG2 molecules.

of normal donors.[94] Intermolecular linkages between the dimers are depicted in **Figure 5**; however, the exact structure has yet to be determined. The natural targets of IgG2 molecules are carbohydrate antigens, and the interactions are generally low affinity. The evolutionary significance of the multi-valence of IgG2 molecules therefore may be to improve avidity of these molecules to their targets.[1,94] The clinical safety issues associated with identical covalent dimers of therapeutic IgG2 molecules having high affinity to their intended targets, or cross-linking of therapeutic IgG2 molecules with other endogenous IgG2 molecules, has yet to be determined. Investigation of mutations in the hinge region to prevent dimerization would help mitigate potential safety issue.

Recent studies have revealed another significant feature of this class of IgG molecules, i.e., the activity of the IgG2 molecule is modulated by disulfide shuffling in serum.[86,95,96] Three structurally different isoforms were identified: IgG2-A, IgG2-B and IgG2-A/B (**Fig. 6**). The isoforms were identified both with native human IgG2 antibodies made from myeloma plasma and from normal serum. Inter-conversion in serum from IgG2-A to IgG2-A/B took about 2 days, and the conversion of IgG2-A/B to

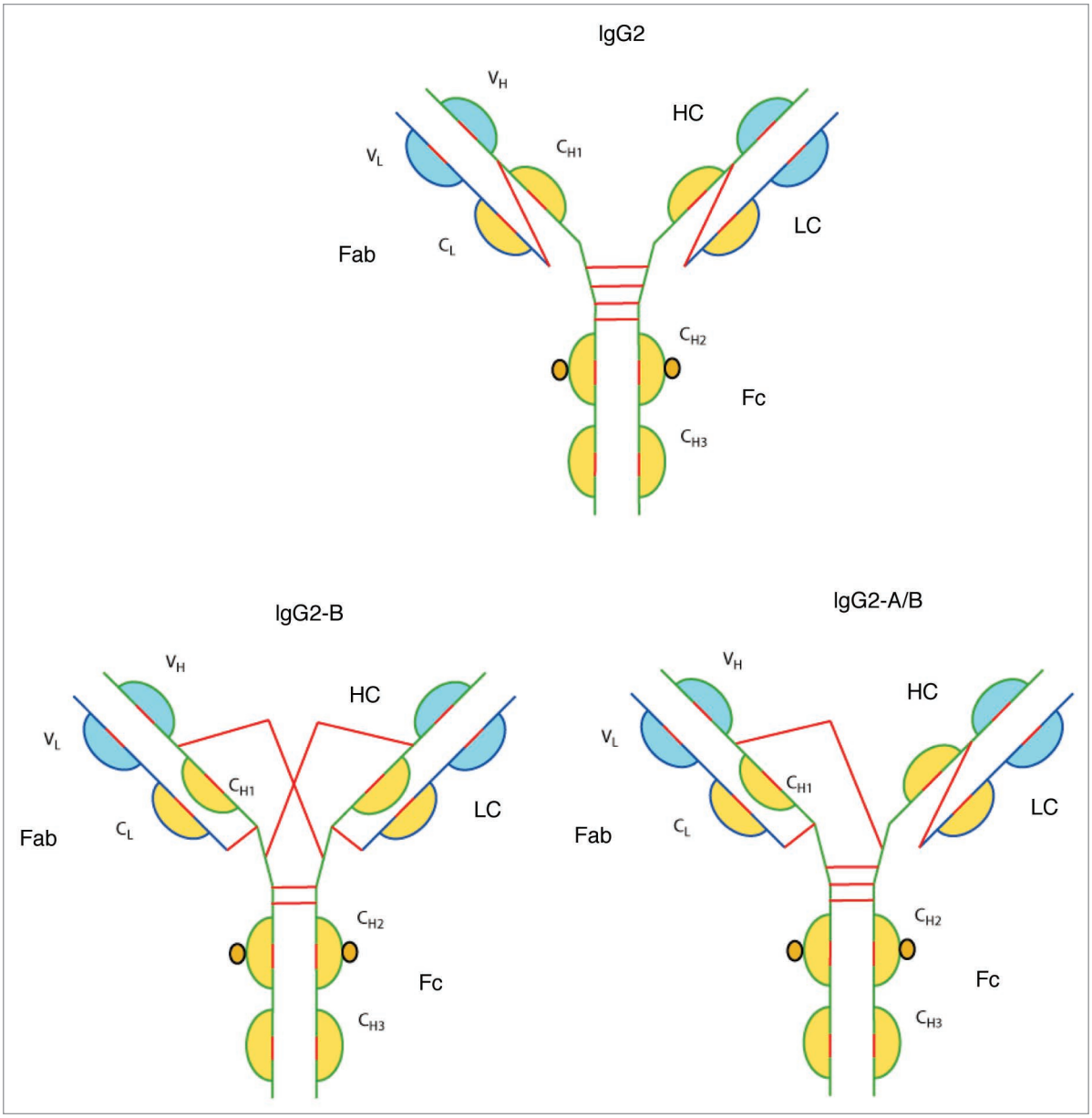

Figure 6. Shuffling of inter-chain disulfide bonds of IgG2 in serum.

IgG2-B, the rate limiting step, took several days. More importantly conversion from IgG2-A to IgG2-B resulted in a decrease in activity of the molecule, probably brought about by restricting movement of the Fab arms. It was also discovered that IgG2 molecules with a lambda light chain were less likely to form the IgG2-B isoforms, possibly due to the extra serine residue in the light chain that sterically hindered cross-linking of the cysteine residues. A side-by-side comparison carried out between an IgG1 and an IgG2 molecule designed to block interleukin-1 cell surface receptor type 1 revealed that the IgG2 molecule underwent a statistically significant decrease in activity when compared to the IgG1 molecule (approximately one third the activity of IgG1).