Biologics: structural heterogeneity and immunogenicity

ABSTRACT

In principle the whole human proteome is available for the generation of recombinant proteins and glycoproteins that may serve as drugs (biologics). Endogenous human proteins and glycoproteins are structurally heterogeneous but are recognized as self by the immune system; however, recombinant protein and glycoprotein molecules are necessarily produced in heterologous systems and may include structural variants that are non-self and potentially immunogenic. The addition of human type oligosaccharides may be critical to function while the addition of non-human sugar residues can render biologics immunogenic. A particular concern is the structure of oligosaccharides attached by the hamster and murine cell lines that provide the dominant production platform. Critical structure and function properties that contribute to optimization of therapeutic potential are illustrated through recombinant erythropoietin and antibody therapeutics.

Biologics are established as potent additions to the drug armamentarium and their number will multiply over the next decade and beyond. While small molecule drugs are defined chemical entities that can be readily replicated (generics), biologics are large complex molecules that are structurally heterogeneous (Wang and Singh, 2013). They are challenging and expensive to produce, and represent the largest cost and cost growth area for the NHS budget (Jacobs, 2016). As a consequence the National Institute for Health and Care Excellence (2013) has developed a formula for recommending the prescription of speciality drugs within the NHS and set a limit of £30 000 per quality-adjusted life year, i.e. extending an acceptable quality of life by 1 year.

The cost base for an approved biologic, marketed under patent protection, is set by the innovator company and reflects the complexity of the biologic and the necessity for production in living organisms, e.g. human, animal or microbial cells. Initially, it was held that it would not be possible to develop a generic biologic and that the innovator company would enjoy patent protection throughout the lifetime of the drug. However, this lucrative market encouraged pharmaceutical companies to attempt to produce a structural and functional 'mimic' (biosimilar) of a given biologic, and regulatory authorities developed guidelines and criteria for their approval. The first biosimilar product approved in the EU in 2006 was somatropin (Omnitrope) (Generics and Biosimilars Initiative, 2017). The production of innovator, biosimilar and biobetter protein and glycoprotein drugs is the focus of this review.

The advent of the biopharmaceutical industry may be linked with the approval of recombinant insulin (Humulin) in 1982 by the European Medicines Agency and Food and Drug Administration, produced in *Escherichia coli*. The first glycoprotein approved by the European Medicines Agency and Food and Drug Administration was the murine monoclonal antibody muromonab in 1986, produced in mouse hybridoma cells, and followed by recombinant erythropoietin (Epogen) in 1989, produced in Chinese hamster ovary cells. Since the addition of defined oligosaccharide chains is essential to the function of glycoproteins they are produced in mammalian cell lines (Chinese hamster ovary, NS0 and Sp2/0 murine cell lines).

Despite extensive clinical experience adverse reactions to recombinant insulin (~2%) (Ghazavi and Johnston, 2011) and erythropoietin (Macdougall et al, 2012) are still encountered and are frequently the result of patients developing anti-drug or anti-therapeutic antibodies that neutralize therapeutic activity. The development of anti-drug antibodies suggests that the therapeutic is being recognized as 'foreign' (non-self) by the patient's immune system, as a result of the presence of molecules that are structurally different to the endogenous protein or glycoprotein, e.g. as a result of denaturation. These issues are illustrated in this article, looking at the production of recombinant erythropoietin and a monoclonal antibody molecule.

Sources of structural heterogeneity

Protein or glycoprotein synthesis in mammalian cells is a multi-step process that results in structural heterogeneity which is error prone. Errors may be introduced during gene transcription, mRNA translation or the formation of the secondary structure, among other steps. Additionally, the nascent polypeptide chain may be subject to co-translational modifications as it is extruded from the ribosome tunnel, e.g. the addition of oligosaccharide, and is edited for correct folding and initial oligosaccharide processing within the endoplasmic reticulum; it is subject to further post-translational modifications during passage through the Golgi apparatus.

