The Carbohydrate at FcγRIIIa Asn-162
An Element Required for High Affinity Binding to Non-Fucosylated IgG Glycoforms

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FcγRIIIa plays a prominent role in the elimination of tumor cells by antibody-based cancer therapies. Non-fucosylated bisected IgGs bind this receptor with increased affinity and trigger FcγRIII-mediated effector functions more efficiently than native, fucosylated antibodies. In this study the contribution of the carbohydrates of both binding partners to the strength of the complex was analyzed. Glycoengineering of the antibody increased affinity for two polymorphic forms of soluble human FcγRIIIa (by up to 50-fold) but did not affect binding to the inhibitory FcγRIIB receptor. While the absence of carbohydrate at FcγRIIIa’s Asn-162 increased affinity for native IgG, presumably due to the removal of steric hindrance caused by the bulky sugars, it unexpectedly reduced affinity for glycoengineered (GE) antibodies by over one order of magnitude, bringing the affinity down to the same level as for native IgG. We conclude that the high affinity between GE antibodies and FcγRIII is mediated by productive interactions formed between the receptor carbohydrate attached at Asn-162 and regions of the Fc that are only accessible when it is nonfucosylated. As FcγRIIa and FcγRIIB are the only human Fcγ receptors glycosylated at this position, the proposed interactions explain the observed selective affinity increase of GE antibodies for only these receptors. Furthermore, we predict from our structural model that only one of the two Fc-fucose residues needs to be absent for increased binding affinity toward FcγRIII. This information can be exploited for the design of new antibodies with altered Fc receptor binding affinity and enhanced therapeutic potential.

Antibodies provide a link between the humoral and the cellular immune system with IgG being the most abundant serum immunoglobulin. While the Fab region antibodies recognize antigens, the Fc part interacts with membrane-bound Fcγ receptors (FcγRs) that are differentially expressed by all immune competent cells. Receptor crosslinking by a multivalent antigen-antibody complex triggers degranulation, cytolysis or phagocytosis of the target cell, and transcriptional activation of cytokine-encoding genes (1).

Recently, the importance of the activating receptor FcγRIIIa for the in vivo elimination of tumor cells in humans has been demonstrated. In follicular non-Hodgkin's lymphoma patients, a relationship was discovered between the FcγRIIIa genotype and clinical and molecular responses to rituximab, an anti-CD20 chimeric antibody used against hematological malignancies (2). The authors demonstrated that the efficacy of rituximab was higher in patients homozygous for the "high affinity" FcγRIIIa, characterized by a valine at position 158 (FcγRIIIa[Val-158]), than in patients heterozygous or homozygous for the "low affinity" FcγRIIIa, which has a phenylalanine residue at this position (FcγRIIIa[Phe-158]) and has lower affinity for IgG (3). Increased survival of lymphoma patients that mount an anti-tumor humoral response after anti-idiotypic vaccination has also been correlated with homogygocity for FcγRIIIa[Val-158] (4).

The above observations imply a crucial role for FcγRIIIa in the elimination of tumor cells and support the idea that therapeutic monoclonal antibodies (mAbs) with increased affinity for FcγRIIIa will have improved biological activity. One route to increase the affinity of monoclonal antibodies toward FcγRIII and consequently to enhance their effector functions is manipulation of their carbohydrate moiety (5–7). The N-glycosylation of the Fc fragment at Asn-297 in both Cγ2 domains is crucial to the affinity for all FcγRs (8, 9) and is required to elicit proper effector functions (10, 11). It is comprised of a conserved pentasaccharide structure with variable addition of fucose and outer arm sugars (12). The N-glycosylation pattern of mAbs can be manipulated by engineering the glycosylation pathway of the production cell line using enzyme activities that lead to naturally occurring carbohydrates. Umaña and co-workers (5, 7) reported the production of glycoengineered (GE) antibodies, which feature high proportions of bisected, non-fucosylated oligosaccharides, improved affinity for FcγRIIIa and enhanced antibody-dependent cellular cytotoxicity. Antibodies with increased binding to FcγRIIIa have also been obtained using a cell line which is unable to add fucose residues to N-linked oligosaccharides (6, 13).

