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Effect of high mannose glycan pairing on IgG antibody clearance

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ABSTRACT

IgG antibodies contain N-linked glycans on the Fc portion of each heavy chain. The glycan on one heavy chain can either match the glycan on the other heavy chain (symmetrical pairing) or be different (asymmetrical pairing). These Fc glycans influence effector functions and can alter clearance rates. Previous studies showing that high mannose forms result in faster mAb clearance in humans were incapable of differentiating the impact of symmetrically vs. asymmetrically paired HM forms, and, therefore, the effect of pairing on clearance was not clear. Traditional analytical methods, which are used to measure glycans in such studies, do not determine the number of HM glycans per antibody. With a sensitive method designed to measure HM pairing, we followed the levels of symmetrically and asymmetrically paired HM on antibodies in human pharmacokinetic serum samples to determine the impact of Fc HM glycan pairing on therapeutic human IgG clearance in humans. The two HM paired forms cleared at the same rate, indicating that the effect on clearance was not proportional to the degree of modification. Since both forms can exist on therapeutic antibodies and the ratio can differ between products, measuring their relative levels is necessary to properly estimate effects on clearance.

1. Introduction

Antibodies play a central role in immune function and are widely used as biotherapeutics. For therapy, developers take advantage of the exquisite selectivity of antibodies to produce recombinantly engineered drugs that bind only to the desired physiological targets. Other endogenous activities, such as antibody dependent cellular cytotoxicity (ADCC), are exploited in therapeutic antibodies to eliminate targeted cells, such as for cancer therapy [1].

Certain activities, such as ADCC, may be altered by the N-linked glycan structures on the Fc portion of antibodies [2]. IgG1, the most common subtype of human IgG antibodies, requires a glycan attached at Asn 297 in the Fc domain for ADCC. Subtle changes in the Fc glycan structure can greatly affect ADCC activity. For example, removal of a single fucose residue from the complex glycan structure increases ADCC activity 50–200 fold by enhancing binding to FcγRIIIa receptors on natural killer cells [3–5]. Glycans can also affect therapeutic efficacy of antibodies by altering serum clearance. A HM type glycan in the Fc domain increases IgG clearance rates in humans compared to IgG with complex glycans or those lacking glycans, due to cellular uptake via the mannose receptor [6,7].

Since antibodies have two heavy chains and two light chains, effects exerted by attributes on efficacy could differ depending on whether they are found on only one or both chains. On EPO, a small glycoprotein, the impact of sialylation on clearance is proportional to the degree of modification [8]. Oxidation of Fc methionines is predicted to be more impactful on antibody clearance when both heavy chains are modified than when only one is [9]. In these two examples, the effect on clearance is proportional to the degree of change per protein. Alternatively, the impact of an attribute could be binary, i.e. independent of the degree of modification on the molecule. Whether the HM effect on antibody clearance is proportional or independent of the number of modified heavy chains is unknown. In the absence of such knowledge, we have assumed that a molecule possessing one HC with HM and one complex glycan (asymmetrically paired) clears at the same rate as a molecule containing HM on both heavy chains (symmetrically paired) [6]. This “worst case” modeling avoids an underestimation of the impact of HM on antibody clearance.

Abbreviations: HM, high mannose type glycan; M5 (or M#), high mannose glycan 5 (or other number); CO, complex type glycan; mAb, monoclonal antibody; ADCC, antibody-dependent cell-mediated cytotoxicity.

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Most analytical methods used to measure the relative levels of glycan forms, such as glycan maps or peptide maps, determine the percentage of each glycan form relative to the total glycan amount, but do not determine the fraction of antibodies containing a particular form. This distinction is only important in cases where glycans on the two heavy chains pair asymmetrically. Fc glycan pairing is illustrated for an antibody containing an overall level of 20% HM in Fig. 1. With symmetrical pairing, the same glycan form exists on both heavy chains within a single antibody. In the example shown with HM glycan undergoing only symmetrical pairing (HM:HM), the percentage of HM (as determined by glycan mapping or peptide mapping) matches the percentage of antibodies containing HM. Antibodies with only asymmetrical pairing of HM (HM:CO) would have HM on one heavy chain and a different type of glycan (such as complex, CO) on the other heavy chain. In that case, the percentage of antibodies containing HM would be double the percentage of overall HM. Mixtures of asymmetric and symmetric HM pairing might also exist in an antibody sample [10]. Percentages of antibodies containing HM would be greater than the overall HM level but less than that found with completely asymmetrical pairing. The example shown is for random pairing, where the glycan processing on one heavy chain is completely independent of the other chain. In this case, the percentages of the asymmetrical and symmetrical pairing would follow a binomial distribution.