Functional activities of a protein or glycoprotein may depend on further chemical modifications throughout

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its life cycle in vivo (Jefferis, 2016a,b). It is presumed that all such natural structural variants are recognized as 'self' by the immune system, so the first step in the quest to produce a recombinant protein or glycoprotein therapeutic is to determine the structure of the natural (endogenous) molecule isolated from body fluids or tissues. This natural heterogeneity may be further compounded when establishing the structure of a protein or glycoprotein since isolation and characterization protocols can result in the introduction of unnatural chemical modifications, i.e. denaturation. Antibodies specific for human protein or glycoproteins cannot be sourced from humans but are generated from mice immunized with human antigens or by selection from phage display libraries (Tomszak et al, 2016).

A critical strategic decision is the choice of production platform since the processes leading to co-translational, post-translational and chemical modifications are species and cell specific. This means that production of a human protein or glycoprotein in an alien cell line, e.g. Chinese hamster ovary cell line, may result in the introduction of non-self structures leading to the production of anti-drug or anti-therapeutic antibody responses (Ghaderi et al, 2012; Varki, 2017). Before clinical trials a candidate recombinant protein or glycoprotein therapeutic has to be extensively characterized in comparison with the endogenous molecule, using multiple orthogonal physicochemical techniques (Parr et al, 2016). A biosimilar protein or glycoprotein has to be similarly characterized but in comparison with the approved innovator drug product. Recombinant monoclonal antibodies present an extra challenge since each is structurally and functionally unique and an endogenous molecule is not available for structural comparison.

Erythropoietin

The development of recombinant erythropoietin as a therapeutic demonstrated that attachment of an oligosaccharide of defined structure (glycoform) is essential for activity and constitutes a critical quality attribute. Erythropoietin comprises 165 amino acid residues and bears three N-linked and one O-linked oligosaccharides, accounting for ~40% of its mass (Jelkmann, 2012). The erythropoietin initially produced in Chinese hamster ovary cells was shown to exhibit enhanced activity in vitro relative to erythropoietin isolated from human urine; however, trials in vivo demonstrated a lack of activity as a result of rapid loss by degradation. Fractionation of bulk product allowed the isolation of a minor glycoform component that proved to be efficacious in vivo and Epoetin received regulatory approval in 1989.

Successful worldwide use of recombinant erythropoietin followed but in 1999 a cohort of patients in Europe developed pure red cell aplasia (failure to generate erythrocytes) as a result of the generation of anti-drug antibodies that neutralized not only the therapeutic but also endogenous erythropoietin. Investigation showed that 'minor' changes had been introduced in the formulation of erythropoietin produced in Europe, in contrast to that produced in the USA, that were presumed to have resulted in denaturation, rendering the product immunogenic. This illustrates the structural fragility of protein or glycoproteins and the need for pharmacovigilance throughout the lifetime of a drug. Cases of pure red cell aplasia continue to be reported around the world for 'biosimilar' erythropoietins produced by multiple manufacturers and approved by regional or national regulatory authorities (Macdougall, 2005; Macdougall et al, 2012). The experience of Thailand is salutary – as of 1 January 2009 fourteen biosimilar erythropoietins, originating from Argentina, China, South Korea and India, were licensed in Thailand. The cost advantage for these biosimilars resulted in widespread usage but was coincident with an increased number of cases of pure red cell aplasia.

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 half-life, that was approved and patent protected. The improvement was achieved by the introduction of two additional N-linked oligosaccharide attachment sites and the generation of glycoforms expressing terminal sialic acid residues.

Recombinant monoclonal antibody therapeutics

The antibody response in humans comprises five immunoglobulin (Ig) classes: IgM, IgG, IgA, IgE and IgD. In addition IgG comprises four subclasses (IgG1, IgG2, IgG3, and IgG4) and IgA two (IgA1, IgA2), generating a total of nine Ig isotypes (Jefferis, 2012; Vidarsson et al, 2014). The IgG1 subclass predominates in serum and has been the focus for structure/function studies and consequently the predominant format adopted for monoclonal antibodies.

Development of a protocol allowing routine generation of monoclonal antibodies exhibiting preselected specificity was dependent on the availability of the immortalised mouse NS1 and NS0 plasma cell lines. Fusion of spleen cell from an immunised mouse with NS0 cells allows selection of an immortal cell line secreting antibody of pre-selected specificity. While monoclonal antibodies produced in these cell lines had value as research and diagnostic reagents their possible use as drugs or therapeutics for humans was severely limited because of their 'foreignness' and the development of anti-drug or anti-therapeutic antibody responses.