Little information is available on the influence of FcγRIIIa carbohydrates on the affinity for IgG. The crystal structure of unglycosylated FcγRIII in complex with the Fc fragment of human (h) IgG1 indicates that a carbohydrate moiety attached at Asn-162 of FcγRIII would point into the central cavity within the Fc fragment (14), where the rigid core glycans attached to IgG-Asn-297 are also located (15). In the present study, binding of glycosylated soluble (s) hFcγRIIa variants to distinct antibody glycovariants was evaluated by surface plasmon resonance (SPR) and in a cellular system to dissect the interaction between IgG1 and glycosylated FcγRIIIa on a molecular level.

EXPERIMENTAL PROCEDURES

Cell Lines, Expression Vectors, and Antibodies—HEK293-EBNA cells were a kind gift from Rene Fischer (Laboratory of Organic Chemistry, Zürich, Switzerland). Additional cell lines used in this study were Jurkat cells (human lymphoblastic T cell, ATCC number TIB-152) and FcγRIIIa[Val-158]– and FcγRIIIa[Val-158]/Gln-162–expressing Jurkat cell lines, generated as described previously (7). The cells were cultivated according to the instructions of the supplier. DNA encoding the
shFcYRIIa[Val-158] and shFcYRIIa[Phe-158] variants were fused after residue 191 to a hexahistidine tag (NH₂-MRTEDL... GYQG(H₆)-COOH, numbering is based on the mature protein) using PCR as described (16). Asn-162 of shFcYRIIa[Val-158] was exchanged for Gln by PCR. All expression vectors contained the replication origin oriP from the Epstein-Barr virus for expression in HEK293-EBNA cells. GE and native anti-CD20 antibodies were produced in HEK-293 EBNA cells and characterized by standard methods. Neutral oligosaccharide profiles for the antibodies were analyzed by mass spectrometry (Autoflex, Bruker Daltonics GmbH, Faellanden, Switzerland) in positive ion mode (17).

Production and Purification of Recombinant shFcYRIIa Receptors—The shFcYRIIa variants were produced by transient expression in HEK-293-EBNA cells (18) and purified using a HitTrap Chelating HP column (Amersham Biosciences, Otelfingen, Switzerland) and a size exclusion chromatography step with HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20). Human sFcYRIIb and mouse (m) sFcYRIIb were produced and purified as described (19). The concentration of proteins was determined as described (20).

SPR—SPR experiments were performed on a Biacore3000 with HBS-EP as running buffer (Biacore, Freiburg, Germany). Direct coupling of around 1,000 resonance units of human IgG glycovariants was performed on a CM5 chip using the standard amine coupling kit (Biacore). Different concentrations of soluble FcRs were passed with a flow rate of 10 µl/min through the flow cells. Increasing the flow rate did not influence the binding curves. Bulk refractive index differences were corrected for by subtracting the response obtained on flowing sample over a bovine serum albumin-coupled surface. The steady state response was used to obtain the dissociation constant Kᵤ by non-linear curve fitting of the Langmuir binding isotherm. Kinetic constants were obtained using the BlAcore evaluation program curve-fitting facility (v3.0, Biacore), to fit rate equations for 1:1 Langmuir binding by numerical integration.

Binding of IgG to FcYRIIa-expressing Cells—The experiment was conducted as described previously (7). Briefly, hFcYRIIa-expressing Jurkat cells were incubated with IgG variants in phosphate-buffered saline, 0.1% bovine serum albumin. After two washes with phosphate-buffered saline, 0.1% bovine serum albumin, antibody binding was detected by incubating with 1:200 fluorescein isothiocyanate-conjugated anti-human F(ab')₂ fragments (Jackson ImmunoResearch, West Grove, PA) (16). The fluorescence intensity of the bound antibody variants was determined on a FACS Calibur (BD Biosciences, Allschwil, Switzerland).

