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# Lack of Galactosylation Enhances the Pathogenic Activity of IgG1 but Not IgG2a Anti-Erythrocyte Autoantibodies

Kiyooki Ito,<sup>\*,†</sup> Jun-ichi Furukawa,<sup>‡</sup> Kazunori Yamada,<sup>\*,†</sup> Ngoc Lan Tran,<sup>\*</sup> Yasuro Shinohara,<sup>‡</sup> and Shozo Izui<sup>\*</sup>

IgG bears asparagine-linked oligosaccharide side chains in the Fc region. Variations in their extent of galactosylation and sialylation could modulate IgG Fc-dependent effector functions, and hence Ab activity. However, it has not yet been clarified whether the pathogenic potential of IgG autoantibodies is consistently enhanced by the absence of galactose residues per se or the lack of terminal sialylation, which is dependent on galactosylation. Moreover, it remains to be defined whether the increased pathogenicity of agalactosylated IgG is related to activation of the complement pathway by mannose-binding lectin, as suggested by in vitro studies. Using a murine model of autoimmune hemolytic anemia, we defined the contribution of galactosylation or sialylation to the pathogenic activity of IgG1 and IgG2a anti-erythrocyte class-switch variants of 34-3C monoclonal autoantibody. We generated their degalactosylated or highly sialylated glycovariants and compared their pathogenic effects with those of highly galactosylated or desialylated counterparts. Our results demonstrated that lack of galactosylation, but not sialylation, enhanced the pathogenic activity of 34-3C IgG1, but not IgG2a autoantibodies. Moreover, analysis of in vivo complement activation and of the pathogenic activity in mice deficient in C3 or IgG FcRs excluded the implication of mannose-binding lectin-mediated complement activation in the enhanced pathogenic effect of agalactosylated IgG1 anti-erythrocyte autoantibodies. *The Journal of Immunology*, 2014, 192: 581–588.

The CH2 domain of the IgG Fc portion carries asparagine-linked biantennary complex-type oligosaccharide side chains (*N*-glycans), which have been shown to be essential for Fc-dependent effector functions of IgG (1–3). These *N*-glycan structures are highly heterogeneous in terms of galactosylation and sialylation (4, 5). Most of them end with either two *N*-acetylglucosamines (i.e., agalactosylated G0 glycoform), one galactose and one *N*-acetylglucosamine (G1), or two galactose residues (G2), but a significant, although minor, fraction of galactosylated glycans bears one or two terminal sialic acids (S1 or S2). It has been shown that the proportion of IgG lacking galactose is significantly increased in patients with a variety of chronic inflammatory diseases, most notably rheumatoid arthritis (4, 6–10). Increased levels of agalactosylated IgG have been shown to correlate with disease severity and also have prognostic value.

More significantly, improvement of rheumatoid arthritis after anti-TNF therapy or during pregnancy was associated with decreases in levels of agalactosylated IgG (11–16). Notably, an enhanced arthritogenic activity of galactosidase-treated anti-collagen Abs has been reported in a murine model of collagen-induced arthritis (17). In contrast, studies with degalactosylated human IgG1 mAbs obtained through treatment with galactosidase failed to show enhanced Fc-dependent effector functions, as compared with their wild-type (WT) counterparts (18, 19). Moreover, studies on autoimmune-prone MRL-*Fas*<sup>lpr</sup> mice displaying the aberrant IgG galactosylation (5, 20) revealed that increases of agalactosylated IgG occurred independently of disease activity (21). Thus, it has been unclear whether the pathogenic potential of IgG autoantibodies is consistently enhanced by the absence of galactosylation.

In addition, it remains to be defined what mechanism may lead to a possible enhancement of pathogenic activity of agalactosylated IgG. It has previously been suggested that agalactosylated IgG might more efficiently activate complement through the lectin pathway as a result of interaction of mannose-binding lectin (MBL) with *N*-acetylglucosamines exposed by the lack of galactosylation (22, 23). However, studies with anti-platelet monoclonal autoantibodies showed a minor role for MBL-dependent complement activation but a dominant one for IgG FcRs (FcγRs) in mediating the pathogenicity of degalactosylated IgG Abs (23). Nevertheless, the interpretation of these results was still tentative, because it has been shown that complement plays a minimal role in the pathogenesis of this model of immune thrombocytopenia (23, 24). Alternatively, because of a decreased interaction of highly sialylated IgG with FcγRs (25), it has been proposed that the enhanced pathogenicity of agalactosylated IgG could be attributed to their inability to undergo terminal sialylation, which is dependent on upstream galactosylation, rather than the lack of galactosylation (23, 26). However, it should be noted that the anti-inflammatory properties of highly sialylated IgG remain a controversial issue (19, 27–30). Notably, a more recent study reported that IgG galactosylation, but not sialylation,

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Abbreviations used in this article: AIHA, autoimmune hemolytic anemia; B6, C57BL/6; FcγR, IgG FcR; Ht, hematocrit; MALDI-TOF MS, MALDI-TOF mass spectrometry; MBL, mannose-binding lectin; *N*-glycan, asparagine-linked biantennary complex-type oligosaccharide side chain; SNA, *Sambucus nigra* agglutinin; WT, wild-type.