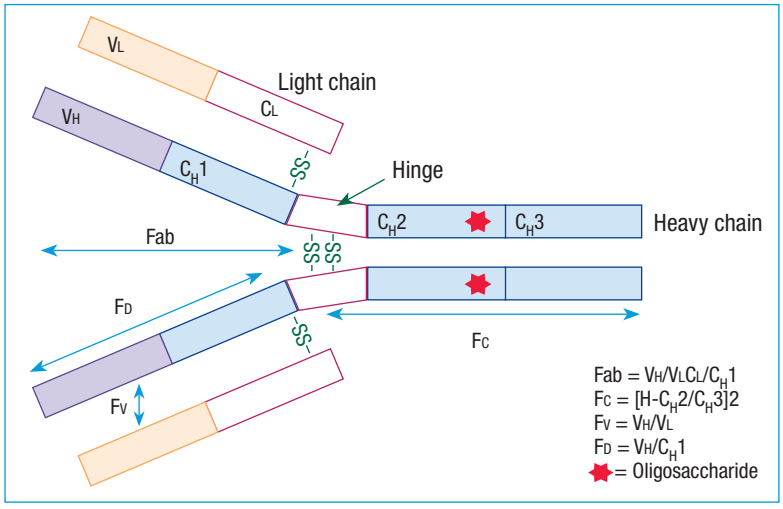
Nevertheless, the first recombinant monoclonal antibody explored for human therapy was a mouse anti-human CD3 (OKT3) reagent that was successfully used to treat patients undergoing an acute rejection episode following liver transplantation. As expected vigorous anti-mouse IgG antibody responses developed in a majority of patients. Over the following years genetic and protein engineering techniques were applied to modify monoclonal antibody structures and limit monoclonal antibody immunogenicity.

These developments can best be appreciated from consideration of the structure of the IgG molecule (*Figure 1*) (Jefferis, 2012; Vidarsson et al, 2014). IgG comprises four polypeptide chains: two identical 'light' chains and two identical 'heavy' or gamma (γ) chains. These chains fold to generate three-dimensional variable regions (VH and VL) and constant regions (C_H1 and CL). Enzymatic cleavage at the 'hinge' (h) region releases two antigen binding fragments (Fab) (VHC_H1/VLCL) and an Fc fragment (h-C_H2/C_H3)2. The hinge allows independent mobility of the Fab regions so that each may bind to its cognate antigen (epitope) to form a three-dimensional immune complex. The mobility of the Fc region likewise allows engagement of endogenous ligands, e.g. cellular Fc receptors, with the C1 component of complement, to activate effector functions that can eliminate immune complexes.

The development that launched the monoclonal antibody therapeutic era was the generation of chimeric mouse/human monoclonal antibodies comprising the variable regions of a mouse antibody combined with the constant regions of human IgG1 to generate a molecule that is ~30% mouse and ~70% human in structure. This resulted in a significant reduction in immunogenicity and a majority of patients could be repeatedly dosed with such monoclonal antibodies.

The next development sought to define the structural elements of the mouse antibody that actually formed the antigen binding site (paratope) and to transplant said paratope sequences into human variable regions, thus generating a 'humanised' monoclonal antibody. This technology supplanted the chimeric protocol, but was itself replaced by the development of protocols for the generation of 'fully' human antibodies. Although fully human monoclonal antibodies are encoded by human Ig genes the methodologies used to generate and select them results in the production of a monoclonal antibody expressing a unique specificity and structure, i.e. the paratope; patients within an outbred human population are unlikely to have encountered these unique structures resulting in anti-drug or anti-therapeutic antibody responses. The first fully human monoclonal antibody therapeutic, adalimumab (Humira), exhibits a ~12% incidence of anti-drug antibodies; however, these responses may be transitory and/or of low titre and, with good patient management, do not necessarily result in significant adverse reactions (Thomas et al, 2015).

Figure 1. The four chain structure of an immunoglobulin G (IgG) molecule.