Modeling—We visualized the interaction of the Fc fragment derived from native IgG and the FcYRII glycans after creating a carbohydrate in silico, attached at the position Asn-162 of the receptor. The glycan unit was modeled on to the crystal structure of FcYRII in complex with Fc-IgG (Protein Data Bank code 1e4k). The interaction between FcYRII and IgG was modeled by directing the Fc-linked pentasaccharide core to the fucose residue of oligosaccharide linked to the Fc-Asn-297. The model was not energy minimized and only created to visualize the proposed binding mode.

RESULTS

Biochemical Characterization of Soluble hFcYRIIa Receptors and Antibody Glycovariants—ShFcYRIIa[Val-158], shFcYRIIa[Phe-158], and shFcYRIIa[Val-158/Gln-162] were expressed in HEK293-EBNA cells and purified to homogeneity. The purified shFcYRIIa[Val-158] and [Phe-158] migrate as broad bands in the apparent molecular weight range of 40–50 kDa when subjected to reducing SDS-PAGE. The apparent molecular weight is slightly lower for the mutant shFcYRIIa[Val-158/Gln-162] (data not shown). This can be explained by the elimination of the carbohydrates linked to Asn-162. Upon enzymatic N-deglycosylation all three receptor variants migrate identically in the apparent molecular weight range of 25–30 kDa and feature three bands as observed previously for the membrane form of FcYRII (21, 22). This heterogeneous pattern may result from the presence of O-linked carbohydrates.

The native antibody glycosylation pattern is characterized by biantennary, fucosylated complex oligosaccharides (Fig. 1, b and c), heterogeneous with respect to terminal galactose content. GE-hlgG1 antibodies were produced in a cell line overexpressing β1,4-N-acetylgalactosaminyltransferase III (GnT-III), an enzyme catalyzing the addition of a bisecting GlcNAc (Fig. 1a) to the β-mannose of the core. Two different GE antibody variants were generated: Glyco-1 was produced by

FIGURE 1. Oligosaccharide characterization of GE and native antibodies. a, carbohydrate moeity attached to Asn-297 of human IgG1-Fc. The sugars in bold define the pentasaccharide core; the addition of the other sugar residues is variable. The bisecting β1,4-linked GlcNAc residue is introduced by GnT-III. b, MALDI-MS spectra of neutral oligosaccharides released from native and GE antibodies. The m/z value corresponds to the carbohydrate type of the antibodies. c, oligosaccharide distributions of the IgG glycovariants used in this study.
overexpression of GnT-III alone and Glyco-2 by co-expression of GnT-III and recombinant Man-II (Ref. 7 and Fig. 1b). Both Glyco-1 and Glyco-2 feature high proportions of bisected, non-fucosylated oligosaccharides (92 and 84%, respectively; Fig. 1c). We have previously shown that both forms give similar increases in affinity for FcγRIIIa and increased antibody-dependent cellular cytotoxicity relative to native IgG1 but differ in their reactivity in complement-dependent cytotoxicity assays (7).

**IgG Oligosaccharide Modifications Lead to Antibodies with Increased Affinity for shFcγRIIIa**—The interaction of antibody glycovariants with shFcγRIIIa variants ([Val-158], [Phe-158], and [Val-158/Gln-162]) was analyzed by SPR. Binding of shFcγRIIIa[Val-158] to the GE antibodies was up to 50-fold stronger than to the native antibody (KD(Glyco-2) 0.015 μM versus KD(native) 0.75 μM, Table 1). The low affinity polymorphic form of the receptor, shFcγRIIIa[Phe-158], also bound to the GE antibodies with significantly higher affinity than to the native antibody (KD(Glyco-1) 0.27 μM (18-fold), KD(shFcγRIIIa) 0.18 μM (27-fold), KD(native) 5 μM (Table 1)).

