

MINIREVIEW

Glycoengineering: The Effect of Glycosylation on the Properties of Therapeutic Proteins

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ABSTRACT: Therapeutic proteins have revolutionized the treatment of many diseases but low activity or rapid clearance limits their utility. New approaches have been taken to design drugs with enhanced *in vivo* activity and half-life to reduce injection frequency, increase convenience, and improve patient compliance. One recently used approach is glycoengineering, changing protein-associated carbohydrate to alter pharmacokinetic properties of proteins. This technology has been applied to erythropoietin and resulted in the discovery of darbepoetin alfa (DA), a hyperglycosylated analogue of erythropoietin that contains two additional N-linked carbohydrates, a threefold increase in serum half-life and increased *in vivo* activity compared to recombinant human erythropoietin (rHuEPO). The increased serum half-life allows for less frequent dosing to maintain target hemoglobin levels in anemic patients. Carbohydrates on DA and other molecules can also increase molecular stability, solubility, increase *in vivo* biological activity, and reduce immunogenicity. These properties are discussed. © 2005 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 94:1626–1635, 2005

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INTRODUCTION

Humoral factors and cytokines are glycosylated with variable numbers of carbohydrate chains each of which has variable carbohydrate structures. While the biological function is typically determined by the protein component, carbohydrate can play a role in molecular stability, solubility, *in vivo* activity, serum half-life, and immunogenicity. The sialic acid component of carbohydrate in particular, can extend the serum

half-life of protein therapeutics (see below). One aspect of glycoengineering is to introduce new N-linked glycosylation consensus sequences into desirable positions in the peptide backbone to generate proteins with increased sialic acid containing carbohydrate, thereby increasing *in vivo* activity due to a longer serum half-life.¹

Darbepoetin alfa (DA) is a hyperglycosylated analogue of recombinant human erythropoietin (rHuEPO) generated by glycoengineering.¹ Recombinant HuEPO is used to treat the anemia associated with chronic kidney disease, cancer, and HIV infection as well as to reduce the requirement for transfusions under certain surgical situations. Recombinant HuEPO consists of a 165 amino acid single polypeptide chain that contains

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three N-linked glycosylation sites at asparagines residues (Asn24, Asn38, Asn83) and one O-linked site at serine residue Ser126.³⁻⁴ The discovery that the sialic acid-containing carbohydrate content of rHuEPO was directly proportional to the serum half-life and *in vivo* bioactivity⁵ led to the hypothesis that additional carbohydrate on rHuEPO might further enhance *in vivo* activity. DA contains two additional N-linked glycosylation sites and has increased *in vivo* activity and prolonged duration of action since serum half-life is increased threefold above rHuEPO.^{1,6,7} This allows for less frequent dosing, with subsequent increased convenience for the patient and caregiver and improved patient compliance. The use of glycoengineering is discussed.

THE PROCESS OF GLYCOSYLATION AND SITE SELECTION

Oligosaccharides play a crucial role in many recognition, signaling, and adhesion events that take place within and between cells. Carbohydrate moieties also aid folding of secreted proteins.⁸ Carbohydrate chains, unlike amino acids and nucleic acid polymers, are assembled enzymatically as opposed to biosynthesis from a defined template, and hence are diverse with respect to both the number and the linkage patterns of the sugar units.⁹ Until very recently successful chemical synthesis had not been reported.¹⁰

Glycoproteins are classified into four categories: O-linked, N-linked, glycosaminoglycans, and glycosylphosphatidylinositol (GPI)-anchored proteins. This review will focus only on O- and N-linked glycosylation. O-linked glycosylation is initiated by the attachment of a single monosaccharide, usually *N*-acetylgalactosamine, to a Ser or Thr residue.¹¹ This proximal sugar is subsequently elaborated by a series of glycosyltransferases to form a mature *O*-glycan structure. It is unknown why certain amino acid residues in a protein are selected for glycan addition as there is no clear consensus sequence for O-linked glycosylation.^{12,13} Instead, an O-linked glycosylation site is defined by secondary structural elements such as an extended β turn.¹⁴⁻¹⁶

N-linked glycosylation takes place with the transfer of a 14-residue oligosaccharide, to a nascent polypeptide at the Asn located within Asn-X-Ser/Thr consensus motif where X is any amino acid except Pro.¹⁷ The attached oligosaccharide then undergoes enzymatic trimming and

maturation via the action of glycosyltransferases that append additional sugar units to the terminal mannose residues.¹⁸

Most naturally occurring consensus sequences in secreted proteins are not glycosylated.¹⁹⁻²¹ Consensus sequences are therefore necessary but not sufficient for N-linked carbohydrate addition. Alteration of the adjacent sequence can convert a non-functional consensus sequence to a functional site, indicating that sequence context or secondary structure affects recognition of the consensus sequence. For example, Pro⁸⁷-Asn⁸⁸-Thr⁹⁰ in rHuEPO was not glycosylated while substitution of the Pro residue for a Ser, Val, or Ala residue in the rHuEPO analog sequence resulted in carbohydrate addition.²¹ Possible secondary structures required for carbohydrate addition within functional glycosylation sites are β or Asn-X turns.⁸ Carbohydrate addition precedes protein folding.^{1,21-23} Thus, sites introduced into normally buried positions of the molecule, e.g., rHuEPO, can be glycosylated however, the resultant proteins have altered protein structures and/or or stabilities due to inhibition of correct protein folding.^{1,21}

