

Protein heterogeneity and the immunogenicity of biotherapeutics.

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Abstract

Cancer and transplant patients will be receiving concomitant cytotoxic drugs that induce various levels of immunosuppression. Patients with chronic diseases that experience long term exposure to recombinant P/GPs are at greater risk but may be protected with mild immunosuppressive agents. Currently, an ever expanding armamentarium of biologics is being developed that includes engineered IgG molecules that differ in structure to endogenous IgG and/or their fragments. Such manipulations increase the propensity for immunogenicity; however, outcomes may differ between acute conditions, for which treatment may be within a relatively short time frame and chronic diseases that may require long term exposure.

Keywords: Human genome, Glycoprotein, Monoclonal antibodies, Immunoglobulin G.

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Introduction

The human genome (HG) is comprised of ~20,000 open reading frame (*ORF*) genes; however the human proteome (HP) is orders of magnitude greater due to alternate *ORF* gene splicing (AS), errors in transcription or translation, the addition of co- and post-translational modifications (CTM; PTM) etc. A recent guesstimate suggested that each *ORF* may be translated to generate 100 structurally distinct proteins, within the outbred human population [1].

Protein and glycoprotein (P/GP) molecules exist *in vivo* as discreet entities within complex multi-component media, e.g. plasma, cell sap etc. and exert their function(s) through specific interactions with target/receptor molecules. In health each individual expresses a unique proteome and personal integrity demands immunological tolerance to all self-molecules. Ordered aggregation of monomer molecules may be essential for normal function; however, inappropriate, or non-native, aggregation is demonstrable and implicated in the pathogenesis of numerous diseases and may give rise to the generation of autoantibodies [2,3]. Similarly, denaturation and aggregation of protein therapeutics may render them immunogenic and result in the development of anti-drug/anti-therapeutic antibodies (ADA/ATA).

The thriving biopharmaceutical industry depends on the production of recombinant P/GPs having structural fidelity with a selected endogenous molecule; therefore, structural variants generated during production, purification, formulation and/or delivery is a major concern and equates to potential immunogenicity [2,3]. Practise has shown that pharmacovigilance must be exercised over the life time of an established drug since incidences of adverse events have been reported for drugs long established in the clinic, e.g. insulin [4] and erythropoietin (EPO) [5].

Loss of efficacy is frequently due to the development of ADA/ATA that neutralise therapeutic activity [6,7]. The

development of ADA suggests the presence of structurally altered/denatured molecules that are recognized as “foreign” (non-self) by the patient’s immune system i.e. are immunogenic. In this mini review I shall discuss properties of native and recombinant P/GPs that have to be controlled throughout the production and administration of recombinant P/GP therapeutics; illustrated for EPO and antibody therapeutics.

Structural Heterogeneity: Post-Translational Modifications (PTMs) *in vivo* and *ex vivo*.

In vivo

P/GPs synthesis in mammalian cells is an error prone multi-step process and the end product(s) inevitably exhibits structural heterogeneity. Lack of fidelity with the sequence encoded by the gene may occur at multiple stages, e.g. transcription, mRNA translation, de novo secondary/tertiary structure formation etc. Additionally, nascent polypeptide chains may be subject to co-translational modifications (CTMs) as it is extruded from the ribosome tunnel, e.g. secondary folding, the addition of oligosaccharide, N-myristoylation etc. When released from the ribosome the P/GP transits to the endoplasmic reticulum where it is edited for correct tertiary folding and initial oligosaccharide processing; further post-translational modifications (PTMs) are effected during passage through the Golgi apparatus [8-11] and throughout its life cycle *in vivo*.

It is presumed that all such molecular entities are recognised as “self” by the immune system; therefore the first step in the quest to produce a recombinant P/GP therapeutic is determination of the structure of the natural (endogenous) molecule isolated from body fluids or tissues. However, the techniques and processes employed may result in denaturation and the introduction of further CMs, e.g. proline isomerisation.

In practice a consensus structure for each endogenous target P/GP will have been established and a candidate recombinant therapeutic will be evaluated, structurally and functionally, in comparison with the endogenous molecule. This approach cannot be realised for a potential recombinant monoclonal antibody (mAb) therapeutic since an endogenous anti-self-antibody is not available for comparison. Candidate mAbs are sourced from inbred mice and engineered to generate chimeric of humanized mAbs, by selection from a phage display library or transgenic mice expressing human immunoglobulin genes [12,13].