If a functional effect of a glycan is only dependent on its presence in one heavy chain, then the impact could be underestimated by measuring overall HM levels. Therapeutic antibodies obtained from expression in mammalian tissue culture cells can contain low levels of HM, from 2 to 12% overall [11]. Conclusions drawn about Fc glycan function are typically associated with the overall glycan percentage and not with the fraction of antibodies containing that form. For example, in published work demonstrating that antibodies with HM clear faster than those without, glycan levels were analyzed by peptide mapping, which does not determine the percentage of antibodies containing the HM glycan [6]. To ascertain whether this analytical gap is significant, both the degree of asymmetrical pairing and whether this pairing affects a given biological parameter must be determined. In this study we describe assays that were developed to measure and test the degree of HM pairing in therapeutic antibody samples and determine whether this pairing affects antibody clearance in human subjects.

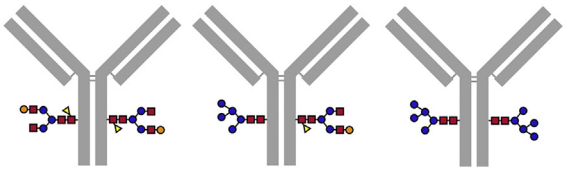
2. Materials and methods

2.1. Materials

Recombinant human monoclonal antibodies, mAbA (IgG1), mAbB (IgG2), and mAbC (IgG2), were expressed in Chinese hamster ovary (CHO) cells and purified by standard manufacturing procedures. Actigel ALD Superflow and sodium cyanoborohydride ($NaCNBH_3$) were purchased from Sterogene Bioseparations. Endoglycosidase S (EndoS) from Streptococcus pyrogenes (IgGZero™) was from Genovis AB (Cambridge, MA). Papain was obtained from Roche (Indianapolis, IN). Trifluoroacetic Acid (TFA) was from Pierce. Dithiothreitol (DTT) and Copper II Chloride ($CuCl_2$) were from Sigma–Aldrich (St. Louis, MO). Phosphate buffered Saline (PBS, pH7.4) was from Invitrogen Corp (Grand Island, NY). All organic solvents were analytical or HPLC grade.

2.2. Preparation of mAbA from human pharmacokinetic (PK) samples

MAbA was administered to adult human patients in a single intravenous injection. Blood samples were collected over several weeks at specific time points. After allowing time to clot, the clot was separated from serum by centrifugation. MAbA concentrations in serum were determined with a sandwich ELISA assay. Each 0.5 mL serum sample was incubated with 1000 U of EndoS at 37 °C for 4 h to cleave complex N-glycans on the Fc domain of IgGs. Afterwards, mAbA was extracted from the treated serum through ligand affinity [12]. Briefly, each serum sample was mixed with 4 mL PBS and 0.2 mL of mAbA-ligand resin. The mixture was rocked at room temperature for 4 h. The mixture was then centrifuged at 350 × g for 5 min to pellet the resin. The supernatant was discarded and the resin containing bound mAbA was gently washed 3 times each with 5 mL of PBS containing 0.5 mL NaCl. After washing the resin once with 200 μL digestion buffer (0.1 M Tris–HCl, 4 mM EDTA, 5 mM cysteine, pH 7.6), the resin with bound mAbA was incubated in 100 μL digestion buffer containing papain. The mabA to enzyme ratio was 10 to 1 (w/w). The digestion proceeded at ambient temperature for overnight. After digestion, the supernatant containing the Fc region was removed and incubated for approximately 4 h at 4 °C. To fragment IgG2 molecules, such as mAbB and mAbC, additional reducing agent (1 mM DTT) was added to improve the digestion [13]. Following digestion, 2 mM $CuCl_2$ was added and the sample was incubated at 4 °C for >4 h to re-oxidize any disulfide bonds



20% HM	CO:CO	HM:CO	HM:HM	Species with HM
Random Pairing	64%	32%	4%	36%
Symmetrical Pairing	80%	0%	20%	20%
Asymmetrical Pairing	60%	40%	0%	40%

Fig. 1. Fc glycan pairing preferences. Figure represents IgG antibodies with different glycan combinations. The structure on the left has complex glycans (CO) on both heavy chains (CO:CO). The middle structure has a complex glycan on one heavy chain and a HM (HM) structure on the other (HM:CO). The structure on the right has HM on both heavy chains (HM:HM). Values shown represent levels of each of these forms that would be present if 20% of the Fc glycans were HM and the distribution on the molecules combined in different ways.