Sites can also be glycosylated to varying degrees, seen within Mpl-ligand and Leptin analogs.¹ This variability in the fraction of molecules containing carbohydrate at particular sites is due to sequence context and secondary structure of the N-linked site. Although molecular 3D modeling would be useful in determining whether site location may be glycosylated or may disrupt tertiary structure, this is not essential. Empirical and predictive analysis of N-linked sites introduction into rHuEPO produced analogs that were glycosylated efficiently whilst maintaining structural integrity.²¹ The carbohydrate composition of rHuEPO N-linked chains are complex and differ in terminal *N*-acetylneuraminic acid (Neu5Ac) content, *O*-acetylation of the Neu5Ac residues, *N*-acetylglucosamine extensions, and degree of branching.^{24,25} However, carbohydrate compositional analysis of glycoengineered rHuEPO analogs found that additional carbohydrates were processed to complex carbohydrates in a manner similar to naturally occurring glycosylation sites and that there were no new associated carbohydrate structures.¹ However, the position of a site may influence the likelihood of carbohydrate attachment and the subset of normal structures of carbohydrate that may be attached. This has been observed in rHuEPO where the carbohydrates added to the naturally occurring glycosylation site at Asn24 tend to be smaller, whereas

carbohydrates added to Asn83 and Asn38 tend to be larger.^{26,27}

PROPERTIES OF GLYCOSYLATED AND GLYCOENGINEERED PROTEINS

Physical Properties-

The stability of protein therapeutics is of prime importance and glycosylation can play an important role in maintaining molecular integrity. Carbohydrate can reduce susceptibility to proteolysis. For example: fibronectin is more sensitive to proteolysis when carbohydrates are removed,²⁸ the sensitivity of glycosylated interferon beta (IFN- β) to thermal denaturation is decreased compared to the unglycosylated form,²⁹ and carbohydrate stabilizes the molecular conformation of interleukin 5 (IL-5) upon exposure to heat.³⁰

Carbohydrate on rHuEPO plays a role in maintaining its molecular stability. Asialo-rHuEPO and fully deglycosylated rHuEPO lost *in vitro* biological activity upon heat treatment, maintaining only 35% and 11% of initial activity, respectively, whereas glycosylated rHuEPO lost no activity.³¹ Unglycosylated rHuEPO is denatured more readily by guanidine-HCl, heat, and pH, resulting in aggregates and precipitates, while glycosylated rHuEPO shows reversible folding and remains soluble.^{32,33} Carbohydrate was also found to protect the peptide component of rHuEPO from oxygen free radical damage.³⁴

Sialic acid containing carbohydrates are highly hydrophilic structures and increase solubility of proteins by shielding hydrophobic residues. They can also affect adhesive properties of proteins.³⁵ For example, mucins are heavily *O*-glycosylated with increased gel-like properties due to their high sialic acid content. When five additional N-linked carbohydrates were added to native sequence leptin via glycoengineering¹ the resultant glycosylated leptin had an over 15-fold increase in solubility over native leptin (Amgen data on file). When glycosylated and unglycosylated *Plasmodium falciparum* merozoite surface protein 1 (MSP1) was expressed in transgenic mouse milk unglycosylated MSP-1 was considerably more insoluble than glycosylated MSP-1 when purified.³⁶ Furthermore, unglycosylated MSP-1 proved to be a significantly more efficient vaccine than glycosylated MSP-1 when monkeys were challenged with a lethal infection of *P. falciparum*.³⁶ This may have been partly due to the formation of

aggregates and precipitates of the unglycosylated protein that mounted an elevated immune response compared to the glycosylated protein. Alternatively, the carbohydrate may have "shielded" the protein from immune surveillance (see below).

Biological Activity

Experiments with EPO glycosylation analogs demonstrated a direct, positive, relationship between number of carbohydrate chains and *in vivo* activity.²⁷ Although position of the attached chains affected activity, the number played the more important role. When intracellular signaling was examined, the kinetics and magnitude of response of rHuEPO to DA were comparable, indicating that additional carbohydrate does not blunt the biological response. DA has a similar conformation and stability compared to rHuEPO.¹ However, due to extra sialic acid, it has significantly enhanced serum half-life and, consequently, *in vivo* activity. Threefold more rHuEPO than DA was required to elicit a similar erythropoietic response at three times per week dosing in mice.⁶ This difference increased to 13-fold when the molecules were administered at weekly intervals. When single injections were compared approximately 30–40 fold more rHuEPO was required to match the activity of the lower dose of DA. Thus, the relative *in vivo* potency of DA compared to rHuEPO increased as the dosing interval increased.³⁷ In a multi-cycle carboplatin chemotherapy/radiotherapy model of anemia in mice,³⁸ a sixfold higher dose of rHuEPO fell short of the effect of DA in stimulating erythropoiesis (Figure 1). This demonstrates that the extended serum half-life yields potent *in vivo* effects.

The ability of increased glycosylation to enhance *in vivo* activity has been investigated for a number of other molecules including mpl ligand and leptin. Leptin is a nonglycosylated protein involved in the control of body weight.³⁹ Using glycoengineering, a leptin analog with five additional carbohydrates (GE-LeptinL4-58) was generated. Obese mice (*ob/ob*) treated with GE-LeptinL4 lost more weight and maintained the reduced weight for a greater length of time than those treated with unglycosylated recombinant human leptin (rHuLeptin).¹ Furthermore, increasing the dose of rHuLeptin 10-fold could not produce the same weight loss induced by hyperglycosylated GE-LeptinL4-58 in normal mice (Figure 2).

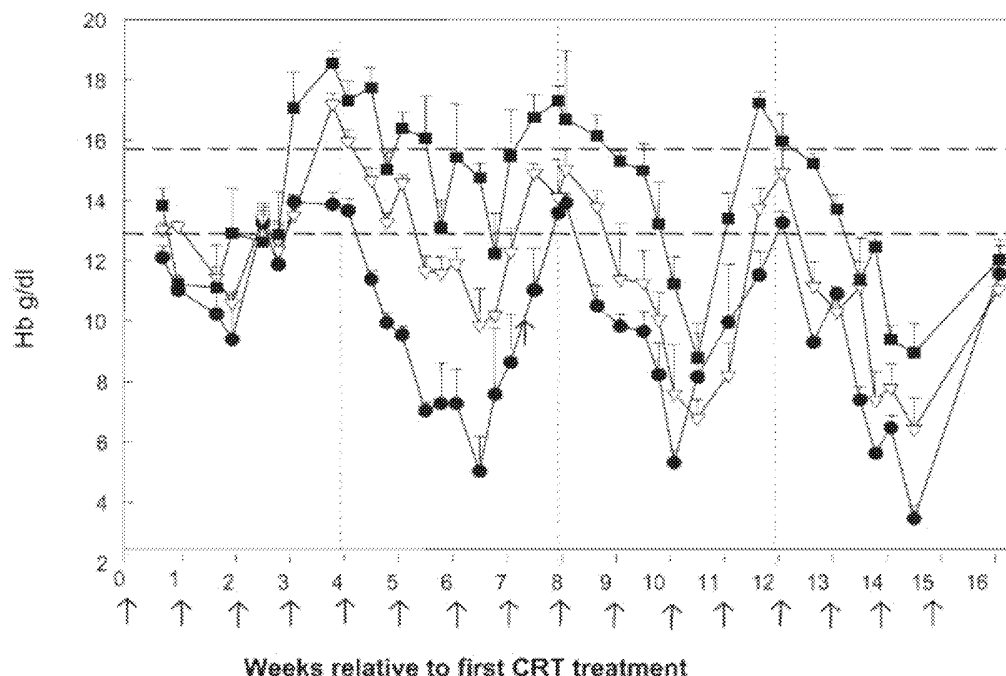


Figure 1. Darbepoetin alfa (DA) improved the kinetics of hemoglobin recovery in mice after dose-intensive chemo/radiotherapy treatment (CRT) in comparison with rHuEPO. Mice were untreated (●) or treated with either 30 µg/kg DA (■) or 180 µg/kg rHuEPO (▽) every 7 days (↑). CRT was performed every 4 weeks (vertical dotted lines). Horizontal broken lines depict upper and lower limits of normal hemoglobin (Hb) in mice. Hb levels are shown in g/dL. Experimental details are described elsewhere.³⁸

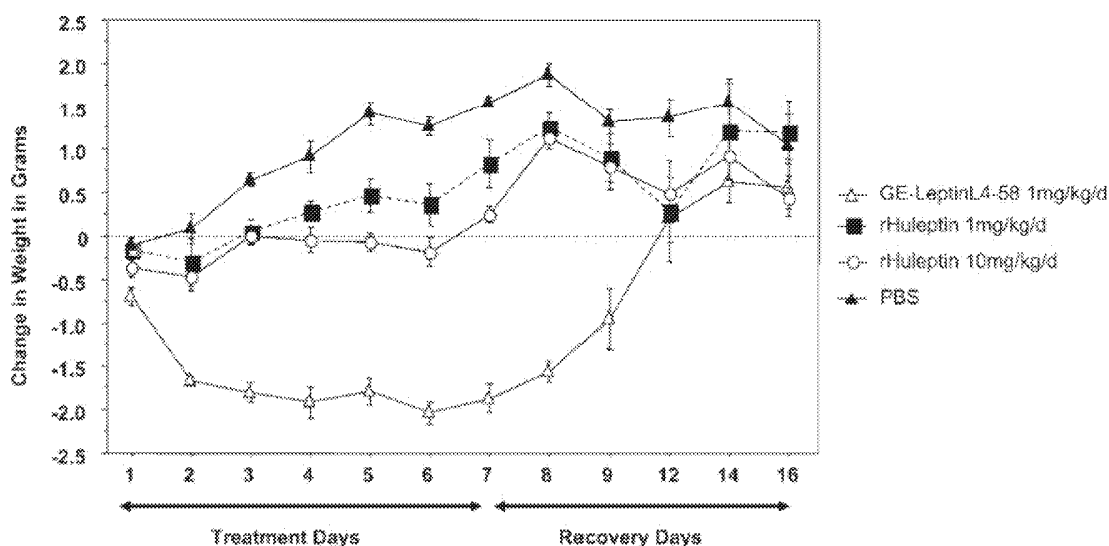


Figure 2. Hyperglycosylated human GE-LeptinL4-58 decreases weight in mice more effectively than unglycosylated, rHuLeptin. Mice (six mice per group) were dosed daily for 7 days with either 1 mg/kg GE-LeptinL4-58 (△), 1 mg/kg unglycosylated rHuLeptin (■), 10 mg/kg unglycosylated rHuLeptin (○), or PBS control (▲). Weights were monitored daily during 7 treatment days then 5 days thereafter during the recovery phase. Changes in weight (g) from baseline are plotted. Experimental details are described elsewhere.¹

Mpl ligand is a glycoprotein hormone involved in megakaryocyte growth, maturation, and platelet production.⁴⁰ Mpl ligand contains two O-linked carbohydrates but lacks N-linked carbohydrates in the amino terminal EPO-like domain.^{41,42} Glycosylation analogs of the Mpl ligand EPO-like domain, were generated and combinations of successfully glycosylated analogs were made. Molecules N39 (four additional N-linked carbohydrates) and N40 (a total of six N-linked carbohydrates) increased the amount and duration of platelet production in normal mice compared to native sequence rHuMpl ligand, and activity correlated with extent of glycosylation.¹ Taken together, these studies demonstrate that the glycoengineering of proteins can enhance *in vivo* activity even of proteins that do not normally contain N-linked carbohydrate moieties.

Serum Half-Life and Clearance

The increase of *in vivo* potency of glycosylated and hyperglycosylated proteins appears to be due to an increase in serum half-life or circulating residence time. The additional two carbohydrate chains in DA conveys a threefold longer serum half-life in rats and dogs compared to rHuEPO.⁶ In EPO-naïve human patients receiving peritoneal dialysis, DA had an approximately threefold

longer mean terminal half-life than rHuEPO, a more than twofold greater area under the curve (AUC) leading to a 2.5-fold lower clearance⁷ (Figure 3). Subcutaneous administration almost doubled the mean terminal half-life in comparison with intravenous administered DA.⁷ Serum half-life extension was also observed for DA compared to epoetin alfa in anemic patients with nonmyeloid malignancies receiving multiple cycles of chemotherapy.⁴³ A more recent study with DA in cancer patients with chemotherapy-induced anemia, where pharmacokinetic samples were collected up to 3 weeks (504 h) after dosing, allowed for full evaluation of the pharmacokinetic profile of DA in this setting. In that study, the mean terminal half-life was estimated as 61–88 h, depending on timing of chemotherapy.⁴⁴

Follicle stimulating hormone (FSH) is a gonadotropin used clinically for the treatment of infertility and of anovulatory conditions including polycystic ovary syndrome and stimulation of multiple follicles for *in vitro* fertilization. FSH, comprised of two subunits, have N-linked glycosylation sites, and isoforms with high sialic acid content were found to have reduced renal clearance and increased *in vivo* bioactivity.^{45,46} Consensus N-linked glycosylation sequence extensions were added onto the N-terminus of one of the subunits to form mutein FSH1208 which was

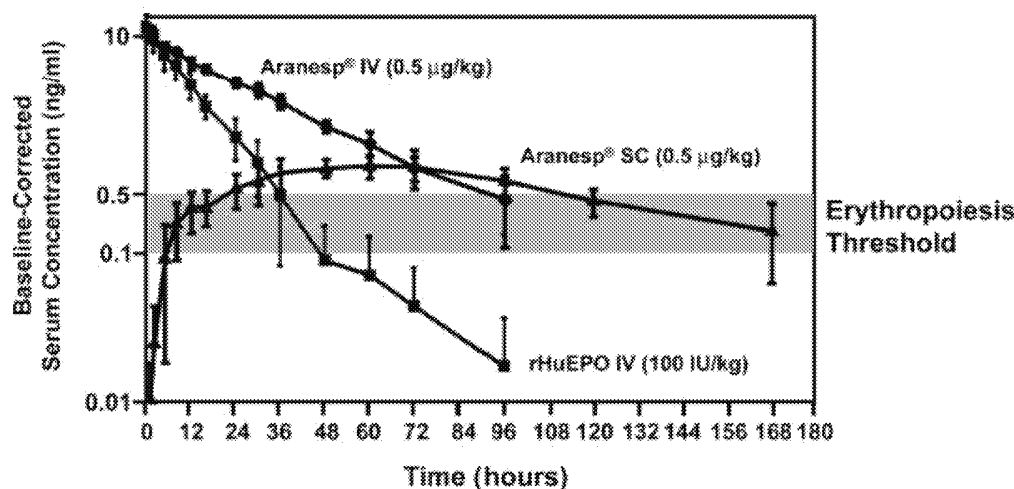


Figure 3. Aranesp[®] (DA) has a threefold increase in serum half-life compared to rHuEPO. Single dose pharmacokinetic profiles of equivalent peptide masses of DA administered intravenously (IV) or subcutaneously (SC), and rHuEPO administered IV in dialysis patients are shown. Serum half-lives were: Aranesp[®] IV, 25.3 ± 2.2 h; Aranesp[®] SC, 48.8 ± 5.2 h; and rHuEPO IV, 8.5 ± 2.4 h. Data reproduced with kind permission of Lippincott-Williams & Wilkins, from Macdougall et al.⁷ Erythropoiesis threshold based on range from Besarab et al.⁶⁹

efficiently glycosylated and had an increased half-life (three to fourfold) and AUC (four to sixfold).⁴⁷ The extended half-life enhanced the *in vivo* bioactivity as a single dose of FSH1208 provided similar increases in ovary weight compared to three daily doses of native FSH. A reduction in renal clearance may explain the increased serum half-life and *in vivo* activity of FSH.⁴⁷

In contrast, glycosylated interleukin 3 (IL-3) is trapped by the extracellular matrix (ECM) and slowly released into the circulation. Glycosylated IL-3 had a 30%–40% increased ability to stimulate histidine carboxylase activity in bone marrow after a single injection compared with non-glycosylated forms due to a twofold increase in serum half-life.⁴⁸ Due to the high volume of distribution for glycosylated IL-3 it was suggested that carbohydrate preferentially trapped IL-3 within tissues by binding to the ECM. Therefore, the slow release of glycosylated IL-3 into the circulation elevated *in vivo* half-life.

It is still unclear why some sialylated glycoproteins are cleared at slower rates from the serum than their desialylated counterparts. Originally it was suggested that the hepatic asialoglycoprotein receptor (ASGPR) bound desialylated glycoproteins targeting them for degradation. Thus, the rate of desialylation of glycoproteins was hypothesized to control clearance. However, studies reported that the saturation of the ASGPR with asialoorosomucoid did not delay the clearance of sialylated ¹²⁵I-orosomucoid, implicating other mechanisms of asialoglycoprotein clearance.⁴⁹ However, no accumulation of desialylated glycoproteins or lipoproteins have been reported in ASGPR knockout mouse circulation, even though they demonstrated reduced clearance rates of asialoorosomucoid or asialofetuin.^{50,51} This raises the possibility that different receptors may influence the clearance of different glycoproteins.

Although rHuEPO has been used in the clinic for over 20 years, the precise mechanism of clearance is unknown. Since there was no apparent difference in rHuEPO clearance between normal and patients with liver cirrhosis, these data suggests that liver, and by implication, ASGPR does not play a significant role in rHuEPO clearance.⁵² The bone marrow has also been suggested to be a clearance organ in addition to the effector organ. Thus, clearance of EPO may be at least partially controlled by binding to an EPO receptor on erythropoietic progenitor cells where it is subsequently internalized and degraded in the lysosomes.⁵³ Although some organs such as liver and

kidney do not play a major role, there may be conditions where they affect rHuEPO clearance.⁵⁴

Immunogenicity

Antibodies raised to protein therapeutics can have deleterious consequences including loss of efficacy through neutralization of activity, for example IFN- α 2B and recombinant IFN- β .^{55,56} In rare cases, antibodies to administered rHuEPO are produced in patients which also recognize and neutralize endogenously produced EPO, resulting in the development of antibody mediated pure red cell aplasia (PRCA).⁵⁷ Although extremely rare before 1998, with only three cases being reported, by 2003 there were over 160 patients worldwide diagnosed with antibody-positive PRCA after receiving rHuEPO with the majority of cases outside the USA.⁵⁸ Since millions of patients had been treated with rHuEPO prior to 1998, it was hypothesized that changes in the manufacturing procedure and or formulation may have accounted for this increase.⁵⁷

One theoretical drawback to the glycoengineering of therapeutic proteins could be immune recognition of the mutated amino acid sequence resulting in the production of neutralizing antibodies. DA differs from rHuEPO at five amino acid positions suggesting that an antibody could theoretically be directed against the "altered" region. However, as of submission of this manuscript, there are no reported cases of neutralizing antibodies in any patients that were caused by DA.⁵⁹

Several possibilities could explain the lack of antibodies in humans to DA. A positive effect of additional carbohydrate on solubility and its effect on stabilizing proteins may reduce the possibility of formation of aggregates. Aggregation has been implicated in increased immunogenicity of proteins.^{60,61} Casadevall et al.⁵⁷ reported that immunoreactivity of antibodies in PRCA patient serums to rHuEPO increases when carbohydrate is removed suggesting that the carbohydrate can inhibit antibody binding by "shielding" the underlying protein sequences from the immune system. In support of this notion, a single triantennary sialylated complex carbohydrate occupies a significant volume, approximately 2542 Å³⁶² thereby reducing the available surface area on the protein to immune surveillance. This is consistent with the observation that carbohydrates reduce polyclonal and monoclonal antibody immunoreactivity to rHuEPO⁶³ and sialic acid on DA inhibited antibody binding in immunoassays.⁶⁴

Other studies have similarly suggested that carbohydrate can shield altered peptide sequences from immune surveillance. For example such reports indicated that chemical addition of polysialic acid to asparaginase results in reduced antigenicity in mice.⁶⁵ Furthermore, wasp venom peptide immunogenicity⁶⁶ and *Plasmodium falciparum* merzoite surface protein 1 immunogenicity³⁶ dramatically decreases when carbohydrate is attached to the molecule. These studies together with the published reports that carbohydrates reduce the immunogenicity of viruses^{67,68} suggests that hyperglycosylated therapeutic proteins may have reduced immunogenicity compared to their non-glycosylated counterparts.

CONCLUSIONS

Carbohydrates confer advantageous physical and biological properties to naturally occurring humeral factors and protein therapeutics. Enhancing the carbohydrate content of proteins through the introduction of consensus N-linked glycosylation sites (glycoengineering) can increase *in vivo* potency of molecules through elevating serum half-life. In theory, altering the peptide sequence raises the potential for generating neutralizing antibodies to the engineered protein therapeutic and endogenous protein. Although this potential should always be taken into consideration in designing protein therapeutics, the likelihood of immune reactions can be minimized by taking advantage of the beneficial properties that carbohydrates add to the molecule (antibody repulsion and shielding, increased solubility and reduced aggregation). Risks can be reduced further by targeting mutations away from receptor binding domains, as has been performed for DA. The glycoengineering approach may therefore be a generally applicable technology to enhance the properties of protein therapeutics.

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REFERENCES

1. Elliott S, Lorenzini T, Asher S, Aoki K, Brankow D, Buck L, Busse L, Chang D, Fuller J, Grant J,

- Hernday N, Hokum M, Hu S, Knudten A, Levin N, Komorowski R, Martin F, Navarro R, Osslund T, Rogers G, Rogers N, Trail G, Egrie J. 2003. Enhancement of therapeutic protein *in vivo* activities through glycoengineering. *Nat Biotechnol* 21:414–421.
2. Browne JK, Cohen AM, Egrie JC, Lai PH, Lin FK, Strickland T, Watson E, Stebbing N. 1986. Erythropoietin: Gene cloning, protein structure, and biological properties. *Cold Spring Harb Symp Quant Biol* 51:693–702.
3. Lin FK, Suggs S, Lin CH, Browne JK, Smalling R, Egrie JC, Chen KK, Fox GM, Martin F, Stabinsky Z. 1985. Cloning and expression of the human erythropoietin gene. *Proc Natl Acad Sci USA* 82: 7580–7584.
4. Egrie JC, Browne J, Lai P, Lin FK. 1985. Characterization of recombinant monkey and human erythropoietin. *Prog Clin Biol Res* 191:339–350.
5. Egrie JC, Browne JK. 2001. Development and characterization of novel erythropoiesis stimulating protein (NESP). *Br J Cancer* 84:3–10.
6. Egrie JC, Dwyer E, Browne JK, Hitz A, Lykos MA. 2003. Darbepoetin alfa has a longer circulating half-life and greater *in vivo* potency than recombinant human erythropoietin. *Exp Hematol* 31:290–299.
7. Macdougall IC, Gray SJ, Elston O, Breen C, Jenkins B, Browne J, Egrie J. 1999. Pharmacokinetics of novel erythropoiesis stimulating protein compared with epoetin alfa in dialysis patients. *J Am Soc Nephrol* 10:2392–2395.
8. Imperiali B, Hendrickson TL. 1995. Asparagine-linked glycosylation: Specificity and function of oligosaccharyl transferase. *Bioorg Med Chem* 3: 1565–1578.
9. Rudd PM, Dwek RA. 1997. Glycosylation: Heterogeneity and the 3D structure of proteins. *Crit Rev Biochem Mol Biol* 32:1–100.
10. Plante OJ, Seeberger PH. 2003. Recent advances in automated solid-phase carbohydrate synthesis: From screening to vaccines. *Curr Opin Drug Discov Dev* 6:521–525.
11. Van den Steen P, Rudd PM, Dwek RA, Van Damme J, Opdenakker G. 1998. Cytokine and protease glycosylation as a regulatory mechanism in inflammation and autoimmunity. *Adv Exp Med Biol* 435: 133–143.
12. Hansen JE, Lund O, Engelbrecht J, Bohr H, Nielsen JO, Hansen JE. 1995. Prediction of O-glycosylation of mammalian proteins: Specificity patterns of UDP-GalNAc:polypeptide N-acetyl-galactosaminyltransferase. *Biochem J* 308:801–813.
13. Thanka Christlet TH, Veluraja K. 2001. Database analysis of O-glycosylation sites in proteins. *Biophys J* 80:952–960.
14. Elliott S, Bartley T, Delorme E, Derby P, Hunt R, Lorenzini T, Parker V, Rohde MF, Stoney K. 1994.

- Structural requirements for addition of O-linked carbohydrate to recombinant erythropoietin. *Biochemistry* 33:11237–11245.
15. Kirnarsky L, Nomoto M, Ikematsu Y, Hassan H, Bennett EP, Cerny RL, Clausen H, Hollingsworth MA, Sherman S. 1998. Structural analysis of peptide substrates for mucin-type O-glycosylation. *Biochemistry* 37:12811–12817.
 16. Van den Steen P, Dwek RA, Opdenakker G. 1998. Concepts and principles of O-linked glycosylation. *Crit Rev Biochem Mol Biol* 33:151–208.
 17. Bause E. 1983. Structural requirements of N-glycosylation of proteins. Studies with proline peptides as conformational probes. *Biochem J* 209: 331–336.
 18. Kornfeld R, Kornfeld S. 1985. Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem* 54:631–664.
 19. Gavel Y, von Heijne G. 1990. Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: Implications for protein engineering. *Protein Eng* 3:433–442.
 20. Apweiler R, Hermjakob H, Sharon N. 1999. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim Biophys Acta* 1473:4–8.
 21. Elliott S, Chang D, Delorme E, Eris T, Lorenzini T. 2004. Structural requirements for additional N-linked carbohydrate on recombinant human erythropoietin. *J Biol Chem* 279:16854–16862.
 22. Nilsson IM, von Heijne G. 1993. Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane. *J Biol Chem* 268:5798–5801.
 23. Kalies KU, Hartmann E. 1998. Protein translocation into the endoplasmic reticulum (ER)—Two similar routes with different modes. *Eur J Biochem* 254:1–5.
 24. Sasaki H, Bothner B, Dell A, Fukuda M. 1987. Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA. *J Biol Chem* 262:12059–12076.
 25. Rush RS, Derby PL, Smith DM, Merry C, Rogers G, Rhode MP, Katta V. 1995. Microheterogeneity of erythropoietin carbohydrate structure. *Anal Chem* 15:1442–14452.
 26. Takeuchi M, Kobata A. 1991. Structures and functional roles of the sugar chains of human erythropoietins. *Glycobiology* 1:337–346.
 27. Elliott S, Egrie J, Browne J, Lorenzini T, Rogers N, Ponting I. 2004. Control of rHuEPO biological activity: The role of carbohydrate. *Exp Hematol* 32:1146–1155.
 28. Bernard BA, Yamada KM, Olden K. 1982. Carbohydrates selectively protect a specific domain of fibronectin against proteases. *J Biol Chem* 257: 8549–8554.
 29. Runkel L, Meier W, Pepinsky RB, Karpusas M, Whitty A, Kimball K, Brickelmaier M, Muldowney C, Jones W, Goelz SE. 1998. Structural and functional differences between glycosylated and non-glycosylated forms of human interferon-beta (IFN-beta). *Pharm Res* 15:641–649.
 30. Kodama S, Tsujimoto M, Tsuruoka N, Sugo T, Endo T, Kobata A. 1993. Role of sugar chains in the *in vitro* activity of recombinant human interleukin 5. *Eur J Biochem* 211:903–908.
 31. Tsuda E, Kawanishi G, Ueda M, Masuda S, Sasaki R. 1990. The role of carbohydrate in recombinant human erythropoietin. *Eur J Biochem* 188:405–411.
 32. Narhi LO, Arakawa T, Aoki KH, Elmore R, Rohde MF, Boone T, Strickland TW. 1991. The effect of carbohydrate on the structure and stability of erythropoietin. *J Biol Chem* 266:23022–23026.
 33. Endo Y, Nagai H, Watanabe Y, Ochi K, Takagi T. 1992. Heat-induced aggregation of recombinant erythropoietin in the intact and deglycosylated states as monitored by gel permeation chromatography combined with a low-angle laser light scattering technique. *J Biochem* 112:700–706.
 34. Uchida E, Morimoto K, Kawasaki N, Izaki Y, Abdu SA, Hayakawa T. 1997. Effect of active oxygen radicals on protein and carbohydrate moieties of recombinant human erythropoietin. *Free Radic Res* 27:311–323.
 35. Gooch CF, Gramer M, Andersen D, Bahr J, Rasmussen JR. 1991. The oligosaccharides of glycoproteins: Factors affecting their synthesis and their influence on glycoprotein properties. In: Sikdar SK, Brier M, Todd P, editors. *Frontiers in bioprocessing II*. Washington DC: American Chemical Society, pp 199–240.
 36. Stowers AW, Chen LH, Zhang Y, Kennedy MC, Zou L, Lambert L, Rice TJ, Kaslow DC, Saul A, Long CA, Meade H, Miller LH. 2002. A recombinant vaccine expressed in the milk of transgenic mice protects Aotus monkeys from a lethal challenge with *Plasmodium falciparum*. *Proc Natl Acad Sci USA* 99:339–344.
 37. Sasu B, Hartley C, Schultz H, McElroy P, Khaja R, Elliott S, Egrie J, Browne JK, Begley CG, Molineux G. 2003. Has the Unit of erythropoietic activity outlived its usefulness? *Blood* 102:4386.
 38. Hartley C, Elliott S, Begley CG, McElroy P, Sutherland W, Khaja R, Heatherington AC, Graves T, Schultz H, Del Castillo J, Molineux G. 2003. Kinetics of haematopoietic recovery after dose-intensive chemo/radiotherapy in mice: Optimized erythroid support with darbepoetin alpha. *Br J Haematol* 122:623–636.
 39. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature* 372:425–432.

40. Kuter DJ. 2004. Thrombopoietin factors. In: Morstyn G, Foote M, Lieschke GJ, editors. Hematopoietic growth factors in oncology. Totowa, NJ: Humana Press. pp 125–152.
41. Bartley TD, Bogenberger J, Hunt P, Li YS, Lu HS, Martin F, Chang MS, Samal B, Nichol JL, Swift S. 1994. Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpl. *Cell* 77:1117–1124.
42. Hunt P, Li YS, Nichol JL, Hokom MM, Bogenberger JM, Swift SE, Skrine JD, Hornkohl AC, Lu H, Clogston C. 1995. Purification and biologic characterization of plasma-derived megakaryocyte growth and development factor. *Blood* 86:540–547.
43. Elliott S, Heatherington AC, Toote M. 2004. Erythropoietic factors. In: Morstyn G, Foote M, Lieschke GJ, editors. Hematopoietic growth factors in oncology. Totowa, NJ: Humana Press. pp 97–124.
44. Glaspy J, Henry D, Patel R, Tchekmedyian S, Berg R, Rossi G, Austin M. 2003. A randomized trial evaluating the pharmacologic and hematologic effects of the timing of darbepoetin alfa and chemotherapy administration. *Blood* 102:9b.
45. Creus S, Chaia Z, Pellizzari EH, Cigorrage SB, Ulloa-Aguirre A, Campo S. 2001. Human FSH isoforms: Carbohydrate complexity as determinant of in-vitro bioactivity. *Mol Cell Endocrinol* 174: 41–49.
46. D'Antonio M, Borrelli F, Datola A, Bucci R, Mascia M, Polletta P, Piscitelli D, Papoian R. 1999. Biological characterization of recombinant human follicle stimulating hormone isoforms. *Hum Reprod* 14:1160–1167.
47. Perlman S, van den HB, Christiansen J, Gram-Nielsen S, Jeppesen CB, Andersen KV, Halkier T, Okkels S, Schambye HT. 2003. Glycosylation of an N-terminal extension prolongs the half-life and increases the *in vivo* activity of follicle stimulating hormone. *J Clin Endocrinol Metab* 88:3227–3235.
48. Ziltener HJ, Clark-Lewis I, Jones AT, Dy M. 1994. Carbohydrate does not modulate the *in vivo* effects of injected interleukin-3. *Exp Hematol* 22:1070–1075.
49. Clarenburg R. 1983. Asialoglycoprotein receptor is uninvolved in clearing intact glycoproteins from rat blood. *Am J Physiol* 244:G247–G253.
50. Ishibashi S, Hamner RE, Herz J. 1994. Asialoglycoprotein receptor deficiency in mice lacking the minor receptor subunit. *J Biol Chem* 269:27803–27906.
51. Tozawa R, Ishibashi S, Osuga J, Yamamoto K, Yagyu H, Ohashi K, Tamura Y, Yahagi N, Iizuka Y, Okazaki H, Harada K, Gotoda T, Shimano H, Kimura S, Nagai R, Yamada N. 2001. Asialoglycoprotein receptor deficiency in mice lacking the major receptor subunit. Its obligate requirement for the stable expression of oligomeric receptor. *J Biol Chem* 276:12624–12628.
52. Jensen JD, Jensen LW, Madsen JK, Poulsen L. 2004. The metabolism of erythropoietin in liver cirrhosis patients compared with healthy volunteers. *Eur J Haematol* 54:111–116.
53. Sawyer ST, Krantz SB, Goldwasser E. 1987. Binding and receptor-mediated endocytosis of erythropoietin in Friend virus-infected erythroid cells. *J Biol Chem* 262:5554–5562.
54. Heatherington AC. 2003. Clinical pharmacokinetic properties of rHuEPO: A review. In: Molineux G, Foote M, Elliott S, editors. Erythropoietins and erythropoiesis—Molecular, cellular, preclinical and clinical biology. Basel, Switzerland: Birkhauser-Verlag. pp 87–112.
55. Zang YCQ, Yang D, Hong J, Tejada-Simon MV, Rivera VM, Zhang JZ. 2000. Immunoregulation and blocking antibodies induced by interferon beta treatment in MS. *Neurology* 55:397–404.
56. Oberg K, Norheim I, Alm G. 1989. Treatment of malignant carcinoid tumors: A randomized controlled study of streptozocin plus 5-FU and human leukocyte interferon. *Eur J Cancer Clin Oncol* 25: 1475–1479.
57. Casadevall N, Nataf J, Viron B, Kolta A, Kiladjian JJ, Martin-Dupont P, Michaud P, Papo T, Ugo V, Teyssandier I, Varet B, Mayeux P. 2002. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *New Engl J Med* 346:469–475.
58. Mayeux P, Casadevall N. 2003. Antibodies to endogenous and recombinant erythropoietin. In: Molineux G, Foote MA, Elliott SG, editors. Erythropoietins and erythropoiesis. Basel, Switzerland: Birkhauser Verlag. pp 229–240.
59. Smalling R, Foote M, Molineux G, Swanson SJ, Elliott S. 2004. Drug-induced and antibody-mediated pure red cell aplasia: A review of literature and current knowledge. *Biotechnol Annu Rev* 10:237–250.
60. Moore WV, Leppert P. 1980. Role of aggregated human growth hormone (hGH) in development of antibodies to hGH. *J Clin Endocrinol Metab* 51: 691–697.
61. Man NT, Cartwright AJ, Andrews KM, Morris GE. 1989. Treatment of human muscle creatine kinase with glutaraldehyde preferentially increases the immunogenicity of the native conformation and permits production of high-affinity monoclonal antibodies which recognize two distinct surface epitopes. *J Immunol Methods* 125:251–259.
62. Imperiali B, O'Connor SE. 1999. Effect of N-linked glycosylation on glycopeptide and glycoprotein structure. *Curr Opin Chem Biol* 3:643–649.
63. Elliott S, Chang D, Delorme E, Dunn C, Egrie J, Giffin J, Lorenzini T, Talbot C, Hesterberg L. 1996. Isolation and characterization of conformation sensitive antierythropoietin monoclonal antibodies: Effect of disulfide bonds and carbohydrate on

- recombinant human erythropoietin structure. *Blood* 87:2714–2722.
64. Wide L, Wilkstrom B, Eriksson K. 2003. A new principle suggested for detection of darbepoetin-alpha (NESP) doping. *Ups J Med Sci* 108:229–239.
 65. Fernandes AI, Gregoriadis G. 2001. The effect of polysialylation on the immunogenicity and antigenicity of asparaginase: Implication in its pharmacokinetics. *Int J of Pharm* 217:215–224.
 66. Ho CL, Lin YL, Chen WC, Rocchi R, Piek T. 1998. Comparison of the immunogenicity of wasp venom peptides with or without carbohydrate moieties. *Toxicon* 36:217–221.
 67. Reitter JN, Means RE, Desrosiers RC. 1998. A role for carbohydrates in immune evasion in AIDS. *Nat Med* 4:679–684.
 68. van Drunen Littel-van den Hurk, Hughes G, Babiuk LA. 1990. The role of carbohydrate in the antigenic and immunogenic structure of bovine herpesvirus type 1 glycoproteins gI and gIV. *J Gen Virol* 71:2053–2063.
 69. Besarab A, Flaharty KK, Erslev AJ, McCrea JB, Vlasses PH, Medina F, Caro J, Morris E. 1992. Clinical pharmacology and economics of recombinant human erythropoietin in end-stage renal disease: The case for subcutaneous administration. *J Am Soc Nephrol* 2:1405–1416.