The choice of production platform is a critical strategic decision since the processes involved in the addition of CTMs, PTMs and CMs are species and cell specific and production of a human P/GP in an alien cell line, e.g. CHO (Chinese hamster ovary) cell line, may result in the introduction of non-self-structures, immunogenicity and the generation of ADA/ATA responses [6-8]. Prior to clinical trials a candidate recombinant P/GP therapeutic has to be extensively characterised in comparison with the endogenous molecule, employing multiple orthogonal physico-chemical techniques [14,15]. Patent protection for numerous recombinants P/GP drugs have now expired and many more are approaching expiry, providing opportunities for the production of biosimilar drugs. Candidate biosimilars must be characterised in comparison with the approved innovator drug product [16,17].

Protein folding in vivo

Proteins are synthesised, within ribosomes, as a linear sequence (string!) of amino acid residues covalently linked through the peptide bond; elements of secondary structure may form, de nova, and can include generation of an acceptor site for the addition of high mannose oligosaccharides N-linked to an asparagine residue present within a glycosylation sequon, i.e. the sequence asparagine-x-serine or threonine (asp-x-ser/thr; N-X-S/T), where x is any amino acid residue other than proline.

Following release from the ribosome the protein transits to the endoplasmic reticulum where the high mannose oligosaccharide is truncated and exerts a quality control function for correct folding; miss-folded proteins being marked for proteasomal degradation [8-11]. Multiple PTMs may be effected during passage through the Golgi apparatus including further oligosaccharide processing, phosphorylation, sulphation etc. Thus a P/GP achieves its evolutionary determined structure that ensures it traffics to the appropriate cellular compartment or is secreted [18-21].

It has been estimated that if a protein of 100 amino acid residues was to undergo random motions in search of the lowest energy form it may need to pass through 1089 conformations that would take 1066 years to sample; however, within the cell the P/GP passes through intrinsic protein folding pathways to achieve the functional tertiary/quaternary conformation in seconds [22].

Our understanding of P/GP structure/function relationships is mostly based the interpretation of x-ray crystallographic studies that tend to represent proteins as having a fixed (solid!) structure [15]. Newer techniques show that proteins are “living, breathing” entities that may exist in conformational equilibria that can include intrinsically unstructured regions [23,24]; *ex vivo* such regions, may act as focal points for aggregation [2,3,9,23,24]. Algorithms that attempt to analyse or predict structural parameters of P/GPs as they exist within *in vivo* environments are in their infancy [10,24].

Protein folding: ex vivo

Proteins are comprised of amino acid residues that bear non-polar, polar uncharged and charged side chains and may fold to generate proteins having an overall hydrophobic or hydrophilic character. Proteins that are soluble in aqueous media have an overall hydrophilic character whilst hydrophobic amino acid side chains are orientated to the internal space and mutual interactions stabilise structure; however, a scan of the surface exposed side chains may reveal hydrophobic patches that can act as centres for aggregation [2,9-11].

This potential is underlined by diseases in which P/GP aggregation results in the deposition of insoluble fibrils in tissues e.g. neurodegenerative disorders such as Alzheimer’s (AD), Parkinson’s (PD), Huntington’s (HD), Transmissible Spongiform Encephalopathies (TSEs), and Amyotrophic Lateral Sclerosis (ALS) [25-31]. Fundamental studies of protein folding and aggregation have focused on the hen egg white lysozyme molecule, the native form of which has high solubility in aqueous media.

However, following exposure to denaturing solvents *in vitro*, followed by restoration to physiologic conditions it can misfold to form aggregates and fibrils, similar to pathogenic species seen in disease. Six spontaneous mutations in human lysozyme have been reported and all except one lead to systemic non-neurogenic amyloidosis involving kidney, liver and spleen [27-29]. Prion disease is an extreme example of the propensity for soluble proteins to form fibrils *in vivo* [30,31]. In its soluble form it has a helical structure; however, in the disease state the protein converts to a beta sheet structure that aggregates to forms fibrils; the denatured prion protein can act as a “catalyst” to induce normal prion protein to convert to a beta sheet structure.