Mechanisms and mode of action

An antibody may be protective and deliver therapeutic benefit solely as a result of its binding specificity for the target, e.g. neutralizing a bacterial toxin or soluble tumour necrosis factor-α (TNFα). However, when the target is a bacterium or a cancer cell actions that result in killing and removal of debris are essential (Jefferis, 2012, 2014; Vidarsson et al, 2014). The immune complex formed in turn become a target 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); immune complexes 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 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 immune complex 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; similarly, the C1 component of complement exhibits selective IgG subclass binding (*Table 1*) (Jefferis, 2012, 2014;

Table 1. Human immunoglobulin (Ig) G subclasses binding FcγR and C1

	IgG1	IgG2	IgG3	IgG4
FcγRI	+++	–	+++	+
FcγRII	+	–	+	–
FcγRIII	++	–	++	+
C1	++	–	+++	–

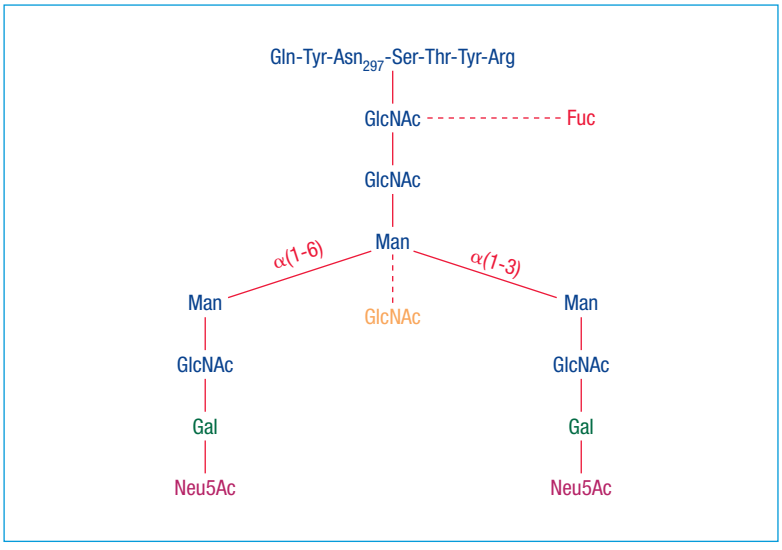
> IgG1 subclass antibodies that bear oligosaccharides devoid of fucose residue can exhibit a 10–100-fold increase in their ability to mediate killing of cancer cells by NK cells.

Vidarsson et al, 2014). 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 monoclonal antibody therapeutic to anticipate the preferred mode of action in vivo when selecting the IgG subclass. To date of the 205 monoclonal antibody therapeutics listed in the international ImMunoGeneTics (IGMT) database 180 are IgG1, nine are IgG2, two IgG3 and 14 IgG4 (ImMunoGeneTics, 2017).

Impact of glycosylation on mode of action of monoclonal antibodies

An IgG molecule comprises ~1440 amino acid residues and two oligosaccharides, each comprising 7–13 sugar residues, attached within the CH2 domains and accounting for 2–3% of its molecular mass. 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 immune complexes to trigger modes of action mediated by activation of FcγR and the C1 complement component, i.e. glycosylation and individual glycoforms are critical quality attributes. A minimum requirement for mode of action activation is the presence of a seven residue oligosaccharide on each heavy chain. Differential addition of other sugars generates a multiplicity of IgG glycoforms that may each modulate the affinity of binding of immune complexes to effector ligands and hence their mode of action (*Figure 2*) (Jefferis, 2012, 2014; Vidarsson et al, 2014; Dashivets et al, 2015).

Figure 2. Representative IgG complex biantennary oligosaccharides with the 'core' heptasaccharide residues, (GlcNAc)2Man3(GlcNAc)2, in blue. Fuc = fucose; Gal = galactose; GlcNAc = N-acetylglucosamine; Man = mannose; Neu5Ac = N-acetyl neuraminic acid.