Although the dissociation of both receptor variants from native IgG was too fast to enable a direct determination of kinetic constants for these interactions, overlaying the experimental data clearly shows that a major effect of glycoengineering the antibodies is decreased dissociation from the receptors (Fig. 2a). To estimate dissociation rates from native IgG dissociation curves were simulated using different rate constants and compared with the experimental data (data not shown). These calculations indicated that the entire increase in affinity upon glycoengineering could be accounted for by decreased dissociation rate constant (k<sub>off</sub>).

The association rate constants (k<sub>on</sub>) values of the two polymorphic forms of shFcγRIIIa for GE antibodies were similar, but the dissociation rate of sFcγRIIIa[Phe-158] was significantly faster and largely accounts for the lower affinity of this receptor (Table 1).

The affinity of the antibodies for human and murine FcγRIIb was also measured. GE and native IgGs bound the human inhibitory receptor shFcγRIIb with similar affinity (K<sub>D</sub> = 1.6–2.4 μM, Table 1). For the murine version of this receptor the affinity for human IgG1 was also unaltered by antibody glycoengineering, but surprisingly was 3.4–5.5 times that of the human FcγRIIb receptor (Table 1). The dissociation constant (K<sub>D</sub>) for the interaction of the native antibody with sh/mFcγRIIb could only be determined by steady state analysis (Table 1) because the equilibrium was attained too quickly for a kinetic evaluation (Fig. 2a).

**FcγRIIIa Glycosylation Regulates Binding to Antibody Glycovariants**—A mutant form of hFcγRIIIa that is not glycosylated at Asn-162 (shFcγRIIIa[Val-158/Gln-162]) was used to analyze the influence of

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**TABLE 1**

Summary of affinity constants determined by equilibrium and kinetic analysis

| IgG1   | Fcγ receptor       | K<sub>D</sub> (μM) | K<sub>off</sub> (s<sup>-1</sup>) | K<sub>on</sub> (μM<sup>-1</sup>s<sup>1/2</sup>) |
|--------|--------------------|--------------------|---------------------------------|---------------------------------|
| Native | shFcγRIIIa[Val-158]| ND<sup>a</sup>     | ND<sup>a</sup>                  | ND<sup>a</sup>                  |
| Glyco-1| shFcγRIIIa[Val-158]| 2.4 ± 0.01         | 5.8 ± 0.01                     | 0.024 ± 0.001                  |
| Glyco-2| shFcγRIIIa[Val-158]| 3.2 ± 0.01         | 5.1 ± 0.01                     | 0.016 ± 0.001                  |
| Native | shFcγRIIIa[Phe-158]| ND<sup>a</sup>     | ND<sup>a</sup>                  | ND<sup>a</sup>                  |
| Glyco-1| shFcγRIIIa[Phe-158]| 1.6 ± 0.09         | 32.1 ± 0.1                     | 0.20 ± 0.001                   |
| Glyco-2| shFcγRIIIa[Phe-158]| 2.3 ± 0.01         | 29.1 ± 0.1                     | 0.13 ± 0.001                   |
| Native | shFcγRIIIa[Val-158/Gln-162]| 5.9 ± 0.05     | 90 ± 0.4                      | 0.16 ± 0.001                   |
| Glyco-1| shFcγRIIIa[Val-158/Gln-162]| 4.7 ± 0.02     | 89 ± 0.5                      | 0.19 ± 0.001                   |
| Glyco-2| shFcγRIIIa[Val-158/Gln-162]| 8.1 ± 0.06     | 72 ± 0.3                      | 0.09 ± 0.001                   |
| Native | shFcγRIIb          | ND<sup>a</sup>     | ND<sup>a</sup>                  | ND<sup>a</sup>                  |
| Glyco-1| shFcγRIIb          | ND<sup>a</sup>     | ND<sup>a</sup>                  | ND<sup>a</sup>                  |
| Glyco-2| shFcγRIIb          | ND<sup>a</sup>     | ND<sup>a</sup>                  | ND<sup>a</sup>                  |
| Native | smFcγRIIb          | ND<sup>a</sup>     | ND<sup>a</sup>                  | ND<sup>a</sup>                  |
| Glyco-1| smFcγRIIb          | ND<sup>a</sup>     | ND<sup>a</sup>                  | ND<sup>a</sup>                  |
| Glyco-2| smFcγRIIb          | ND<sup>a</sup>     | ND<sup>a</sup>                  | ND<sup>a</sup>                  |

<sup>a</sup> Kinetic too fast for exact determination.