As previously stated we do not have means of determining the fine structure of P/GPs as they exist *in vivo* and are limited to extrapolation from structural studies of isolated P/GPs purified from human fluids and tissues employing multiple physico-chemical techniques that may introduce further structural heterogeneity e.g. deamidation of asparagine and glutamine residues, oxidation of methionine and tryptophan residues, glycation of lysine etc. [10,14,24].

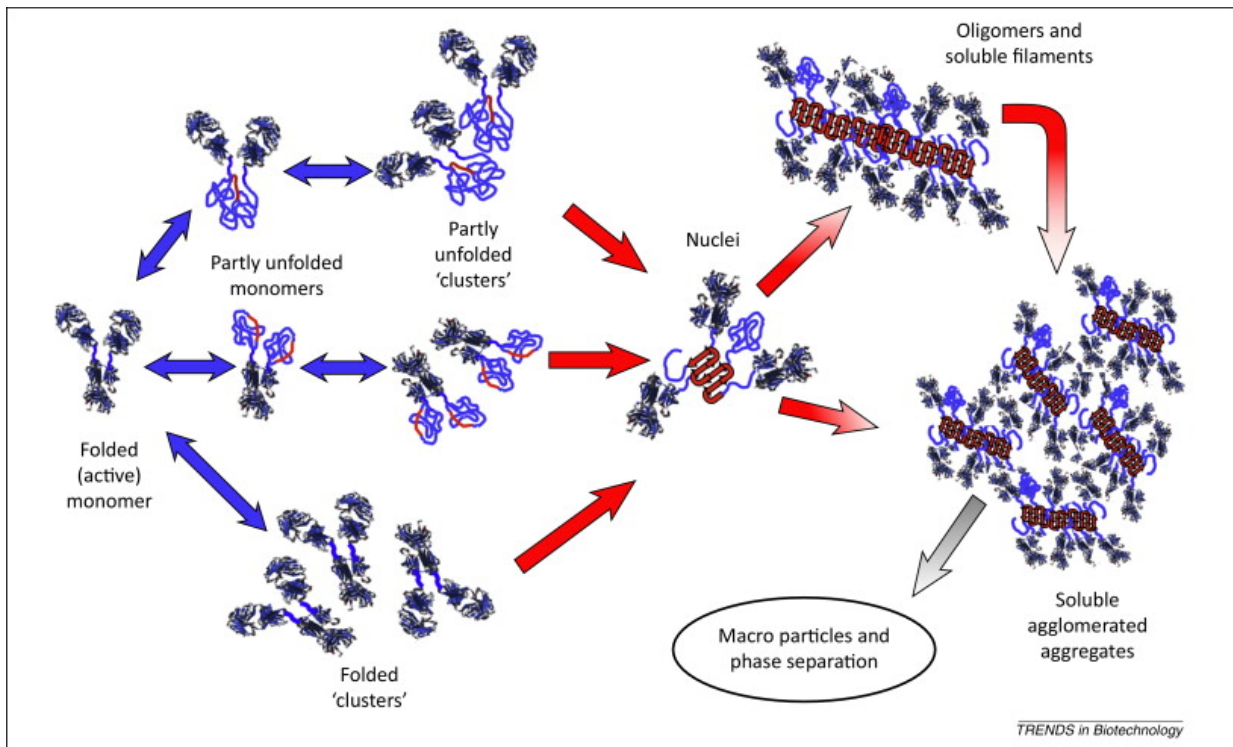


Figure 1. Schematic diagram illustrating multiple non-native aggregation pathways for a multi-domain protein such as a monoclonal antibody composed of a single Fc fragment and two identical Fab fragments [25]. Reproduced with permission.

Additionally proteins may undergo subtle reversible conformational changes that results in momentary exposure of hydrophobic regions that can be mutually attractive with formation of “partly unfolded clusters” i.e. aggregates Figure 1 [3,25-27]. Such clusters can act as nuclei for the formation of larger aggregates, possibly extending to precipitation. Structural heterogeneity is compounded by differing susceptibilities of individual amino acid residues to modifications depending on its position within the molecule and the immediate micro-environment.