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was associated with the improvement of disease activity during pregnancy in patients with rheumatoid arthritis (16). Thus, it remains to be clarified whether it is the level of galactosylation of IgG autoantibodies per se or the level of terminal sialylation that modulates their pathogenic potential.

To better define the contribution of galactosylation and sialylation of *N*-glycans to the pathogenic potential of IgG autoantibodies, we have used a murine model of autoimmune hemolytic anemia (AIHA) induced by 34-3C IgG anti-RBC monoclonal autoantibodies, specific against erythrocyte Band 3 protein (31), established from autoimmune-prone NZB mice (32, 33). The previous analysis of IgG switch variants of 34-3C mAb demonstrated the remarkably different pathogenic potential of four IgG subclasses, as a function of their respective capacities to interact with different classes of Fc $\gamma$ R and to activate complement in vivo (33–35). Indeed, the IgG2a subclass of 34-3C mAb, which activates three different Fc $\gamma$ Rs (Fc $\gamma$ RI, Fc $\gamma$ RIII, and Fc $\gamma$ RIV) and complement, displayed the highest pathogenic activity, whereas its IgG1 variant capable of activating only Fc $\gamma$ RIII, but not complement, exhibited the weakest pathogenic potential (33, 36, 37). In this study, we generated degalactosylated or highly sialylated variants of 34-3C IgG1 and IgG2a mAbs, and compared their pathogenic potentials with those of highly galactosylated or desialylated counterparts. Our results demonstrated that the pathogenic activity of 34-3C IgG1, but not IgG2a anti-RBC autoantibodies was enhanced after degalactosylation, whereas the mere extent of sialylation had no effect on pathogenic potential. Moreover, we observed that agalactosylated IgG1 anti-RBC mAb failed to display complement activation in vivo, thereby arguing against the contribution of MBL-dependent complement activation to the enhanced pathogenic activity of degalactosylated IgG1 anti-RBC mAb.

## Materials and Methods

### Mice

C57BL/6 (B6) mice deficient in FcR  $\gamma$ -chains (i.e., lacking functional expression of Fc $\gamma$ RI, Fc $\gamma$ RIII, and Fc $\gamma$ RIV involved in erythrophagocytosis) were generated with B6-derived embryonic stem cells (38). C3-deficient mice, generated by gene targeting in 129-derived embryonic stem cells (39), were backcrossed for five generations on a B6 background, as described previously (33). B6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animal studies described in this report have been approved by the Ethical Committee for Animal Experimentation of the University of Geneva.

### Anti-RBC mAbs

Hybridoma-secreting 34-3C IgG2a anti-RBC mAb was established from unmanipulated NZB mice (32). The generation of a 34-3C IgG1 class-switch variant and a 34-3C IgG2a F243A mutant carrying phenylalanine instead of alanine at position 243 in the CH2 domain was previously described (33, 40). All the hybridoma and transfectoma cells were grown in DMEM supplemented with 1% Ultrosor HY (PALL Life Sciences, Cergy, France), and IgG mAbs were purified from culture supernatants by protein G column chromatography. The purity of IgG was >95%, as documented by SDS/PAGE.

### Experimental AIHA

AIHA was induced by a single i.v. injection of purified anti-RBC mAb into 2- to 3-mo-old mice. The injection of mAb was controlled by assessing the level of Ab opsonization of RBCs 24 h later using biotinylated rat anti-mouse *k*-chain mAb (H139.52.1.5), followed by PE-conjugated streptavidin, as described previously (35). The deposition of C3 on RBCs was determined with biotinylated goat anti-mouse C3 (Cappel Laboratories, Durham, NC), followed by PE-conjugated streptavidin (33). Hematocrit (Ht) values were determined after centrifugation of blood samples collected into heparinized microhematocrit tubes.

### Degalactosylation and desialylation of IgG mAb

34-3C IgG1 and IgG2a mAbs in 0.05 M sodium phosphate buffer (pH 6.0) were incubated with  $\beta$ 1,4-galactosidase (15 mU for 1 mg IgG) cloned from *Streptococcus pneumoniae* (EMD Chemicals, San Diego, CA) at 37°C for

48 h. 34-3C IgG2a F243A mutant in 0.05 M acetate buffer (pH 5.5) was incubated with  $\alpha$ 2,3/ $\alpha$ 2,6-neuraminidase (200 U for 1 mg IgG) cloned from *Clostridium perfringens* (New England Biolabs, Herts, U.K.) at 37°C for 48 h. Then galactosidase- or neuraminidase-treated IgG was dialyzed against PBS and purified by protein G column chromatography. As a control, mAbs were treated similarly in the absence of galactosidase or neuraminidase.

### Sambucus nigra agglutinin lectin affinity chromatography

To enrich sialylated 34-3C IgG2a F243A, we used *Sambucus nigra* agglutinin (SNA) lectin fractionation as described by Kaneko et al. (25). In brief, 30 mg 34-3C IgG2a F243A was dialyzed against TBS containing 0.1 mM CaCl<sub>2</sub> and applied to 2 ml of an agarose-bound SNA lectin column (Vector Laboratories, Burlingame, CA). The flow-through fraction was collected by washing the column with TBS containing 0.1 mM CaCl<sub>2</sub>. Then 34-3C IgG2a F243A bound to SNA lectin column was eluted in two steps using 0.5 M lactose in TBS, followed by 0.5 M lactose in 0.2 M acetic acid. All fractions were dialyzed against PBS.