<sup>b</sup> No duplicate.
the receptor's carbohydrate on complex formation with IgG. Interestingly, upon removal of N-glycosylation at Asn-162, native IgG showed a 3-fold increase ($K_d = 0.24$ versus $0.75 \mu M$) in affinity for the receptor, whereas GE antibodies showed an over 10-fold increase in affinity (Table 1). For binding to GE antibodies, removal of the receptor glycosylation site resulted in an almost 2-fold increase in $k_{on}$ but an over 14-fold increase in $k_{off}$ (Table 1). Steady state and kinetically determined $K_d$ values differed by 1.6–2.2-fold for binding of shFcγRIIIa[Val-158/Gln-162] to the antibodies. This discrepancy most likely results from a high error in fitting the very fast dissociation phase.

The SPR-based results were corroborated using Jurkat cells expressing membrane bound FcγRIIIa, which represents a natural environment for FcγRIIIa expression (23). We used the anti-FcγRIII mAb 3G8, which does not discriminate between FcγRIIIa[Val-158] and FcγRIIIa[Val-158/Gln-162] (24), to monitor FcγRIII expression in these cell lines. In this experiment GE antibodies bound FcγRIIIa[Val-158] better than the native antibody (Fig. 3c). Binding to FcγRIIIa[Val-158/Gln-162] was, however, significantly reduced for all IgG variants, including native IgG (Fig. 3c). The very fast dissociation rate constants found in the SPR experiment for binding of FcγRIIIa[Val-158/Gln-162] to all three IgG variants could explain the lower binding in the cellular assay.

**DISCUSSION**

**Kinetic Analysis of the FcγRIIIa/IgG Interaction**—Overall our measured $K_d$ values for the interaction of IgG1 with glycosylated FcγRIIIa agree with those previously published by Okazaki et al. (25). These authors concluded that the affinity increase of the non-fucosylated (GE) antibody is predominantly caused by an increase in $k_{on}$. In contrast, although we could not quantify $k_{on}$ and $k_{off}$ for binding to native IgG due to the high velocity of the reaction, comparison of the binding curves for native and GE antibodies clearly shows significantly faster dissociation of the receptor variants from native IgG (Fig. 2a). We conclude that upon antibody glycoengineering either new interactions between the binding partners are formed or the present ones are improved. Importantly, we showed that glycoengineered antibodies bind with significantly higher affinity to the more common low affinity variant of FcγRIIIa than native antibodies do to the less common high affinity variant of the receptor. This gives the hope of improving anti-cancer antibody therapies for people with this allelic variant.

**The Glycosylation of FcγRIIIa at Asn-162 Modulates Binding to Antibodies**—FcγRIIIa of mammalian origin is a highly glycosylated protein with five N-linked glycosylation sites. From the crystal structure of IgG1-Fc in complex with unglycosylated FcγRIII (14), glycosylation at Asn-162 in FcγRIII has been hypothesized to reduce affinity for native IgG due to steric hindrance exerted by the hFcγRIIIa[Asn-162] carbohydrate moiety. This has been confirmed with the appropriate glycosylation mutant of FcγRIII, while removal of carbohydrates at the other four N-glycosylation sites did not affect affinity for native IgG (24).
High Affinity IgG Binding by FcγRIIa

To further investigate the importance of glycosylation of IgG and FcγRIIa for their interaction, a mutant version of the high affinity receptor which is unglycosylated at position 162 (shFcγRIIa[Val-158/Gln-162]) was constructed. As expected, removal of the carbohydrate at Asn-162 of the receptor increased binding affinity for the native antibody (3-fold, Table 1). On the other hand, removal of the FcγRIIa’s carbohydrate at Asn-162 unexpectedly led to reduced binding affinity for GE antibodies by over an order of magnitude, bringing the affinity down to the level observed for the native antibody. The data were corroborated in a cellular assay system, where GE antibodies bound significantly better to hFcγRIIa[Val-158]- than to hFcγRIIa[Val-158/Gln-162]-expressing cells (Fig. 3c).