Prediction of aggregation prone regions (APR)

Aggregation prone regions (APRs) may be classified as structural or critical. Structural APRs contribute to the stability of the native protein core structure but may be exposed following denaturation *ex vivo* and form aggregates under refolding conditions; critical APRs are exposed in the native state and may contribute to physiological protein/protein interactions *in vivo* and *in vitro*.

Multiple physiochemical techniques and algorithms have been developed to identify APRs and inform protein engineering to reduce a propensity for aggregation [32-34]; a concomitant increases in recombinant proteins productivity has been reported [35]. Since hydrophobic binding contributes to protein/protein interactions APRs may be anticipated as a feature of functional sites and much attention has been focused on the antigen binding site (i.e. the paratope) of antibody molecules [35,36].

However, antibodies are multi-functional molecules and the formation of antigen/antibody complexes is an essential

prelude to the activation of downstream effector functions activated by interactions of the Fc region with soluble and/or cell bound and ligands, e.g. cellular Fc receptors (FcγR, FcRn), the C1 component of complement etc. [37,38] Interaction sites for these ligands have been identified and includes the hydrophobic sequence 231-APELLGGPSVFLFPP-245 [15,20,37,38]. Protein engineering has been employed to reduce the propensity for aggregation whilst retaining activation of effector molecules that determines their mechanism of action (MoA).

Immunogenicity

In health an individual is tolerant to their proteome; however, multiple autoimmune diseases manifest the potential for loss of tolerance to self-molecules or aberrant (mutant) forms of self-molecules arising *in vivo*. The potential for immunogenicity of biotherapeutics in humans may vary depending on the character of the disease being treated; three broad categories may be identified [39-41]:

A disease in which a patient fails to express an essential P/GP or expresses a mutant inactive form, e.g. enzyme deficiencies. In each case an active therapeutic is non “self” and has potential to be immunogenic.

Therapeutics that augment the patient’s endogenous production, eg. insulin, erythropoietin. The patient may be expected to be tolerant unless there is a mismatch between P/GP polymorphic variants present in outbred population or the therapeutic has been subject to denaturation/aggregation, with exposure of altered structure, during production, storage and/or delivery.

Antibody therapeutics are a special case in that, in addition to polymorphisms within the “constant” regions, the unique specificity is reflected in unique antigen binding site (paratope) structure, i.e. is non-self.

Monoclonal Antibodies: Commercial Evolution

The antibody response in humans is comprised of five immunoglobulin (Ig) classes: IgM, IgG, IgA, IgE and IgD; in addition IgG is comprised of four subclasses (IgG1, IgG2, IgG3, and IgG4) and IgA two (IgA1, IgA2) generating nine Ig isotypes [15,42,43]; each antibody isotype expresses a unique profile of effector mechanisms. The IgG1 subclass predominates in serum and has been the focus for structure/function studies and the predominant format adopted for approved mAb therapeutics.

Following binding to its target, with the formation of antibody/antigen complexes, antibodies of the IgG1 subclass may trigger a cascade of inflammatory effector mechanisms that constitute its “mechanism of action” (MoA). Activation of IgG1 mAbs provides natural protection in the killing and removal of bacteria and other “foreign bodies” but may similarly be activated for the killing and removal of cancer cells. Each IgG subclass may be exploited to offer MoA profile appropriate to differing disease indications.

The antibody landscape is developing rapidly as new engineered constructs are customised to optimise treatment protocols, e.g. antibody fragments that enhance solid tumour penetration, antibody-drug conjugates that are internalised into target cells where drug release is effected [12,13]. It should be noted that the binding of a divalent antibody to a multivalent antigen, e.g. a bacterium, results in the formation of an immune complex (IC) that is itself an aggregated form of the antibody. ICs are removed and degraded by leucocytes that are also antigen presenting cells and may therefore, present peptides derived from the paratope of a mAb [44].

