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Quantitative *in vivo* comparisons of the Fcγ receptor-dependent agonist activities of different fucosylation variants of an immunoglobulin G antibody

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Abstract

Although it has been shown that functions of immunoglobulin G (IgG) antibodies (Abs) that depend on binding to certain Fcγ receptors (FcγR) can be influenced by Fc glycan fucosylation, quantitative *in vivo* analyses comparing the effects of different levels of fucose are still lacking. We used a simple mouse model to compare FcγR-dependent T cell activation induced by different fucosylation variants of a hamster/human IgG1 chimeric version of anti-mouse CD3 monoclonal Ab, 145-2C11 (2C11). Initial studies supported the expectation that this agonist activity by 2C11 was a reflection of FcγR binding, including comparisons of human IgG1 and IgG4 variants of 2C11 that showed the IgG4 to be dramatically less active at inducing T cell activation. Dose–response analyses in mice then showed that a sample of the human IgG1 version of 2C11 Ab in which 40% of the Fc glycans in the population of Ab molecules were fucosylated was 3–5 times more potent than a sample with 90% of its Fc glycans fucosylated. A sample with 10% fucosylation showed the same activity as the 40% fucosylated sample, revealing that complete absence of fucose was not necessary to achieve maximal Fc function in this model. *In vitro* binding to recombinant mouse FcγRs by the 2C11 variants revealed interesting relationships between fucose content and receptor affinity, and suggested the involvement of FcγRIV in mediating 2C11 activity *in vivo*. These analyses showed that low-fucose human IgG1 Abs indeed show greater FcγR-dependent activities in mice, but that Abs with moderate levels of fucose may be just as potent as Abs with very low or no fucose.

Keywords: Immunoglobulin G; Fucosylation; Fcγ receptors; T cell activation; anti-CD3 antibody

1. Introduction

Several groups have reported recent progress in improving immune effector functions of human IgG Abs by re-engineering either the amino acid sequences or the glycan structures in their Fc domains [1–6]. These efforts were accelerated after it was reported that cancer patients who expressed a polymorphic variant of Fcγ receptor (FcγR) IIIa known to have greater affinity for IgG showed better clinical responses to rituximab, a mouse/human IgG1 chimeric Ab against CD20 [7,8]. Those results offered evidence that Ab variants with enhanced affinity for FcγRIIIa would be more efficacious in some applications. The *in vitro* functional evaluations of engineered IgG variants associated with

Abbreviations: 2C11, 145-2C11; Ab, antibody; FcγR, Fcγ receptor; ADCC, Ab-dependent cellular cytotoxicity.

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such optimization efforts are not particularly cumbersome, but *in vivo* evaluations are typically lengthy, labor-intensive, and may be costly. In addition, because there is often little known about which FcγRs are relevant to the mechanism of action for a given Ab, *in vivo* analyses may be more meaningful for evaluating IgG variants than *in vitro* studies performed with a restricted number of FcγRs. For these reasons, and because there may be many different IgG structural variants that one may wish to evaluate for Fc function in rodents, we sought to establish a quick and non-labor-intensive means of studying *in vivo* interactions between IgG Abs and FcγRs.

Previous reports have shown that activation of CD3-positive T cells by monoclonal anti-CD3 Abs is dependent on the capacity of the anti-CD3 Abs to bind FcγRs [9–11]. The reasons for the dependence on FcγR binding are not completely clear, but presumably have to do with enabling more efficient clustering of CD3 molecules on the T cell surface, creating a higher avidity of the Ab for CD3 as a result of simultaneous Fc domain anchoring, and/or prolonging interactions between the T cell and neighboring FcγR-expressing cell. Regardless of the exact reasons, if the dependence is quantitative in the sense that increasing affinity for FcγR translates into increased levels of T cell activation, then it should be possible to exploit this agonist activity in an *in vivo* model for studying Fc–FcγR interactions.

An example of the type of question that can be addressed in such a model is to what extent mice show differential FcγR-dependent responses to different human IgG isotypes, such as IgG1 and IgG4. This is a question that has invited some confusion over the years, since very different results have been reported using different experimental systems. Although *in vitro* assays have more often shown human IgG1 versions of a particular Ab to be far superior to their human IgG4 versions at triggering Ab-dependent cellular cytotoxicity (ADCC) [12], there are also published *in vitro* ADCC data in which human IgG4 was about as active as IgG1 [10,13]. Even *in vivo* studies have shown results ranging from the IgG1 version of an anti-tumor Ab having much greater anti-tumor activity than the IgG4 version of the same Ab [14], to an IgG4 version of an anti-tumor Ab being nearly as active as the IgG1 version of the same Ab [13], to an IgG4 version of an anti-T cell Ab being just as active at mediating clearance of T cells as an IgG1 version of the same Ab [15]. Evidence suggests that the different results are at least in part a reflection of different FcγRs being involved with the particular activity being measured.

Another question that could be addressed is whether mice respond differently to IgG glycoform variants. With the recent development of Fc glycan-related technologies that enhance Fc function of Abs [4–6,16,17], an important question becomes whether FcγR-dependent activities of glycoform variants in mice parallel what has been observed using human FcγR-bearing effector cells. Human IgG1 Abs with low levels of fucose in their Fc glycans have been reported to have greater affinity for human FcγRIIIa and dramatically enhanced *in vitro* activity in ADCC assays using human PBMC effector cells [1,4,6]. However, following reports that the affinity of such low-fucose Abs for mouse FcγRIII and FcγRII was no higher than that of high-fucose Abs [5], there was seemingly less incentive to study low-fucose Abs in mice. Nevertheless, Niwa et al. [18] compared the anti-tumor activity in mice of a high-fucose and a low-fucose version of a chimeric human IgG1 Ab against CC chemokine receptor 4 and observed that the low-fucose variant appeared to be more potent than the high-fucose variant, even in the absence of endogenous mouse NK cells. A possible reason is the recently-discovered mouse FcγR referred to as CD16-2 by Mechetina et al. [19] and as FcγRIV by Nimmerjahn et al. [20]. The extracellular domain of this receptor has significantly higher sequence identity to human FcγRIIIa than those of the previously-studied mouse FcγRIII receptor, and hence may be hypothesized to be sensitive to IgG fucose levels for that reason alone. The sensitivity of this receptor to mouse IgG fucosylation was recently confirmed [21]. In addition, it was reported that mouse FcγRII, a receptor that mediates inhibiting signals, also shows enhanced binding to low-fucose variants of mouse IgG2a and IgG2b Abs [21], an observation subsequently reported for a human IgG1 Ab binding to human FcγRIIb and mouse FcγRII [22]. Although both groups demonstrated that *in vitro* binding correlated with Fc functions *in vivo*, their studies compared just single dose levels in mice and only high- and low-fucose variants, which leaves open the question of the nature of the quantitative relationship between fucose content and Fc functionality.

We sought to evaluate in a quantitative manner how the level of Fc glycan fucosylation impacts Fc functionality of a human IgG1 Ab in mice using a model that offers timely results.

2. Materials and methods

2.1. Materials

A plasmid encoding a single-chain Fv version of hamster anti-mouse CD3 ε-chain Ab, 145-2C11 (2C11) was kindly provided by Dr. Jeffrey Bluestone (University of California, San Francisco). The heavy and light chain variable region coding sequences in this plasmid were previously PCR-