### Analysis of oligosaccharide structures

Purification of *N*-glycans from different IgG mAbs was performed based on chemoselective glycoblotting technique, as described previously (40–42). In brief, IgG samples were reductively alkylated under the presence of detergent and then digested with trypsin and peptide *N*-glycosidase F (Roche Diagnostics, Penzberg, Germany) (43). The digested samples were mixed with a novel hydrazide-functionalized glycoblotting polymer (42), and sialic acids were methyl-esterified to render sialylated oligosaccharides chemically equivalent to neutral oligosaccharides (44). The IgG oligosaccharides recovered as derivatives of  $N\alpha$ -[amino-oxy]acetyltryptophanylarginine methyl ester were subjected to MALDI-TOF mass spectrometry (MALDI-TOF MS) using an Ultraflex II mass spectrometer (Bruker Daltonik, Bremen, Germany) controlled by the FlexControl 3.0 software package. The analytical procedure was proved to be reproducible with a coefficient of variation <15% by analysis of *N*-linked oligosaccharides prepared from normal human serum and from human IgG (42). Some glycan signals were further analyzed by TOF/TOF as described previously (45). Estimation of oligosaccharide structures was obtained by input of peak masses into the GlycoMod Tool (<http://au.expasy.org/tools/glycomod/>) or GlycoSuite Tool (<http://www.unicarbk.org/query>) and by referring literatures describing the *N*-glycan of IgG (25, 46).

### Statistical analysis

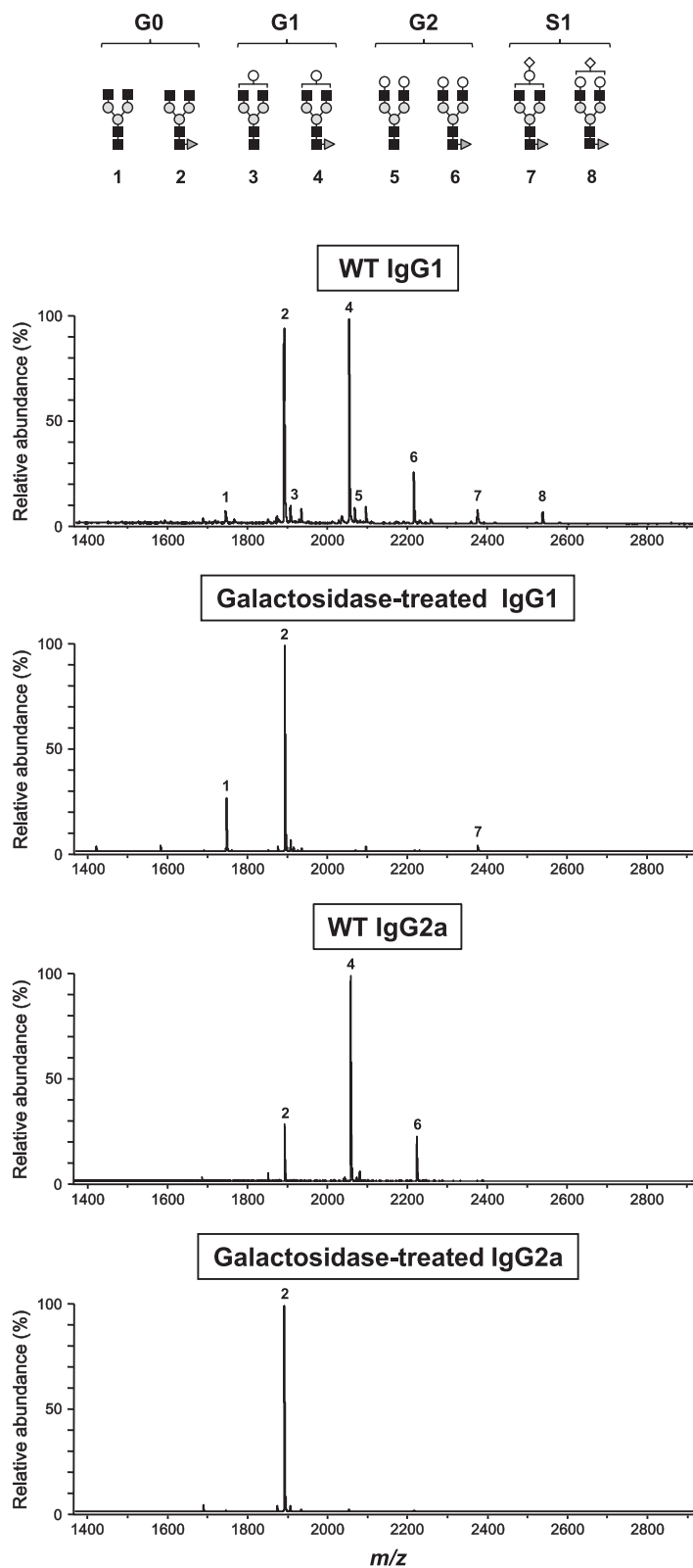
Unpaired comparison for Ht values was analyzed by Student *t* test. The *p* values <5% were considered significant.