The first GP approved by the EMA and FDA was the murine monoclonal antibody (mAb) Muromonab (1986, anti-human CD3 OKT3), produced in mouse hybridoma cells; it was administered to patients undergoing acute rejection of a liver transplant. Whilst successfully suppressing the rejection episode vigorous anti-mouse IgG antibody responses developed in a majority of patients; excluding the possibility of exposing patients to the therapeutic on a subsequent occasion. Over succeeding years genetic and protein engineering techniques were employed to limit immunogenicity by successively increasing the human IgG character of mAbs.

The commercial mAb therapeutic era may be identified with the development of chimeric mouse/human mAbs comprised of the variable regions of a mouse antibody linked to the constant regions of human IgG1, generating a molecule that is ~30% mouse and ~70% human in structure [6-8,15]. A significant reduction in immunogenicity resulted and a majority of patients could be repeatedly dosed with these mAbs. Further developments defined the amino acid residues of the mouse antibody that formed the antigen binding site (paratope) and transplanted them into selected human variable

regions; generating a “humanised” mAb [6-8,15]. This technology is being replaced by protocols for the generation of “fully human” antibodies. These mAbs are products of rearranged human variable region genes; however, by virtue of the fact that they are selected to be anti-self their unique paratope structure may provoke ADA/ATA responses [12,13] in an outbred human population.

Meta-analysis of the incidence of ADA for the first approved “fully human” anti-TNF- α antibody (Adalimumab, Humira), generated by phage display, ranged from 1-54%; when administered across multiple inflammatory diseases [6,7]. The ADA responses may be transitory and/or of low titre and, with good patient management, do not necessarily result in significant adverse reactions [45]; a threshold for immunogenicity is evidenced by the fact that ADA responses are reduced when patients are concomitantly receive a mild immunosuppressant, e.g. methotrexate [46]. Antibodies generated from phage display depend on the pairing of VH and VL sequences that would be forbidden *in vivo* and may express non-self-epitopes. The alternative technology for generating fully human antibodies from mice rendered transgenic for human immunoglobulin genes results in a natural pairing of VH and VL sequences and the incidence of ADA for the anti-TNF- α Golimumab is reported as 0-19% [6].

Glycosylation: Recombinant Erythropoietin and IgG Antibodies

A majority of proteins are generated utilising the standard 20 amino acids linked through the peptide bond between alpha carbon atoms; in contrast oligosaccharides utilise multiple linkages with a potential to generate enormous glycome and glyco-proteome diversity; it is estimated that six sugar residues can be assembled to generate 10^{12} unique hexa saccharides [47]. The repertoire of sugars utilised varies between species, gender, cell line etc. In addition oligosaccharides may be N-linked, as previously discussed, or O-linked through serine, threonine or mannose residues. Importantly, CHO and NS0 (murine) cell lines may add immunogenic non-human oligosaccharide structures to intended “fully” human recombinant therapeutics [13,48,49]. Protein engineering and gene “knock-out”/“knock-in” techniques have been employed to modulate the glycoform profile of GPs; as illustrated in this text for EPO and IgG.

Erythropoietin

Recombinant EPO produced in CHO cells was initially shown to exhibit enhanced activity *in vitro*, in comparison with approved therapeutic isolated from urine. However; trials *in vivo* revealed a lack of therapeutic efficacy due to its rapid clearance from the circulation. It was later shown the attached oligosaccharides bore terminal galactose sugar residues, rather than the required sialic acid, resulting in clearance in the liver *via* the asialoglycoprotein receptor. Fractionation of the CHO derived EPO allowed preparation of an active sialylated glycoform establishing this parameter as a Critical Quality Attribute (CQA); recombinant EPO (Epoetin) received regulatory approval in 1989, is comprised of 165 amino acid

residues and bears three N-linked and one O-linked oligosaccharide that accounts for ~40% of its mass [50-53].

Successful world-wide use of recombinant EPO followed but in 1999 a cohort of patients in Europe developed pure red cell aplasia (failure of erythrocyte production) due to the generation of ADA that neutralised not only the therapeutic but also endogenous EPO. Investigation showed that “minor” changes had been introduced in the formulation of EPO produced in Europe, in contrast to the US, that were presumed to have resulted in denaturation rendering the product immunogenic [51]. This illustrates the structural fragility of P/GPs and the need for pharmacovigilance throughout the lifetime of a drug. Incidences of PRCA continue to be reported around the world and include “biosimilar” EPOs produced by multiple manufacturers and approved by regional or national regulatory authorities [52]. Experiences of Thailand are salutary; as of the 1st January 2009 fourteen [14] biosimilar EPOs, originating from various countries, was licensed in Thailand [53]. The cost advantage for these biosimilars resulted in widespread usage but was coincident with an increase in reports of PRCA due to the generation of ADA [53].