[96] This decrease in activity was speculated to be due to inter-conversion of the species—a plausible mechanism of dampening activity of endogenous IgG2 molecules over time. However, a significant decrease in activity during circulation of therapeutic IgG2 antibodies may not be desired, and investigations into mutations that prevent disulfide shuffling would also help mitigate potential safety issues.

IgG1 in serum (stabilization of disulfide bonds). IgG1 is the isotype most frequently chosen for development of an antibody therapeutic.[1] The Fc domain of an IgG1 molecule can trigger effector function by binding to its Fc receptor (FcγRI, FcγRII or FcγRIII), the C1q component of the complement pathway or the FcRn receptor. Effector functions such as ADCC (antibody dependent cell mediated cytotoxicity) are mediated via FcγRs, CDC (complement dependent cytotoxicity) occurs via binding to the C1q protein, and long half-life in serum results

from interaction of IgG1 and the neonatal Fc receptor (FcRn). Depending on the therapeutic target of the antibody, mutations in the Fc region can be carried out to either enhance or reduce effector functions.[3,4,97]

A total of 16 disulfide bonds (4 inter-chain and 12 intra-chain) are found in IgG1 molecules (**Fig. 1**). Correct pairing of the 16 disulfide bonds takes place in the ER, and the process is aided by chaperones and enzymes such as protein disulfide isomerase. However, IgG1 molecules will occasionally have a population of molecules where the intra-chain disulfide bond in the V_H domain between Cys22-Cys96 residues is not paired.[98,99] Further, the unpaired cysteine residues can be found in molecules extracted from the ER/Golgi, suggesting that, despite the unpaired cysteine residues, they are not targeted for degradation via the ubiquitin pathway (Correia IR and Alessandri L, unpublished results).