## Results

### Development of more severe anemia after injection of degalactosylated 34-3C IgG1 glycovariant than WT 34-3C IgG1 mAb

To determine the contribution of galactosylation of IgG anti-RBC autoantibodies to the development of AIHA, a degalactosylated 34-3C IgG1 anti-RBC glycovariant was generated. Because this mAb was very poorly sialylated (40), we treated it directly with  $\beta$ 1,4-galactosidase in vitro without pretreatment with neuraminidase. When the extent of degalactosylation was controlled by MALDI-TOF MS analysis on *N*-glycans released from 34-3C IgG1, we observed that >95% of galactose residues were successfully removed after treatment with  $\beta$ 1,4-galactosidase (Fig. 1, Table I). Accordingly, the content of agalactosylated G0 glycoform amounted to 96.4%, 2.2-fold higher than that of WT 34-3C IgG1. It should be stressed that the nucleotide sequence analysis of the variable regions of 34-3C mAb excluded the presence of potential *N*-glycosylation sites in its Fab region (47).

The pathogenic potential of degalactosylated 34-3C IgG1 variant was then compared with that of its more highly galactosylated WT counterpart by assessing the development of AIHA in B6 mice. As reported previously (33), a single injection of 500  $\mu$ g 34-3C IgG1 anti-RBC mAbs resulted in transient decreases in Ht values peaking on day 4 (Fig. 2A). However, we observed that the degalactosylated 34-3C IgG1 variant induced a more severe anemia than WT 34-3C IgG1. Indeed, 4 d after the injection, mice injected with the



**FIGURE 1.** Degalactosylation of *N*-glycans of 34-3C IgG1 and IgG2a mAbs treated with  $\beta$ 1,4-galactosidase. Structures of different nonsialylated and sialylated glycoforms are depicted in the upper panel, with the numbers corresponding to the respective peaks on MALDI-TOF MS profiles of *N*-glycans released from 34-3C IgG1 and IgG2a mAbs treated or not with galactosidase. Peaks 1 and 2 correspond to G0 glycoforms without or with a fucose, peaks 3 and 4 to G1 glycoforms without or with a fucose, peaks 5 and 6 to G2 glycoforms without or with a fucose, and peaks 7 and 8 to S1 glycoforms with a fucose. Note almost complete disappearance of G1 and G2 glycoforms (peaks 3–6) in 34-3C IgG1 and IgG2a treated with galactosidase. Black squares represent *N*-acetylglucosamine; gray circles represent mannose; white circles represent galactose; white diamonds represent *N*-glycolylneuraminic acid; dark gray triangles represent fucose. *m/z*, Mass-to-charge ratio.

degalactosylated variant displayed a mean Ht value ( $\pm$  SD) of  $23.8 \pm 2.4\%$ , which was significantly lower than that observed with its WT counterpart ( $33.0 \pm 0.7\%$ ;  $p < 0.0001$ ).

#### *No complement activation by agalactosylated 34-3C IgG1 variant*

An increased pathogenic activity of degalactosylated 34-3C IgG1 variant could be a result of the possible activation of complement

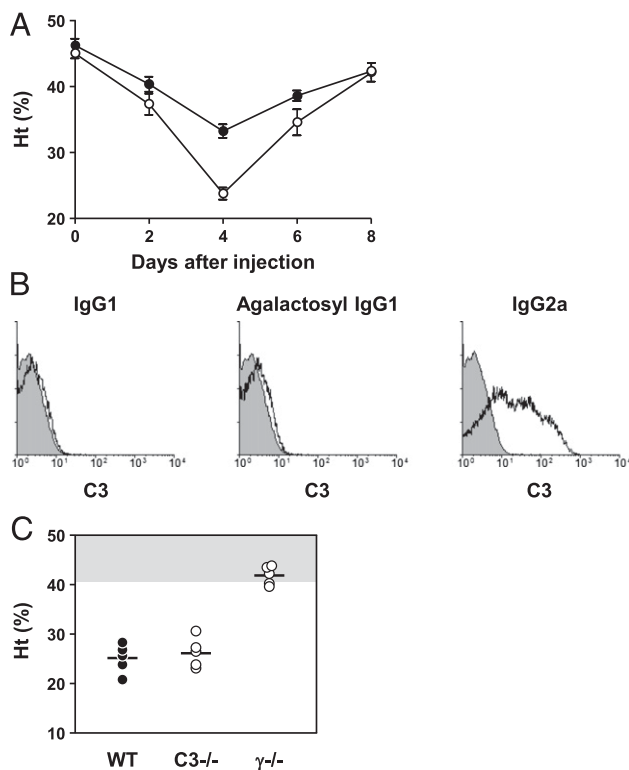
pathway by MBL, as suggested by in vitro studies (22). However, circulating RBCs of B6 mice injected with the degalactosylated 34-3C IgG1 variant displayed only marginal C3 deposits (Fig. 2B), as was the case of mice injected with the WT 34-3C IgG1 mAb, which is known not to activate complement efficiently (33). Moreover, the pathogenic effect of degalactosylated 34-3C IgG1 variant remained unchanged in  $C3^{-/-}$  B6 mice but was completely abrogated in B6 mice deficient in FcR  $\gamma$ -chains lacking