Anticipating expiration of patent protection and the advent of biosimilars the innovator company (Amgen) developed an

improved (biobetter) product (Darbepoetin alfa), exhibiting increased efficacy and an extended *in vivo* half-life; it was approved and received patent protection [54,55]. The improvement was achieved by the introduction of two additional N-linked oligosaccharide attachment sites resulting in the production of glycoforms bearing additional N-linked oligosaccharides expressing terminal sialic acid residues.

Antibodies

An IgG molecule is comprised of ~1440 amino acid residues and two N-linked oligosaccharides each comprised of 7-13 sugar residues. For decades little account was taken of this “minor” structural feature until it was shown that removal of the oligosaccharide resulted in loss of the ability of ICs to trigger MoAs mediated by activation of FcγR and the C1 complement component, i.e. glycosylation of IgG is a CQA [44,45]. A minimum requirement for MoA activation is the presence of a seven residue oligosaccharide on each heavy chain. Differential addition of sugar residues generates a multiplicity of IgG glycoforms that may each modulate the affinity of binding of ICs to effector ligands and hence MoAs, Figure 2 [44,45,56-58].

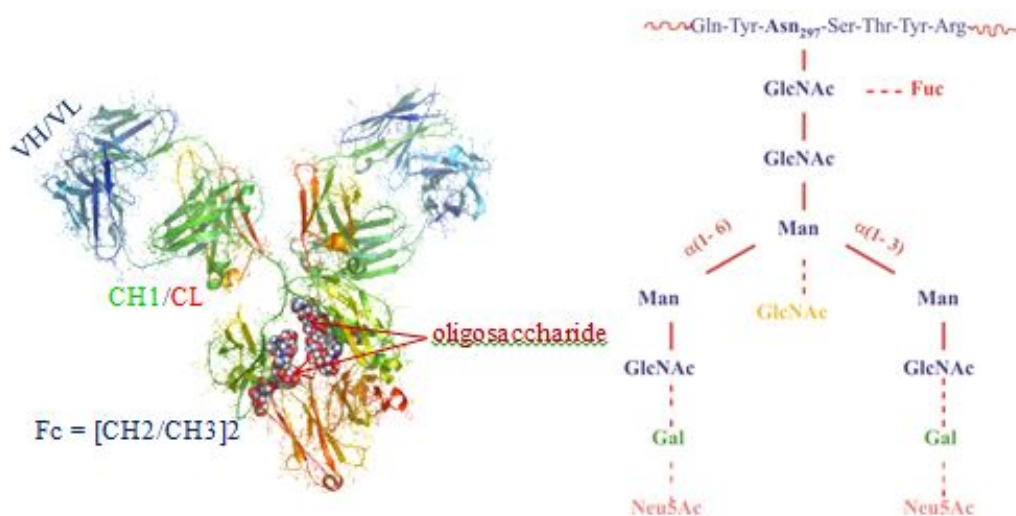


Figure 2. Representative IgG complex diantennary oligosaccharides. The “core” heptasaccharide residues, (GlcNAc)₂Man₃(GlcNAc)₂, in blue.

The glycoform heterogeneity of human serum IgG is not mirrored by the glycoform profile of mAbs produced in CHO, NS0 or Sp2/0 cells; in contrast these cells express a restricted glycoform profile that may include immunogenic non-human glycoforms. The glycoform profile cannot be significantly manipulated by changes in culture conditions; therefore, the contribution of individual glycoforms to MoAs has been investigated by *in vitro* enzymatic modification of mAb or genetic engineering of the producer cell line. A dramatic outcome from these studies has been the demonstration that IgG antibodies that bear oligosaccharides devoid of fucose residues can exhibit a 10-10² folds increase in their ability to mediate killing of cancer cells by NK (natural killer) cells; similar increases can be achieved for mAb expressing a

bisecting N-acetylglucosamine residue. New production CHO cell lines have been established following the “knock-out” of the fucosyltransferase gene or “knock-in” of the bisecting N-acetylglucosamine transferase gene [48,56-58]. These cell lines have been used to generate approved “biobetter” versions of previously approved mAbs.