In a significant finding, Ouellette et al. recently reported that the unpaired cysteine residues reformed their disulfide bonds when the mAb is exposed to serum.[98] Unlike IgG2 and IgG4 molecules, analytical evaluation of the mAb after recovery from serum, from both in vivo studies and after spiking the mAb in serum, showed rapid pairing of the cysteine residues in serum. It is possible that thiol molecules present in serum provide an environment outside the ER for correct cysteine pairing. This is an important study because incomplete disulfide bond formation has been shown to affect antigen recognition and reduce potency of the molecule.[100]

Influence in Serum on Higher Order Structure of mAbs

Altered antigen binding in serum. The behavior of protein therapeutics in solution is routinely assessed by analytical ultracentrifugation (AUC), where the velocity of sedimentation (SV) provides first principle hydrodynamic information.[101,102] Such studies enable an investigator to obtain information on size, shape and binding properties of the molecule. The principal measurement taken during sedimentation velocity is the radial distribution of concentration over time, where the measurements of concentration are obtained either by UV absorbance or using Rayleigh interference detectors. Recent advance in optics, have incorporated fluorescence detectors that not only improve sensitivity, but also allow analysis of labeled molecules in complex matrices such as serum.[29,103] Studies in serum will not only monitor aggregate formation, but also evaluate interaction of the antibody therapeutic with its target antigen.

Studies of Alexa-488 labeled omalizumab, an anti-IgE therapeutic, revealed that incorporation of 4.5 moles of dye per mole of protein did not affect potency of the molecule, nor did it result in increased aggregate or fragment formation.[29] The researchers did find that the affinity of omalizumab for its antigen was not only increased in serum, but the complexes observed in serum were different from those characterized in PBS.[29] A number of explanations for this discrepancy are possible; however, the most plausible one takes into account the highly crowded environment in serum, where the excluded volume leads antibody therapeutics to adopt a compact structure and show enhanced protein association with antigen in serum.[29]

Fragmentation and aggregation of mAbs in blood. Fragmentation and aggregation of antibody therapeutics are commonly monitored during formulation studies at storage temperature, as well as during stressed stability studies.[104] While both AUC and SEC enable analysis of labeled proteins in serum, current initiatives in drug development call for "rapid" evaluation and selection of candidate proteins, peptides or mAbs that not only behave well in blood, but also show good PK properties. A high throughput analytical technique with good precision, sensitivity and ease of use is required to identify poorly behaved molecules early in development. Discontinuing studies of candidates that aggregate or fragment in blood or those with poor PK properties can reduce costs, but also facilitate screening of larger numbers of molecules.

The LabChip GXII platform (Caliper Life Sciences) is a high throughput CE based analytical technique that can be used to analyze pre-labeled mAbs directly from whole blood (Correia IR, unpublished results). Samples of blood are simply spun (2,000 rpm for 5 minutes) and the supernatant (3–5 μL) collected for analysis. The GXII will size proteins between 14 to 200 kDa, and the assay has a 4 log linear range (50 pg/μL to 100 ng/μL). Analysis of each sample is performed in about 40 seconds, and can be done directly from a 96 or 384 well plate. Example electropherograms from three separate analysis of a labeled mAb after 4 and 24 hours in whole blood are shown in **Figure 7**; **Figure 8** shows electropherograms obtained for a labeled mAb quantifying aggregate and fragment levels after incubation for 0, 4 and 24 hours. The LabChip GXII is a high throughput analytical technique with very good precision and sensitivity and mAbs can be easily analyzed directly from blood, providing real time information on the behavior of candidate mAbs in blood.

Conclusion

The past two decades has seen therapeutic mAbs evolve from murine-based products to chimeric to fully human products. The most spectacular advances have been made in the fields of oncology and immunology, although mAb products for cardiovascular, ophthalmic and infectious diseases are on the market. As of March 2008, 21 mAbs were marketed in the US, and of these, 15 (1 murine, 5 chimeric, 8 humanized and 1 human) were the IgG1 isotype.[105] In 2009, four more human IgG1 molecules were approved by the US Food and Drug Administration (ustekinumab, golimumab, canakinumab, ofatumumab).[2] However, non-IgG1 isotypes are increasingly being developed as therapeutics, with an IgG2 (panitumumab), and IgG2/4 hybrid (eculizumab) and two IgG4 (natalizumab, gemtuzumab ozogamicin) currently approved in the US, and a number at various stages in development.