Table I. Structural analysis of *N*-glycans purified from WT and galactosidase-treated 34-3C IgG1 and IgG2a anti-RBC mAbs

34-3C	Galactosidase	Nonsialylated Glycoforms			Sialylated Glycoforms	
		G0	G1	G2	S1	S2
IgG1	–	43.9	42.4	7.6	3.4	<0.1
IgG1	+	96.4	0.2	0.4	2.0	0.1
IgG2a	–	14.3	65.9	15.1	0.3	<0.1
IgG2a	+	97.0	0.7	0.4	<0.1	<0.1

Results are expressed as relative abundance of nonsialylated (G0, G1, and G2) and sialylated (S1 and S2) glycoforms among total oligosaccharides. Some of the carbohydrate moieties could not be assigned to the glycoforms defined in Fig. 1. However, these represent very minor fractions.

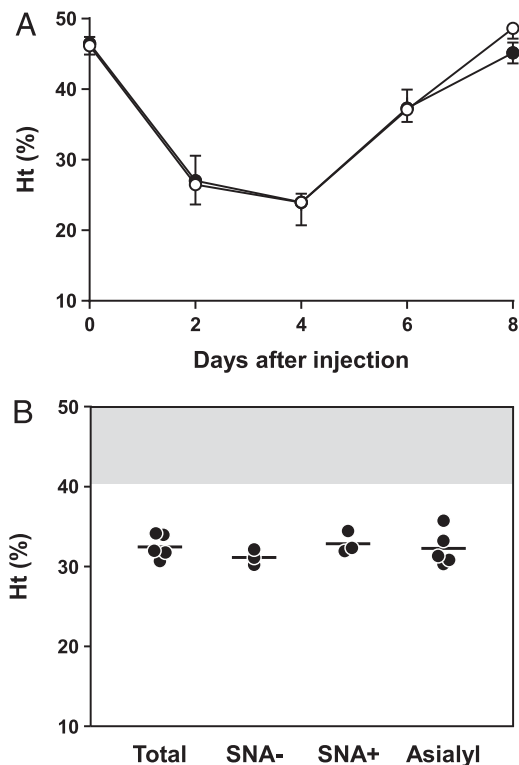
Fc $\gamma$ Rs (Fig. 2C). These data indicated the lack of contribution of MBL-dependent activation of complement to the development of AIHA induced by degalactosylated 34-3C IgG1 mAb.



**FIGURE 2.** Development of more severe anemia without induction of complement activation after injection of degalactosylated 34-3C IgG1 glycovariant than WT 34-3C IgG1 mAb. (A) A total of 500  $\mu$ g degalactosylated (○) or WT (●) 34-3C IgG1 was i.v. injected into B6 mice. Results are expressed as mean Ht values ( $\pm$  SD) of seven mice. (B) Flow cytometric analysis of complement activation in vivo 24 h after an i.v. injection of degalactosylated or WT 34-3C IgG1 (500  $\mu$ g) or of control 34-3C IgG2a (200  $\mu$ g) anti-RBC mAb into B6 mice. RBCs were stained with biotinylated goat anti-C3 Abs, followed by PE-conjugated streptavidin. Shaded areas indicate the background staining obtained with untreated B6 mice. Note marginal C3 deposits on RBCs in mice injected with degalactosylated 34-3C IgG1 variant, with levels as low as in those injected with WT 34-3C IgG1, in contrast with a marked C3 deposition on RBCs in those injected with 34-3C IgG2a mAb. (C) A total of 500  $\mu$ g degalactosylated 34-3C IgG1 variant was i.v. injected into WT, C3<sup>-/-</sup>, and Fc $\gamma$ R<sup>-/-</sup> B6 mice. Ht values of individual mice determined 4 d after injection are shown. Mean Ht values are indicated by horizontal lines. The normal range of Ht values (mean  $\pm$  3 SD) of control B6 mice is represented as shaded area.

*No enhanced pathogenic activity by degalactosylated 34-3C IgG2a glycovariant as compared with WT 34-3C IgG2a*

We have previously shown that the IgG2a subclass of the 34-3C anti-RBC mAb is far more potent in inducing anemia than its IgG1 variant, which is due to an engagement of multiple phagocytic receptors (Fc $\gamma$ RI, Fc $\gamma$ RIII, Fc $\gamma$ RIV, and complement receptors), as compared with the engagement of Fc $\gamma$ RIII alone in IgG1-induced anemia (33, 36, 37, 48). Because 34-3C IgG2a mAb is much more galactosylated than its IgG1 variant (Table I), we determined whether the pathogenic effect of this highly potent IgG2a subclass of 34-3C mAb could also be enhanced through degalactosylation. MALDI-TOF MS analysis on *N*-glycans released from  $\beta$ 1,4-galactosidase-treated 34-3C IgG2a confirmed an efficient removal of galactose residues, as the content of agalactosylated G0 glycoform amounted to 97.0%, 6.6-fold higher than that of WT 34-3C IgG2a (Fig. 1, Table I). However, in contrast with the 34-3C IgG1 subclass, degalactosylated 34-3C IgG2a variant failed to induce more severe anemia than its more highly galactosylated WT counterpart (Fig. 3A). Four days after the injection, mice injected with the degalactosylated variant displayed a mean Ht value ( $\pm$  SD) of 24.0  $\pm$  3.2%, which was indistinguishable from that observed with its WT counterpart (24.0  $\pm$  1.2%).