The glycoform heterogeneity of serum IgG is not mirrored by the glycoform profile of monoclonal antibodies produced in Chinese hamster ovary, 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 manipulated by changes in culture conditions but the contribution of individual glycoforms to modes of action has been investigated by in-vitro enzymatic modification of the monoclonal antibody or genetic engineering of the producer cell line. A dramatic outcome of these studies has been the demonstration that IgG1 subclass antibodies that bear oligosaccharides devoid of fucose residue can exhibit a 10–100-fold increase in their ability to mediate killing of cancer cells by NK (natural killer) cells; similar increases can be achieved for monoclonal antibody expressing a bisecting N-acetylglucosamine residue. Production Chinese hamster ovary cell lines have been established following the 'knock-out' of the fucosyltransferase gene or 'knock-in' of the bisecting N-acetylglucosamine transferase gene (Jefferis, 2016a; Lalonde and Durocher, 2017). These cell lines have been used to generate approved 'biobetter' versions of previously approved monoclonal antibodies.

Examples of approved biobetter monoclonal antibodies

Anti-CD20

The anti-CD20 monoclonal antibody rituximab (Rituxan) that targets the CD20 antigen expressed on the surface of B cells was initially approved for treatment of patients diagnosed with non-Hodgkin's lymphoma. A principal mode of action was elimination of B cells by NK cells expressing the FcγRIII receptor. Subsequently approval was extended to other B cell cancers and autoimmune diseases, e.g. rheumatoid arthritis. However, rituximab was not effective for all forms of B cell cancer, particularly when CD20 expression was low, and a biobetter was sought.

Subsequently, the biobetter anti-CD20 monoclonal antibody obinutuzumab (Gazyva), that bears a non-fucosylated oligosaccharide and exhibits enhanced B cell killing, was approved (Jefferis, 2016b; Lalonde and Durocher, 2017; Yu et al, 2017). A further anti-CD20 monoclonal antibody ocrelizumab (Ocrevus) has been approved for treatment of multiple sclerosis. Interestingly, ocrelizumab also reached phase III clinical trials for rheumatoid arthritis before these were terminated because of the development of neutropenia and deaths from overwhelming infection. These examples demonstrate that the unique specificity of each anti-CD20 monoclonal antibody impacts its mode of action and heterogeneity within and between diseases, i.e. comorbidities.

Anti-TNFα

A breakthrough in the treatment of rheumatoid arthritis was achieved with the development and approval of the chimeric anti-TNFα monoclonal antibody infliximab (Remicade); however, meta-analysis of 14 651 patient responses showed that ~25% developed anti-drug or anti-therapeutic antibodies (Thomas et al, 2015). It was anticipated that development of the fully human antibody adalimumab (Humira) would be a biobetter and eliminate the development of anti-drug or anti-therapeutic antibodies; however, studies reported an anti-drug or anti-therapeutic antibody incidence of ~14%. In each case the incidence could be reduced if patients received the mild immunosuppressive methotrexate (Fechtenbaum et al 2014; Thomas et al, 2015). Adalimumab is currently the world's largest selling drug with 2016 sales amounting to $16.1 billion.

Conclusions

This article illustrates the application of science to achieve 'bench-to-bedside' advances in health care, currently termed translational medicine. Advances in gene sequencing techniques are allowing identification of polymorphisms permitting stratification of diseases which were previously treated as a single entity. Stratification allows identification of cohorts of patients responsive to a given therapeutic while similarly identifying patients that will not benefit. Further stratification of 'common' diseases may identify increasingly small cohorts of patients such that their condition may be classified as an orphan disease responsive to a customised biologic. This generates a conflict between the high cost of development of biologics and a diminishing market. Some respite may be offered by the development of biosimilars, although they are currently only providing 15–30% reduction in cost. The conflict between our ability to deliver ever-expanding therapies for human health care, from conception to death, and equity in delivery will continue and become ever more contentious. **BJHM**

Conflict of interest: none.

KEY POINTS

- Biologic drugs represent the largest cost and cost growth area for the NHS budget.
- Structural heterogeneity of recombinant proteins may result from errors in transcription, translation and/or post-translational modifications.
- Oligosaccharides covalently attached to a protein (i.e. glycoproteins) can have a profound influence on function.
- Structural heterogeneity of recombinant protein or glycoprotein drugs produced in non-human cells may result in immunogenicity.

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