It is important to understand the altered physicochemical properties of IgG2 and IgG4 molecules in serum discussed here; however, engineering stabilizing mutations in the hinge region can help mitigate potential issues associated with dimerization or Fab exchange with endogenous molecules. The influence of

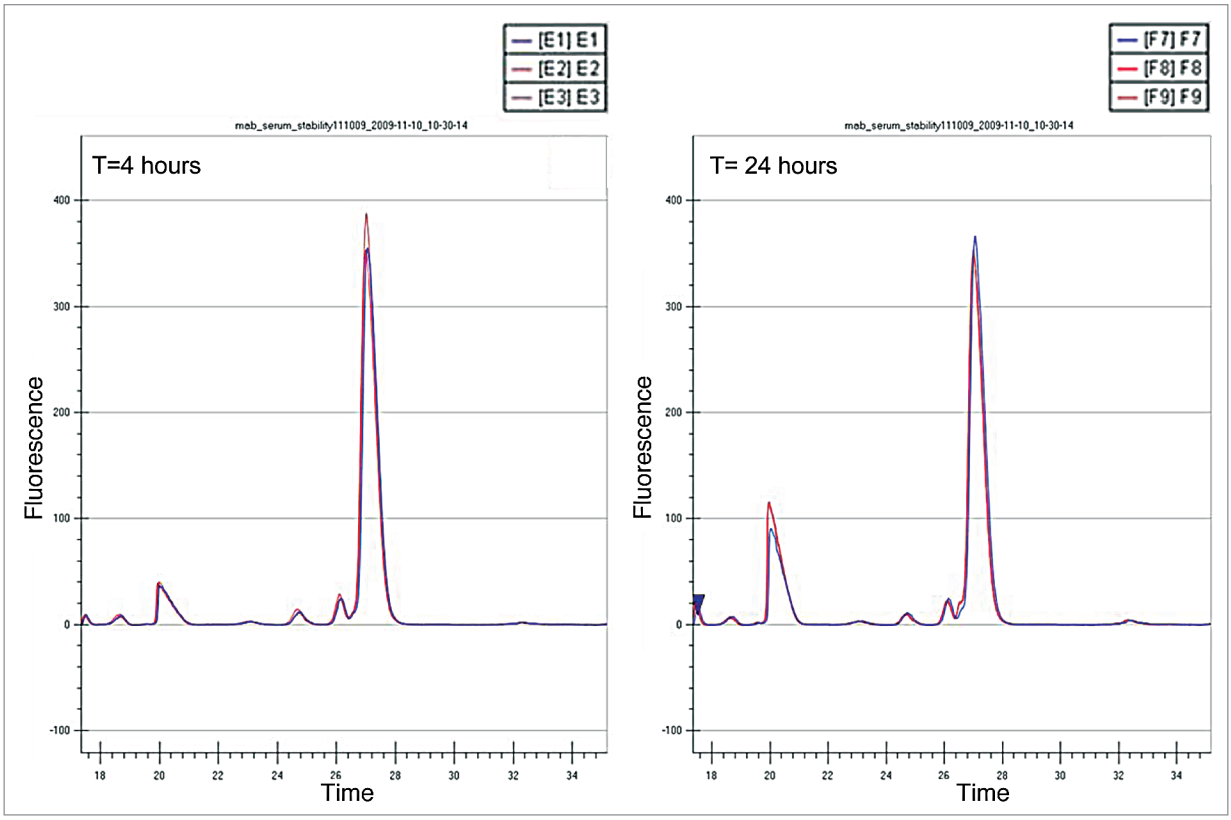

Figure 7. Analysis on LabChip GXII showing recovery of mAbs from blood—precision data (n = 3) after 4 and 24 hours.