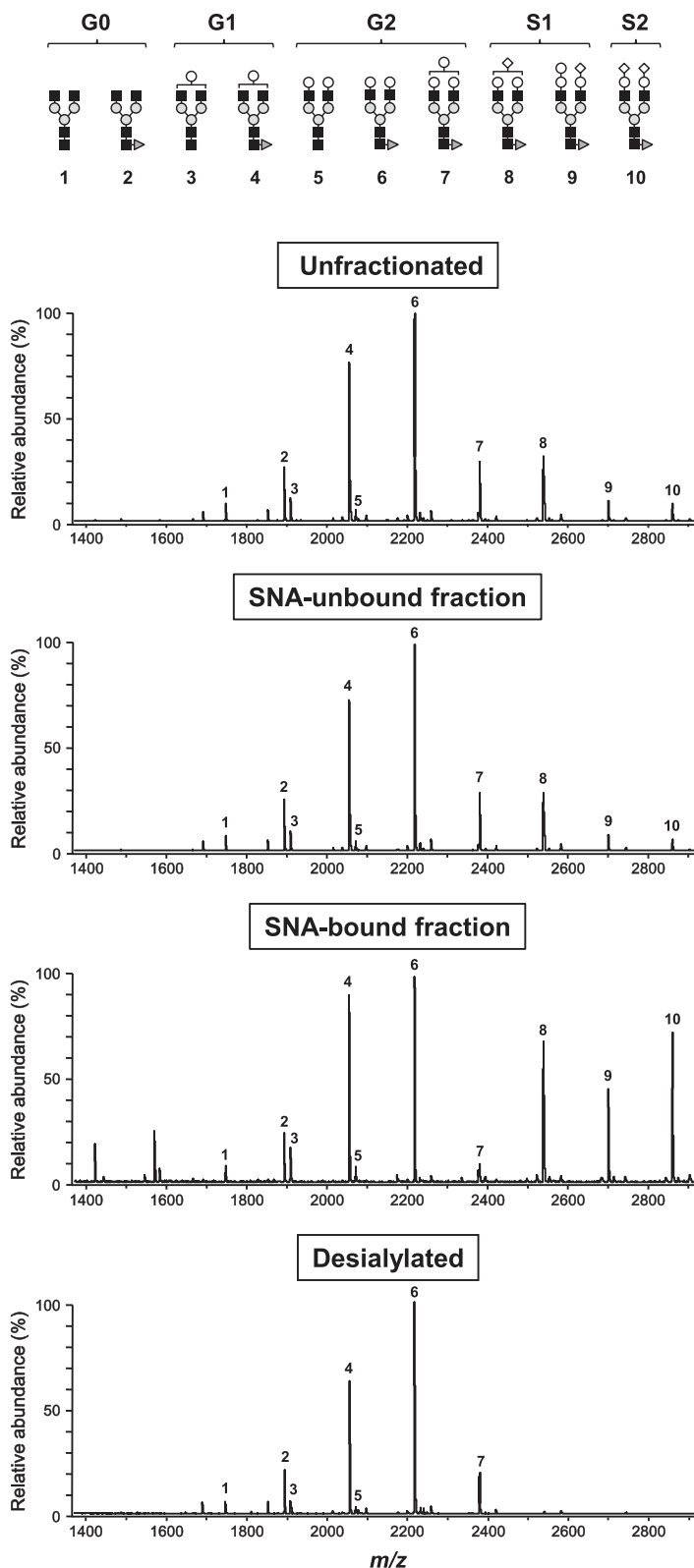


**FIGURE 3.** No enhanced pathogenic activity by degalactosylated IgG2a of 34-3C mAb, and efficient induction of anemia by various 34-3C IgG2a F243A glycovariants independently of the extent of sialylation. (A) A total of 100  $\mu$ g degalactosylated (○) or WT (●) 34-3C IgG2a was i.v. injected into B6 mice. Results are expressed as mean Ht values ( $\pm$  SD) of seven mice. (B) A total of 100  $\mu$ g 34-3C IgG2a F243A of unfractionated (Total) and fractionated preparations on SNA lectin column (SNA<sup>-</sup> and SNA<sup>+</sup>), as well as desialylated 34-3C IgG2a F243A mAb (asialyl), was injected i.v. into B6 mice. Ht values of individual mice measured 4 d after injection of different 34-3C IgG2a F243A glycovariants are shown. Mean Ht values are indicated by horizontal lines. The normal range of Ht values (mean  $\pm$  3 SD) of control B6 mice is represented as shaded area.

*No modulation of pathogenicity by sialylation or desialylation of 34-3C IgG2a F243A variant*

In view of anti-inflammatory properties of IgG Abs enriched with sialic acids (25–27), we have previously generated a more sialylated 34-3C IgG2a F243A variant carrying phenylalanine instead of alanine at position 243 in the CH2 domain, because this replacement has been shown to result in a remarkable increase in Fc sialylation of human IgG3 (49, 50). Indeed, 34-3C IgG2a F243A

displayed a markedly increased content of sialylated glycoforms, which accounted for ~20% of total oligosaccharides, which contrasted with essentially absence of sialylated glycoforms (<1%) in WT 34-3C IgG2a mAb (28). Despite these remarkable differences in the content of sialylated glycoforms, the pathogenic activity of 34-3C IgG2a F243A mutant was comparable with that of WT 34-3C IgG2a. However, the significance of these results could be dismissed by arguing that the content of sialylated glycoforms of



34-3C IgG2a F243A was not high enough to downmodulate the Fc-dependent effector function, as more sialylated variants of 6A6 IgG1 and IgG2b anti-platelet mAbs displaying a lesser pathogenic activity were prepared through fractionation on SNA lectin affinity column (25).

Therefore, we further enriched sialylated species of 34-3C IgG2a F243A through SNA lectin affinity column. In the end, 3.1% of total 34-3C IgG2a F243A charged on SNA lectin column was eluted using 0.5 M lactose. As documented by increases in S1 and S2 glycoforms and concomitant decreases in the G2 glycoform through MALDI-TOF MS analysis, the fractionation on SNA lectin column resulted in an enrichment of sialylated glycoforms of 34-3C IgG2a F243A, which accounted for 50.7% of total oligosaccharides, as compared with 16.7% in the unfractionated counterpart (Fig. 4, Table II). When the content of sialic acid residues was expressed as  $([\% \text{ of S1}] + 2 \times [\% \text{ of S2}])$ , the SNA-bound fraction had a 3.8-fold higher value than the unfractionated 34-3C IgG2a F243A mAb (70.7 versus 18.8). The observed sialic acid species in 34-3C IgG2a F243A was mostly (>90%) *N*-glycolylneuraminic acid, rather than *N*-acetylneuraminic acid (Fig. 4). A recovery of only 3.1% of 34-3C IgG2a F243A and a remarkable enrichment of S2 glycoform through SNA lectin affinity column chromatography is consistent with the finding that the presence of two sialic acid residues in *N*-glycans attached to the two H chains of an IgG molecule (either because of the presence of one S2 glycoform or two S1 glycoforms) is required for the efficient binding of sialylated IgG to SNA lectin columns (51).

The pathogenic potential of the sialic acid-enriched SNA-bound fraction of 34-3C IgG2a F243A was then compared with that of the flow-through SNA-unbound fraction and of the unfractionated counterpart. As shown in Fig. 3B, the extent of anemia provoked by the injection of 100  $\mu$ g of these three different preparations of 34-3C IgG2a F243A was similar (Ht values on day 4: unfractionated,  $32.4 \pm 1.5\%$ ; SNA unbound,  $31.1 \pm 1.0\%$ ; SNA bound,  $32.9 \pm 1.3\%$ ). Moreover, we generated desialylated 34-3C IgG2a F243A variant through treatment with  $\alpha$ 2,3/ $\alpha$ 2,6-neuraminidase in vitro. Structural analysis of *N*-glycans released from neuraminidase-treated 34-3C IgG2a F243A confirmed a complete removal of terminal sialic acid residues (Fig. 4, Table II). However, again, the pathogenic effect of desialylated 34-3C IgG2a F243A (Ht values on day 4:  $32.3 \pm 2.2\%$ ) was indistinguishable from that of its highly sialylated counterpart (Fig. 3B).

## Discussion

This study was designed to more clearly define the respective contributions of galactosylation and sialylation of *N*-glycans to the pathogenic potential of IgG autoantibodies in a murine model of AIHA. Comparative analyses of degalactosylated or desialylated variants versus their highly galactosylated or sialylated counterparts of

34-3C IgG1 and IgG2a anti-RBC mAbs revealed that the pathogenic activity of IgG1, but not IgG2a anti-RBC autoantibodies was modulated by galactosylation, but not sialylation. In addition, the lack of any sign of increased complement activation in vivo by degalactosylated 34-3C IgG1 variant clearly ruled out MBL-mediated complement activation as a molecular mechanism responsible for the enhanced pathogenic effect of agalactosylated IgG.

Our demonstration that degalactosylated 34-3C IgG1 anti-RBC mAb induced a more severe anemia than its WT counterpart shed new light on the pathogenic potential of agalactosylated and asialylated IgG. Because both WT and degalactosylated 34-3C IgG1 mAbs were hardly sialylated, it is clear that the difference in their pathogenic activity cannot be attributed to differences in the extent of terminal sialylation. Previous in vitro studies claimed that agalactosylated IgG could enhance the activation of complement through its interaction with MBL, thereby promoting its pathogenic potential (22). However, we failed to see a significant activation of complement by degalactosylated 34-3C IgG1 in vivo, as judged by the lack of C3 deposits on RBCs in mice after injection. Moreover, the absence of its pathogenic effect in mice deficient in Fc $\gamma$ Rs, as well as no reduction of its pathogenicity in C3<sup>-/-</sup> mice, further argue against the implication of MBL-dependent complement activation in the pathogenic activity of agalactosylated 34-3C IgG1. These data indicate a dominant role of Fc $\gamma$ Rs in promoting the pathogenic effect of agalactosylated IgG Abs, in agreement with observations made in the murine model of immune thrombocytopenia induced by 6A6 anti-platelet mAb (23).