In summary, two requirements have to be met for high affinity interaction between GE IgG and FcγRIIa: a carbohydrate has to be attached at FcγRIIa’s Asn-162, and productive contacts of this receptor carbohydrate with the IgG-Fc can only be made if the latter is non-fucosylated. Based on these results we propose a model in which the Asn-162-linked carbohydrate of FcγRIIa contacts a region of the IgG-Fc where a fucose residue is attached in native antibodies. This fucose residue protrudes from the continuous surface of the Fc into open space and may prohibit a close approach of the Fc receptor carbohydrate core, thereby precluding additional productive interactions (Fig. 4). It should be noted that a complete overlap with the mentioned Fc region is attained by a receptor carbohydrate with as few three monosaccharide units (Fig. 4). Furthermore, the model predicts that only one of the two Fc-fucose residues needs to be absent for increased binding affinity toward FcγRIIa.

In a recent study Okazaki et al. (25) proposed that non-fucosylated antibodies bind FcγRIIa with increased affinity as a result of a newly formed bond between Tyr-296 of the Fc and Lys-128 of the FcγRIIa. However, we found that the increased affinity of non-fucosylated antibodies depends on glycosylation of the receptor which implies that an IgG-Fc[Tyr-296/FcγRIIa[Lys-128]-bond is insignificant to the affinity between GE antibodies and FcγRIIa.

FcγRIIa and FcγRIIib forms are the only forms of the human FcγRs that possess N-glycosylation sites within the binding region to IgG. We therefore conclude that affinity for IgG will be influenced by receptor glycosylation only for these two FcγRs. Comparison of the amino acid sequences of FcγRII from other species indicates that the N-glycosylation site Asn-162 is shared by FcγRII from macaca, cat, cow, and pig, whereas it is lacking in the known rat and mouse FcγRII. Recently mouse (CD16-2) and rat (GenBank™ accession number AY219230) genes with high homology to the human FcγRIIa and which encode proteins containing the Asn-162 glycosylation site were identified (26), and functional expression of the murine protein was recently reported (27). The presence of a FcγRIIa-Asn-162 glycosylation site may enable the immune system to tune the affinity toward FcγRII by differential FcγRII glycosylation (21) and by modulation of the fucose content of IgG.

The Immunological Balance between Activating and Inhibitory FcγRs—It has been proposed that an improvement in the ratio of activating to inhibitory signals should enhance the efficacy of therapeutic antibodies (28). In the current study, the inhibitory shFcγRIIb receptor was found to have a similar affinity for native and GE antibodies (Table 1). The inhibitory receptors sFcγRIIbs from mouse and human are not glycosylated at Asn-162. The lack of discrimination for GE antibodies displayed by FcγRIIb is consistent with glycosylation of activating FcγRII at Asn-162 being essential for increased binding to non-fucosylated IgGs and suggests that these GE antibodies could show enhanced therapeutic efficacy.

The finding that murine FcγRII has significantly higher affinity than human FcγRIIb for both native and GE IgG1 may be important for the correct interpretation of in vivo experiments using mouse models. Enhanced binding to the inhibitory receptor in a mouse model may result in a different threshold of the immune response than that observed in humans.

CONCLUSION

We demonstrated the importance of the carbohydrate moieties of both FcγRII and IgG for their interaction. Our data provide further insight into the complex formation and identified an important interaction between the Asn-162 carbohydrate of FcγRII and the Fc of non-fucosylated IgG glycoforms. This finding should allow the design of new antibody variants that make further productive interactions with the carbohydrate of FcγRIIa, which may impact on future therapies with monoclonal antibodies.

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