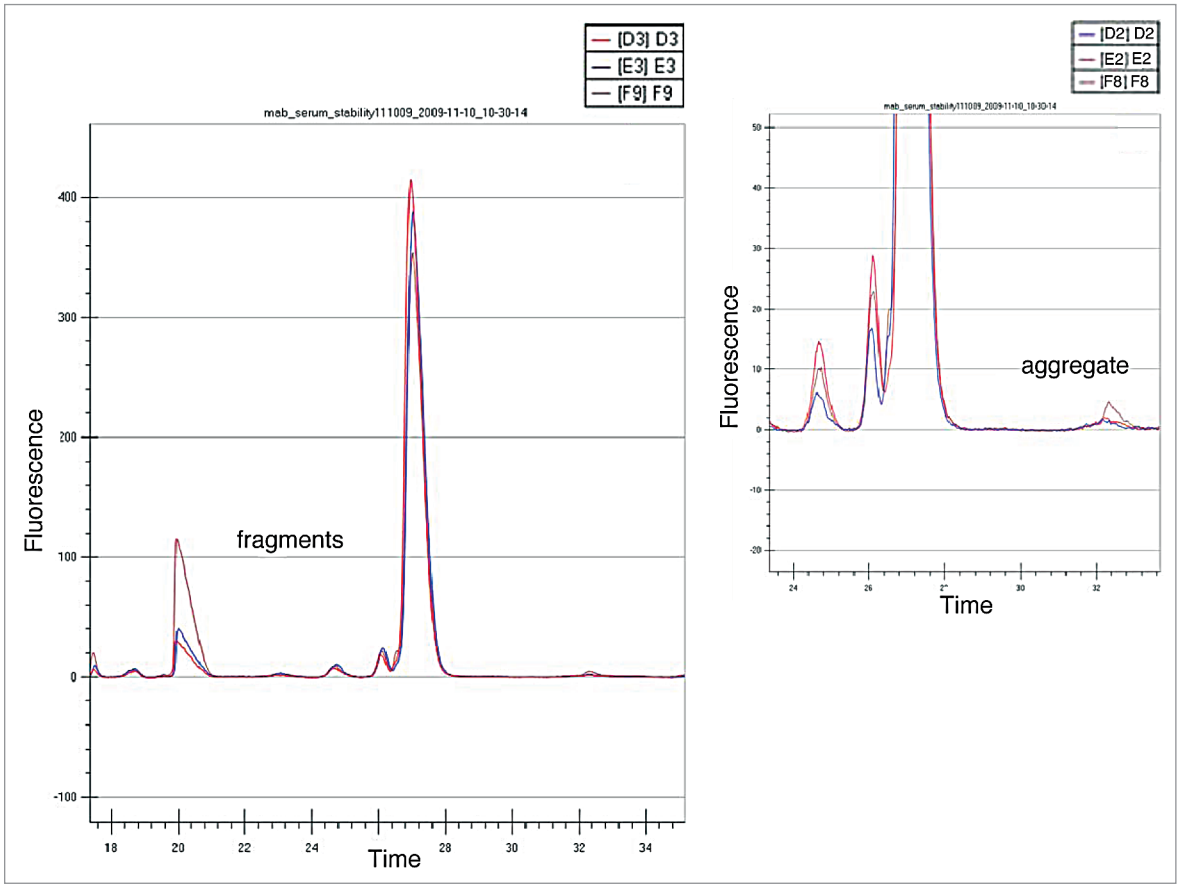

Figure 8. (See opposite page). Monitoring aggregate and fragment formation of a mAb in whole blood using the LabChip GXII (T = 0, 4 and 24 hrs).

serum on other physicochemical properties of antibody therapeutics was also discussed; it is apparent that much information can be obtained during PK studies in monkeys or humans. Analysis of PTM can be performed after the antibody therapeutic is recovered from serum using cross-linked antigen or an anti-human Fcγ antibody. These studies are relevant for not only assessing the exposure of patients to a PTM, but also establishing bioequivalence for the therapeutic; this is especially relevant if a particular isoform is preferentially cleared from serum. The influence of glycation of therapeutic IgG molecules in serum has not been fully explored and future studies must include investigations of this PTM, as well as others that were beyond the scope of this review. In summary, the data discussed here clearly indicate that decisions made during the development of an antibody therapeutic must be based not only on an understanding of the basic biology of the isotype chosen, but also on its stability in serum, which is the intended environment of the therapeutic.

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