Mechanisms/Mode of Action (MoA)

An antibody may be protective and deliver therapeutic benefit solely due to its binding specificity for target, e.g. neutralising an exogenous bacterial toxin or endogenous TNF-α; however, when the target is a bacterium or a cancer cell MoAs that result in killing and removal of debris are essential. [56-58]. The IC

formed in turn become targets for leucocytes that bear cell surface receptors (FcγR) specific to the IgG heavy chain Fc region. The cross-linking of multiple FcγR results in leucocyte activation with the release of toxic agents and/or ingestion (phagocytosis); ICs may also activate the C1 component of the complement system to trigger a cascade of enzymatic reactions resulting in the formation of a membrane attack complex (MAC) that inserts into the cellular membrane with the formation of pores that allow the ingress of water and egress of cellular constituents. Molecules released from the complement cascade also adhere to the IC and engage complement receptors expressed on leucocytes to further enhance cellular activation.

There are three families of FcγR (FcγRI, FcγRII, FcγRIII) that are differentially expressed on leucocytes and bind the IgG subclasses selectively, Table 1; similarly, the C1 component of complement exhibits selective IgG subclass binding. An important parameter that contributes to mAb efficacy is the long half-lives of ~21 days, for IgG1, IgG2 and IgG4, this allows for extended intervals between administered doses; IgG3 has a shorter half-life of ~7 days. Clearance of IgG is mediated *via* the neonatal Fc receptor (FcRn) that is expressed on many cell types and is independent of the IgG glycoform [56-58]. Antibodies of the IgG1 and IgG3 subclass have very similar functional profiles but the IgG2 and IgG4 subclasses exhibit unique profiles. It is important therefore when developing a mAb therapeutic to anticipate the preferred MoA *in vivo* and produce mAbs of an appropriate IgG subclass. To date of the 160 mAb listed in the IGMT database 136 are IgG1, 8 IgG2, 2 IgG3 and 14 IgG4 [58,59].

Table 1. Human IgG subclasses binding FcγR and C1.

	IgG1	IgG2	IgG3	IgG4
FcγRI	+++	-	+++	+
FcγRII	+	-	+	-
FcγRIII	++	-	++	+
C1	++	-	+++	-
FcRn	++	++	++	++
Half-life (days)	21	21	7	21

Conclusion

It is posited that all recombinant P/GP therapeutics may be immunogenic, at least in a proportion of patients, and result in loss of efficacy and/or adverse events. This prediction should be assessed with respect to the disease being treated, thus cancer and transplant patients will be receiving concomitant cytotoxic drugs that induce various levels of immunosuppression. Patients with chronic diseases that experience long term exposure to recombinant P/GPs are at greater risk but may be protected with mild immunosuppressive agents. Currently, an ever expanding armamentarium of biologics is being developed that includes engineered IgG molecules that differ in structure to endogenous IgG and/or their fragments. Such manipulations

increase the propensity for immunogenicity; however, outcomes may differ between acute conditions, for which treatment may be within a relatively short time frame and chronic diseases that may require long term exposure.

Advances in gene sequencing techniques are allowing identification of polymorphisms in “susceptibility” genes that allows for stratification of patients. Stratification can contribute to the development of personalised medicine through identification of cohorts of patients responsive to a given therapeutic whilst similarly identifying patients that are not likely to benefit. Stratification of “common” diseases may identify increasingly small cohorts of patients such that they their condition may be classified as an orphan disease, indicative of a need for treatment with expensive customised biologics, i.e. personalised medicine. This may result in a conflict between the high cost of development of specialist biologics and the diminished market that stratification may identify. Some “respite” may be offered by the development of biosimilars; however, they are currently providing only ~15-30% reduction in cost. The conflict between our ability to deliver ever expanding therapies for human health care, from conception to death, and to provide equity in delivery will continue and become ever more contentious.

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