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This information is current as of January 6, 2022.

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J Immunol 2008; 181:4107-4112; ;
doi: 10.4049/jimmunol.181.6.4107
<http://www.jimmunol.org/content/181/6/4107>

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Impact of a Three Amino Acid Deletion in the CH2 Domain of Murine IgG1 on Fc-Associated Effector Functions¹

Lucie Baudino,* Falk Nimmerjahn,[†] Yasuro Shinohara,[‡] Jun-Ichi Furukawa,[‡] Franz Petry,[§] J. Sjeef Verbeek,^{||} Shin-Ichiro Nishimura,[‡] Jeffery V. Ravetch,^{||} and Shozo Izui^{2*}

Four murine IgG subclasses display markedly different Fc-associated effector functions because of their differential binding to three activating IgG Fc receptors (FcγRI, FcγRIII, and FcγRIV) and C1q. Previous analysis of IgG subclass switch variants of 34-3C anti-RBC monoclonal autoantibodies revealed that the IgG1 subclass, which binds only to FcγRIII and fails to activate complement, displayed the poorest pathogenic potential. This could be related to the presence of a three amino acid deletion at positions 233–235 in the CH2 domain uniquely found in this subclass. To address this question, IgG1 insertion and IgG2b deletion mutants at positions 233–235 of 34-3C anti-RBC Abs were generated, and their ability to initiate effector functions and their pathogenicity were compared with those of the respective wild-type Abs. The insertion of amino acid residues at positions 233–235 enabled the IgG1 subclass to bind FcγRIV but did not improve the binding to C1q. Accordingly, its pathogenicity was enhanced but still inferior to that of IgG2b. In contrast, the IgG2b deletion mutant lost its ability to bind to FcγRIV and activate complement. Consequently, its pathogenicity was markedly diminished to a level comparable to that of IgG1. Our results demonstrated that the initiation of FcγR- and complement-mediated effector functions of IgG2b was profoundly affected by the three amino acid deletion at positions 233–235, but that this natural three amino acid deletion could only partially explain the poor binding of IgG1 to FcγRIV and C1q. This indicates the lack in the IgG1 subclass of as yet unknown motifs promoting efficient interaction with FcγRIV and C1q. *The Journal of Immunology*, 2008, 181: 4107–4112.

The pathogenesis of autoantibody-mediated cellular and tissue lesions in autoimmune diseases relies on the self-Ag binding properties associated with the Fab region and the effector functions associated with the Fc regions of the different Ig isotypes. This is best illustrated by the remarkable differences among IgG subclass switch variants of 4C8 and 34-3C anti-RBC monoclonal autoantibodies in their ability to induce autoimmune hemolytic anemia (1, 2). These differences are indeed dependent on their respective capacity to interact with different FcγRs and to activate complement in vivo (1–3).

Murine immune effector cells express three classes of activating FcγRs (FcγRI, FcγRIII, and FcγRIV) and one inhibitory receptor, FcγRIIB (4). Activating FcγRs are hetero-oligomeric complexes, in which the respective ligand-binding α-chains are associated

with the common FcR γ-chain. FcR γ-chains containing an immunoreceptor tyrosine-based activating motif are required for the assembly and cell surface expression of these activating FcγRs and for the triggering of their various effector functions, including phagocytosis by macrophages, degranulation by mast cells, and Ab-dependent cell-mediated cytotoxicity by NK cells (5). In contrast, FcγRIIB is a single α-chain receptor bearing an ITIM motif. Upon its coligation to activating FcγRs or BCR, it recruits the inositol polyphosphate phosphatase, thereby down-regulating the action of cellular activation mediated by activating FcγRs or BCR (6). FcγRI is capable of binding only IgG2a with high affinity, FcγRIV binds IgG2a and IgG2b immune complexes with intermediate affinity, and the low-affinity FcγRIIB and FcγRIII bind polymeric forms of three different IgG subclasses (IgG1, IgG2a, and IgG2b) but not IgG3 (7–9). Moreover, IgG2a, IgG2b, and IgG3, but not IgG1, efficiently activate complement (2, 10).

Analysis of the pathogenicity of IgG subclass switch variants of the 34-3C anti-RBC mAb revealed that the pathogenic potential of the IgG1 subclass is very poor as compared with those of IgG2a and IgG2b (2). This is due to the fact that murine IgG1 binds only to FcγRIII among three different activating FcγRs and fails to activate complement. A unique structural feature of murine IgG1 is the presence of a deletion of three amino acid residues at positions 233–235 in the CH2 domain. In view of the critical role of the amino acid residues in this region for the interaction of IgG2a with the high-affinity FcγRI (3, 11), the absence of these three amino acid residues in IgG1 could be responsible for the limited Fc-associated effector functions of this IgG subclass. Notably, the IgG2b subclass, which does not have this deletion, displays a higher affinity interaction with FcγRIV compared with FcγRIII (9). To determine the contribution of the deletion at positions 233–235 to IgG effector functions, we generated a 34-3C IgG1 insertion mutant, in which three amino acid residues at positions 233–235 of the IgG2b subclass were inserted, and a 34-3C IgG2b deletion

*Department of Pathology and Immunology, University of Geneva, Geneva, Switzerland; [†]Laboratory for Experimental Immunology and Immunotherapy, Nikolaus-Fiebiger-Center for Molecular Medicine, University of Erlangen-Nuremberg, Erlangen, Germany; [‡]Laboratory of Advanced Chemical Biology, Graduate School of Advanced Life Science, Hokkaido University, Sapporo, Japan; [§]Institute of Medical Microbiology and Hygiene, Johannes Gutenberg-University, Mainz, