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Mini-Review

Theme: ADME of Therapeutic Proteins
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Physiochemical and Biochemical Factors Influencing the Pharmacokinetics of Antibody Therapeutics

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Abstract. Monoclonal antibodies are increasingly being developed to treat multiple disease areas, including those related to oncology, immunology, neurology, and ophthalmology. There are multiple factors, such as charge, size, neonatal Fc receptor (FcRn) binding affinity, target affinity and biology, immunoglobulin G (IgG) subclass, degree and type of glycosylation, injection route, and injection site, that could affect the pharmacokinetics (PK) of these large macromolecular therapeutics, which in turn could have ramifications on their efficacy and safety. This minireview examines how characteristics of the antibodies could be altered to change their PK profiles. For example, it was observed that a net charge modification of at least a 1-unit shift in isoelectric point altered antibody clearance. Antibodies with enhanced affinity for FcRn at pH 6.0 display longer serum half-lives and slower clearances than wild type. Antibody fragments have different clearance rates and tissue distribution profiles than full length antibodies. Fc glycosylation is perceived to have a minimal effect on PK while that of terminal high mannose remains unclear. More investigation is warranted to determine if injection route and/or site impacts PK. Nonetheless, a better understanding of the effects of all these variations may allow for the better design of antibody therapeutics.

KEY WORDS: antibody; FcRn; glycosylation; isoelectric point; pharmacokinetics.

INTRODUCTION

Antibody therapeutics are becoming increasingly important in fighting many diseases. Antibodies are tetrameric glycoproteins composed of two heavy chains (HC) and two light chains (LC) held together by disulfide linkages (Fig. 1) (1). When properly folded into the "Y" shape motif, the constant domains C_H1 and C_L and variable domains V_H and V_L comprise the antigen binding fragment (Fab), with the variable domain containing the complementarity-determining region that is highly specific for a target antigen. The C_H2 and C_H3 domains of the heavy chains form the Fc fragment (fragment crystallizable) of the antibody and can bind to Fc receptors on cells to illicit immune effector functions or to the neonatal Fc receptor (FcRn) to protect it from degradation (2). Human immunoglobulin Gs (IgGs) are divided into four subclasses, i.e., IgG1, IgG2, IgG3, and IgG4, with structural differences in their heavy chains characterizing each subclass (1). It should be noted that IgG3 antibodies are known to be less stable than the other IgG subclasses (3) and, to date, have not been developed as therapeutic antibodies. Examining human and cynomolgus monkey (cyno) clearance values for a wide range of IgG1 antibodies with similar Fc domains demonstrates that different IgG1s have different clearance rates ranging from 0.4 to 40 mL/h (4). Antibody clearance can be driven by target expression and biology, FcRn binding, proteolytic catabolism, and cellular uptake. Furthermore, binding of antibodies to FcRn on epithelial and endothelial cells through their Fc domain regulates their metabolism and partially accounts for their relatively long serum half-lives (5,6).

There are a number of other factors that can impact the pharmacokinetics (PK) of antibodies, such as antibody structural characteristics, delivery and formulation, target properties, and physiological parameters. Research on how manipulating antibody charge, size, valence, FcRn-binding affinity, and degree and type of glycosylation can influence their metabolism, clearance, penetration, and lymphatic absorption has been conducted (7–15). Furthermore, a need exists to further optimize drug delivery methods through formulation, dose, injection volume, and injection site (16–19). Antibody target expression levels and affinity as well as the local physiology of the target-expressing tissue, such as

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ABBREVIATIONS: Cyno, Cynomolgus monkey; Fab, Antigen binding fragment; Fc, Fragment crystallizable; FcγR, Fc gamma receptor; FcRn, Neonatal Fc receptor; GlcNAc, *N*-acetylglucosamine; HC, Heavy chain; IgG, Immunoglobulin G; LC, Light chain; p*I*, Isoelectric point; PK, Pharmacokinetics.

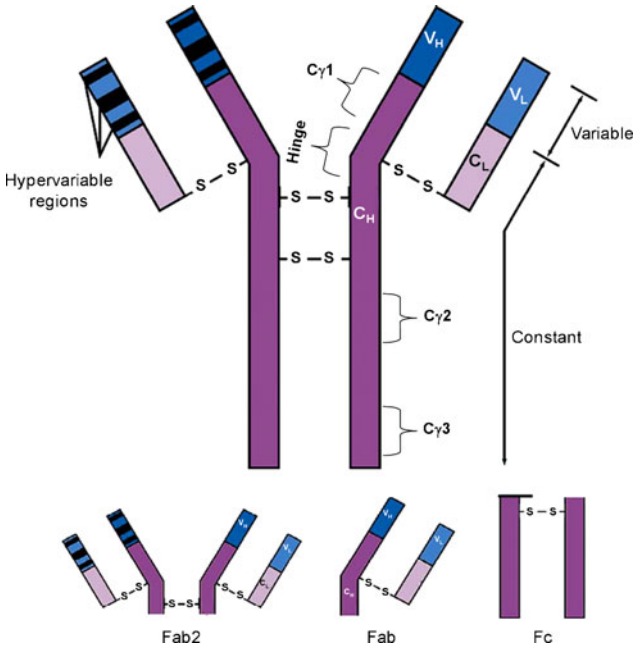

Fig. 1. A diagram of a typical IgG1 antibody along with its constituent parts. Reproduced from Mould and Sweeney (1). Originally published by BioMed Central

vascularity, blood flow, interstitial pressure, degree of necrosis, and/or fibrosis, may also have an impact on antibody PK (20–23). This minireview focuses only on the physiochemical and biochemical factors affecting antibody PK and aims to provide a better understanding about how antibody charge, size/valence, FcRn affinity, degree and type of glycosylation, and delivery route could affect its PK. It is a summary of a presentation given at an AAPS Focus Group Workshop (ADME of Protein Therapeutics Introductory Workshop: Scientific, Technical Concepts and Case Studies, August 15, 2011, Buffalo, NY, USA).

CHARGE AND ISOELECTRIC POINT (p*I*)

Charge is a crucial determinant of the interactions of biological drug material with biosystems (11). Therefore, there is a strong rationale that charge modifications will alter the electrostatic (or nonspecific) interactions with negatively charged components of the cell surface (i.e., sialic acids and glycosaminoglycans). These in turn can play a role in dictating both serum and tissue PK of antibodies. Chemical modifications of antibodies can modulate their net charge and/or hydrophobicity resulting in noticeable changes in biological activity. Typical p*I* values for antibodies fall in the range of 8–9 (11). Cationized proteins have a high propensity to adhere to anionic sites of cell surfaces (i.e., heparan sulfate proteoglycans and phospholipids), so the chemical modification of antibodies or antibody fragments with cationized residues that increase their p*I* by >1 units has resulted in increased plasma clearance, with a higher disposition to normal tissues and higher target tissue uptake (Table I) (24,25). Conversely, modification of Fabs with anionic groups causing a reduction in p*I* by 1–2 units was shown to result in decreased blood clearance and tissue accumulation relative to the unmodified Fab (26). It has been observed that a neutral charge modification that decreased an antibody's pI by more than 1 unit can exhibit faster blood clearance compared to its unmodified counterpart with less activity in normal tissues and improved localization in the target (27). More recently, a study has shown that antibodies with higher p*I* values also tended to exhibit faster systemic clearance rates and lower subcutaneous bioavailabilities in both human and minipig than antibodies with lower p*I* values (28).

To further elucidate the relationship between the pharmacokinetics and the p*I* of antibodies, Igawa *et al.* (29) generated antibody variants with modified p*I* values using site-directed mutagenesis. Specific substitutions were chosen within the surface residues of the heavy chain variable region to modify the p*I* without affecting the antigen-binding property of the antibody. Variants with p*I* values of 1–2 units lower than wild type were shown to display longer half-lives and clearance rates, whether they were administered intravenously or subcutaneously into mice. These results suggest that altering the p*I* through modification of the variable region could offer an alternative to Fc engineering.

As part of many posttranslational events, antibodies undergo chemical or enzymatic degradation via several different mechanisms, including oxidation, deamidation, isomerization, and fragmentation, which result in the formation of several charge variants (12). The presence of charge variants in all antibody preparations is consistent, but the percentage of each variant differs per antibody. However, until recently, the impact of those variations on PK was largely unknown. In a recent study, acidic and basic antibody variants of an IgG1 monoclonal antibody with a range of p*I* values between 8.7 and 9.1 were isolated and administered intravenously and subcutaneously to rats (12). There was no significant difference in the observed serum PK profile, demonstrating that p*I* differences among charge variants were not large enough to result in PK changes and corroborating that at least a net difference of one p*I* unit is needed to exert any influence on systemic PK and tissue distribution.

SIZE AND VALENCE

Antibody fragments, such as single-chain Fv, diabody, triabody, Fab, $F(ab')_2$, and full length antibodies, ranging in size from 30 to 150 kDa and valence from one to three binding sites (9,10) can be derived via molecular engineering

Table I. Serum Exposure (AUC) and Tissue Uptake (%ID/g) of a Radiolabeled Antibody and its Cationized Counterpart

Parameter	[^{111}In]-native 528 MAb	[^{111}In]-cationized 528 MAb
AUC(60 min) (%ID·min/mL)	4,180±210	3976±192
AUC_{ss}(%ID·min/mL)	25,887±3,380	17,770±2,301
Liver uptake(%ID/g)	11.0±0.5	18.6±0.8
Kidney uptake(%ID/g)	0	4.8±0.4
Heart uptake(%ID/g)	0	0
Lung uptake(%ID/g)	2.5±1.6	4.9±0.5

The tissue uptake has been blood background corrected, so the values represent the extravascular tissue radioactivity levels. Reprinted (adapted) with permission from Lee and Pardridge (24). Copyright 2003 American Cancer Society
AUC area under the concentration–time curve, AUC_{ss} AUC at steady state, *%ID/g* percent injected dose per gram of tissue

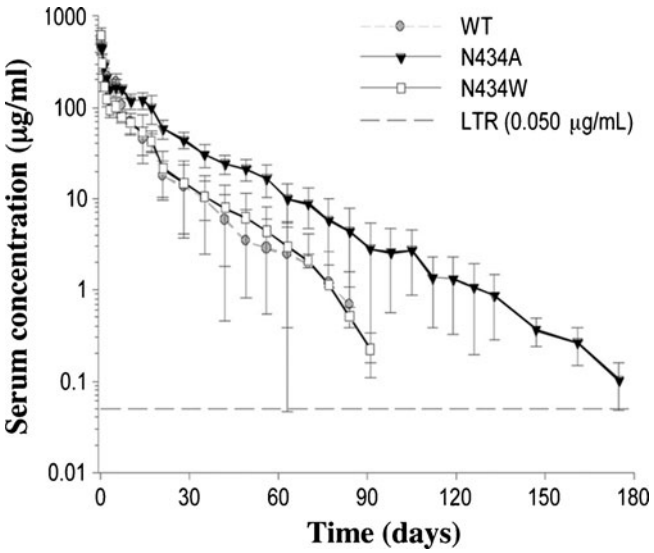

Fig. 2. Serum concentrations following 20 mg/kg i.v. administration in cynos of an antibody (WT) and its two FcRn variants, N434A and N434W. Both variants had improved binding to FcRn at pH 6.0, but N434W also had increased binding to FcRn at pH 7.4. N434A cleared slower from the serum than the wild-type antibody and N434W had the same clearance. Reproduced with permission from Yeung *et al.* (7). Copyright 2009. The American Association of Immunologists, Inc

or controlled enzymatic digestion. While retaining their antigen-binding capabilities, these fragments displayed different *in vivo* serum and tissue PK. The smallest fragments not only cleared the fastest but were also shown to have much higher tumor/organ ratios compared to their larger counterparts. The small fragments also reached their peak tumor levels earlier than the full length antibody.

FcRn BINDING AFFINITY

The role of FcRn in prolonging the half-life of serum IgG has been well characterized (30). It has been noted that murine FcRn binds with high affinity to IgG from different species, including human, whereas human FcRn is more selective. This receptor is expressed within intestinal epithelial cells, endothelial cells, and circulating monocytes and at the maternal–fetal barrier, although species differences in expression have been observed (30). Generally, fluid phase uptake internalizes serum IgG into intracellular endosomes. IgG binds FcRn via pH-dependent electrostatic interactions at the endosomal acidic pH (<6.5) and is recycled and released extracellularly into systemic circulation at the more neutral pH 7.4 (30). This interaction with FcRn protects antibodies from lysosomal degradation. The effect of altering the affinity for FcRn either at pH 6 or 7.4 or even at both pH values has been investigated. In both cynos and mice, increasing binding affinity to FcRn at pH 6 with negligible change to binding at pH 7.4 resulted in decreased serum clearance and increased half-life compared to that of the wild-type antibody (Fig. 2) (7,8). In contrast, increasing the binding to FcRn at both pH 6 and 7.4 exhibited very similar PK to that of the wild-type antibody, indicating that there was no improvement in PK with increased affinity at pH 7.4 (7,8). Despite the different mechanisms underlying the ADME determinants following intravenous and subcutaneous dosing, the same trend was observed regardless of injection route. These data suggest that while it is important to increase binding affinity at pH 6.0, it is also critical to preserve the efficient pH-dependent release of IgG at pH 7.4 when engineering antibodies with a slower clearance and longer half-life than the wild type. Interestingly, prolonged serum PK of high affinity FcRn antibody variants has been obtained in mice expressing human FcRn (30); however, this benefit has yet to be observed in the clinic (31).

GLYCOSYLATION

In general, glycans in the Fc domain are important for specific effector functions. All human IgGs contain carbohydrates at Asn^{297} in the C_H2 domain of the Fc region (32). These *N*-glycans are structurally heterogeneous containing a combination of terminal galactose and *N*-acetylglucosamine (GlcNAc) residues. The G0 structure is the most basic form of the *N*-glycan structures, terminating in two GlcNAc units (Fig. 3). The G1 and G2 designations refer to the modification of the G0 structure with either one or two galactose units, respectively. The GlcNAc unit conjugated directly to Asn^{297} is linked to a fucose moiety (called the "core fucose") that is in the Fc gamma receptor (FcγR) binding region, and the absence of this fucose (G0-F) has been shown to improve binding of the antibody to FcγRIIB and FcγRIIIA in addition to enhancing ADCC activity (33). Additionally, there are high mannose, but low galactose and GlcNAc containing glycans, designated Man5, Man8, and Man9, which have been observed and have shown differential effects on FcγR binding and ADCC or CDC activity (33).

The role of glycans in modulating clearance is not well understood (34). Glycans are buried in the interior surface of the Fc domain, making them inaccessible to asialoglycoprotein and mannose receptors that could recognize the moieties and clear the antibody (13). Consistently, differences in murine PK were not observed between a wild-type and aglycosylated chimeric IgG1 (13). The effect of terminal mannose (Man5-8) on PK has also been examined. In a more recent study, high mannose (Man5) showed increased clearance in humans (15). However, earlier studies with omalizumab (IgG1) showed that Fc glycan structure, including high mannose, did not alter the antibody's clearance in mice, but this could be due to the fact that only a small percentage of the antibody contained high mannose (14).

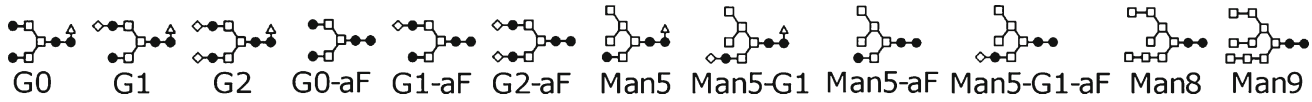

Fig. 3. Schematic structures of various glycan oligosaccharides and their designations. The individual glycans are denoted as follows: galactose (*open diamond*), GlcNac (*closed circle*), mannose (*open square*), and fucose (F, *open triange*). Reproduced from Kanda *et al.* (33), by permission of Oxford University Press

Furthermore, the degree of Fv sialylation did not appear to impact antibody PK (35) nor did complete desialylation (36). There is limited published information about the impact of glycosylation modifications on antibody tissue PK (37).

DELIVERY ROUTES AND SITES

Conflicting results have been reported on the effects of the delivery route and site of administration on PK. A comparison between the bioavailability of Alefacept (Fc fusion protein) following 0.075 mg/kg intramuscular and subcutaneous administration resulted in higher bioavailability with the intramuscular dosing (38). However, in a later bioequivalence study at a flat 15 mg dose in healthy volunteers, nearly identical exposures following intramuscular and subcutaneous administration were observed, demonstrating bioequivalence between the two routes. Conversely, within an injection route, no effect was seen in PK following subcutaneous injection in the upper arm, abdomen, or thigh of 100 mg of golimumab in healthy male volunteers, suggesting that injection site has little impact on PK (39), although more research in this area is warranted. Indeed, in a recent review by McDonald and colleagues (40), injection site was identified as a possible factor influencing the bioavailabilities of molecular therapeutics.

CONCLUSIONS AND FUTURE PERSPECTIVE

In summary, studies have been conducted evaluating the various effects of charge, size, glycosylation, and FcRn affinity at different pH values on antibody PK and tissue distribution. However, more investigation is needed to elucidate how systemic PK is altered by charged amino acid substitutions in the variable or constant regions of the antibody, different IgG subclasses, and different injection routes and sites. Furthermore, very little is known about the PK drivers of the subcutaneous space, and there is a particular need for a systematic evaluation of charge alteration to address such issues as saturation of subcutaneous electrostatic binding sites. Another area that remains unclear is if FcRn in the lymphatic and subcutaneous space could be saturated following administration of high concentration formulations. The effect of glycosylation on subcutaneous PK is also not well understood. Although a great deal has been done to examine the many factors governing antibody PK, more work in this area is needed.

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REFERENCES

1. Mould DR, Sweeney KR. The pharmacokinetics and pharmacodynamics of monoclonal antibodies—mechanistic modeling applied to drug development. Curr Opin Drug Discov Dev. 2007;10 (1):84–96.
2. Sarmay G, Lund J, Rozsnyay Z, Gergely J, Jefferis R. Mapping and comparison of the interaction sites on the Fc region of IgG responsible for triggering antibody dependent cellular cytotoxicity (ADCC) through different types of human Fc gamma receptor. Mol Immunol. 1992;29(5):633–9.
3. Correia IR. Stability of IgG isotypes in serum. mAbs. 2010;2 (3):221–32.
4. Dong JQ, Salinger DH, Endres CJ, Gibbs JP, Hsu CP, Stouch BJ, *et al*. Quantitative prediction of human pharmacokinetics for monoclonal antibodies: retrospective analysis of monkey as a single species for first-in-human prediction. Clin Pharmacokinet. 2011;50(2):131–42.
5. Wang W, Wang EQ, Balthasar JP. Monoclonal antibody pharmacokinetics and pharmacodynamics. Clin Pharmacol Ther. 2008;84(5):548–58.
6. Tabrizi MA, Tseng CM, Roskos LK. Elimination mechanisms of therapeutic monoclonal antibodies. Drug Discov Today. 2006;11 (1–2):81–8.
7. Yeung YA, Leabman MK, Marvin JS, Qiu J, Adams CW, Lien S, *et al*. Engineering human IgG1 affinity to human neonatal Fc receptor: impact of affinity improvement on pharmacokinetics in primates. J Immunol. 2009;182(12):7663–71.
8. Deng R, Loyet KM, Lien S, Iyer S, DeForge LE, Theil FP, *et al*. Pharmacokinetics of humanized monoclonal anti-tumor necrosis factor-{alpha} antibody and its neonatal Fc receptor variants in mice and cynomolgus monkeys. Drug Metab Dispos Biol Fate Chem. 2010;38(4):600–5.
9. Khawli LA, Biela B, Hu P, Epstein AL. Comparison of recombinant derivatives of chimeric TNT-3 antibody for the radioimaging of solid tumors. Hybridoma Hybridomics. 2003;22(1):1–9.
10. Dennis MS, Jin H, Dugger D, Yang R, McFarland L, Ogasawara A, *et al*. Imaging tumors with an albumin-binding Fab, a novel tumor-targeting agent. Cancer Res. 2007;67(1):254–61.
11. Boswell CA, Tesar DB, Mukhyala K, Theil FP, Fielder PJ, Khawli LA. Effects of charge on antibody tissue distribution and pharmacokinetics. Bioconjug Chem. 2010;21(12):2153–63.
12. Khawli LA, Goswami S, Hutchinson R, Kwong ZW, Yang J, Wang X, *et al*. Charge variants in IgG1: Isolation, characterization, *in vitro* binding properties and pharmacokinetics in rats. mAbs. 2010;2(6):613–24.
13. Tao MH, Morrison SL. Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. J Immunol. 1989;143(8):2595–601.
14. Harris RJ. Heterogeneity of recombinant antibodies: linking structure to function. Dev Biol. 2005;122:117–27.
15. Goetze AM, Liu YD, Zhang Z, Shah B, Lee E, Bondarenko PV, *et al*. High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans. Glycobiology. 2011;21(7):949–59.
16. Barrett JS, Wagner JG, Fisher SJ, Wahl RL. Effect of intraperitoneal injection volume and antibody protein dose on the pharmacokinetics of intraperitoneally administered IgG2a kappa murine monoclonal antibody in the rat. Cancer Res. 1991;51 (13):3434–44.
17. Xu Z, Wang Q, Zhuang Y, Frederick B, Yan H, Bouman-Thio E, *et al*. Subcutaneous bioavailability of golimumab at 3 different injection sites in healthy subjects. J Clin Pharmacol. 2010;50(3):276–84.
18. Ternant D, Paintaud G. Pharmacokinetics and concentration–effect relationships of therapeutic monoclonal antibodies and fusion proteins. Expert Opin Biol Ther. 2005;5 Suppl 1:S37–47.
19. Andya JD, Hsu CC, Shire SJ. Mechanisms of aggregate formation and carbohydrate excipient stabilization of lyophilized humanized monoclonal antibody formulations. AAPS pharmSci. 2003;5(2):E10.
20. Jain RK. Transport of molecules, particles, and cells in solid tumors. Annu Rev Biomed Eng. 1999;1:241–63.
21. Minchinton AI, Tannock IF. Drug penetration in solid tumours. Nat Rev Cancer. 2006;6(8):583–92.
22. Tabrizi M, Bornstein GG, Suria H. Biodistribution mechanisms of therapeutic monoclonal antibodies in health and disease. AAPS J. 2010;12(1):33–43.
23. Thurber GM, Schmidt MM, Wittrup KD. Factors determining antibody distribution in tumors. Trends Pharmacol Sci. 2008;29 (2):57–61.

24. Lee HJ, Pardridge WM. Monoclonal antibody radiopharmaceuticals: cationization, pegylation, radiometal chelation, pharmacokinetics, and tumor imaging. Bioconjug Chem. 2003;14(3):546–53.
25. Herve F, Ghinea N, Scherrmann JM. CNS delivery via adsorptive transcytosis. AAPS J. 2008;10(3):455–72.
26. Kobayashi H, Le N, Kim IS, Kim MK, Pie JE, Drumm D, *et al*. The pharmacokinetic characteristics of glycolated humanized anti-Tac Fabs are determined by their isoelectric points. Cancer Res. 1999;59(2):422–30.
27. Khawli LA, Glasky MS, Alauddin MM, Epstein AL. Improved tumor localization and radioimaging with chemically modified monoclonal antibodies. Cancer Biother Radiopharm. 1996;11 (3):203–15.
28. Zheng Y, Tesar DB, Benincosa L, Birnböck H, Boswell CA, Bumbaca D, *et al*. Minipig as a potential translatable model for monoclonal antibody pharmacokinetics after intravenous and subcutaneous administration. mAbs. 2012;4(2):0–1.
29. Igawa T, Tsunoda H, Tachibana T, Maeda A, Mimoto F, Moriyama C, *et al*. Reduced elimination of IgG antibodies by engineering the variable region. Protein Eng Des Sel PEDS. 2010;23(5):385–92.
30. Roopenian DC, Akilesh S. FcRn: the neonatal Fc receptor comes of age. Nat Rev Immunol. 2007;7(9):715–25.
31. Zheng Y, Scheerens H, Davis Jr JC, Deng R, Fischer SK, Woods C, *et al*. Translational pharmacokinetics and pharmacodynamics of an FcRn-variant anti-CD4 monoclonal antibody from preclinical model to phase I study. Clin Pharmacol Ther. 2011;89 (2):283–90.
32. Kaneko Y, Nimmerjahn F, Ravetch JV. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. Science. 2006;313(5787):670–3.
33. Kanda Y, Yamada T, Mori K, Okazaki A, Inoue M, Kitajima-Miyama K, *et al*. Comparison of biological activity among nonfucosylated therapeutic IgG1 antibodies with three different N-linked Fc oligosaccharides: the high-mannose, hybrid, and complex types. Glycobiology. 2007;17(1):104–18.
34. Deng R, Jin F, Prabhu S, Iyer S. Monoclonal antibodies: what are the pharmacokinetic and pharmacodynamic considerations for drug development? Expert Opin Drug Metab Toxicol. 2012;8 (2):141–60.
35. Millward TA, Heitzmann M, Bill K, Langle U, Schumacher P, Forrer K. Effect of constant and variable domain glycosylation on pharmacokinetics of therapeutic antibodies in mice. Biol J Int Assoc Biol Stand. 2008;36(1):41–7.
36. Wright A, Morrison SL. Effect of C2-associated carbohydrate structure on Ig effector function: studies with chimeric mouse-human IgG1 antibodies in glycosylation mutants of Chinese hamster ovary cells. J Immunol. 1998;160(7):3393–402.
37. Wright A, Sato Y, Okada T, Chang K, Endo T, Morrison S. *In vivo* trafficking and catabolism of IgG1 antibodies with Fc associated carbohydrates of differing structure. Glycobiology. 2000;10(12):1347–55.
38. Sweetser MT, Woodworth J, Swan S, Ticho B. Results of a randomized open-label crossover study of the bioequivalence of subcutaneous *versus* intramuscular administration of alefacept. Dermatol Online J. 2006;12(3):1.
39. Xu ZH, Wang QM, Zhuang YL, Frederick B, Yan H, Marini JC, *et al*. Bioavailability of golimumab, an anti-tumor necrosis factor-α human monoclonal antibody, administered subcutaneously at three different injection sites in healthy subjects. Clin Pharmacol Ther. 2009;85(S1):S24.
40. McDonald TA, Zepeda ML, Tomlinson MJ, Bee WH, Ivens IA. Subcutaneous administration of biotherapeutics: current experience in animal models. Curr Opin Mol Ther. 2010;12 (4):461–70.