It should be stressed, however, that we did not find any effect of degalactosylation on the pathogenic activity of the IgG2a subclass of 34-3C anti-RBC mAb. This indicates that the modulation of the pathogenic potential of IgG autoantibodies through the extent of galactosylation is dependent on the IgG subclass. Notably, the Fc-dependent effector function of the IgG2a subclass is much more efficient than that of the IgG1 subclass. Thus, it is likely that the contribution of hypogalactosylation to the pathogenic potential of IgG2a can be masked because of its potent engagement of multiple phagocytic receptors (Fc $\gamma$ RI, Fc $\gamma$ RIII, Fc $\gamma$ RIV, and complement receptor) involved in AIHA, which contrasts with the involvement of a single receptor, Fc $\gamma$ RIII, by IgG1 (33, 36, 37). Our data could explain the findings that no increased Fc-dependent effector functions were observed after treatment with galactosidase of two different human IgG1 mAbs (18, 19), because the IgG1 subclass in humans functionally corresponds to the IgG2a subclass in mice, as it displays the highest Fc effector functions among the four different human IgG subclasses (52). This issue needs to be addressed through the analysis of different human IgG subclasses.

We have previously shown that the 34-3C IgG2a F243A mutant, which contained ~20% sialylated glycovariants, efficiently interacted with Fc $\gamma$ Rs and activated complement, and was as pathogenic as barely sialylated WT Ab (28). However, sialylated 6A6 IgG1 and IgG2b antiplatelet glycovariants enriched through SNA lectin affinity column chromatography were shown to be less pathogenic as compared with their poorly sialylated counterparts (25). Although detailed information on the yield and precise content of sialylated glycoforms in these SNA-fractionated 6A6 Abs was not reported in that study, we assumed that the maintained pathogenic activity of the unfractionated 34-3C IgG2a F243A could be because of its possible lesser content of sialylated glycoforms compared with SNA-fractionated 6A6. However, this analysis also failed to show any difference in the pathogenic potential between a 34-3C IgG2a F243A glycovariant containing more (~50%) sialylated glycoforms (obtained through SNA column fractionation) and its enzymatically desialylated variant. These data rather argue against the notion that sialylated IgG is less pathogenic because of

Table II. Structural analysis of *N*-glycans purified from different glycovariants of 34-3C IgG2a F243A anti-RBC mAb

F243A	Nonsialylated Glycoforms			Sialylated Glycoforms	
	G0	G1	G2	S1	S2
Unfractionated	8.8	26.8	42.8	14.3	2.4
SNA unbound	8.4	27.0	45.3	12.8	1.8
SNA bound	5.5	20.6	22.4	30.7	20.0
Desialylated	9.5	29.5	54.7	<0.1	<0.1

Results are expressed as relative abundance of nonsialylated (G0, G1, and G2) and sialylated (S1 and S2) glycoforms among total oligosaccharides. Some of the carbohydrate moieties could not be assigned to the glycoforms defined in Fig. 4. However, these represent very minor fractions.

reduced binding to Fc $\gamma$ Rs, at least in our murine model of AIHA. In this regard, it is worth noting that the ability of more highly sialylated human IgG1 (obtained through lectin column chromatography and containing 67% sialylated glycoforms) to interact with Fc $\gamma$ Rs was comparable with that of a poorly (5%) sialylated variant (27).

At this moment, we cannot offer the straightforward explanation why our results in the 34-3C anti-RBC mAb model contrast with those obtained with the 6A6 anti-platelet mAb model, in which the extent of sialylation, but not galactosylation, modulated pathogenic potential (23, 25). However, it may be worth emphasizing that the pathogenic mechanism responsible for the development of thrombocytopenia induced by 6A6 IgG anti-platelet mAb appears to be not totally identical to that for the development of AIHA induced by 34-3C IgG anti-RBC mAb. First, the kinetics of depletion of platelets and RBCs are markedly different because 6A6 anti-platelet mAb induced an efficient and rapid elimination of platelets within 4 h, whereas the maximal drop of Ht was observed only 4 d after injection of 34-3C anti-RBC mAb. Second, it has been shown that blood monocytes were responsible for the elimination of platelets in 6A6-induced immune thrombocytopenia (53), whereas Kupffer cells in the liver are the major cell type involved in RBC destruction in 34-3C-induced AIHA (32, 35). Third, complement can play a substantial role in the development of AIHA induced by complement-fixing IgG2a and IgG2b subclasses of 34-3C (33), but only a little role, if any, in immune thrombocytopenia induced by 6A6 IgG2b (23).

In conclusion, our study indicates that the extent of galactosylation per se, but not galactosylation-dependent terminal sialylation, of *N*-glycans attached to the Fc region contributes to the pathogenic potential of IgG anti-RBC autoantibodies, but its effect is dependent on the IgG subclass. Clearly, a more thorough analysis of Fc-dependent effector functions of the different human IgG subclasses in relation to the extent of galactosylation would help clarify the relevance of our observations to the human disease. Moreover, a further better understanding of the interplay between the structural characteristics of agalactosylated glycoforms of IgG and their proinflammatory properties should provide useful guiding principles for the engineering of mAbs for in vivo application.

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## Disclosures

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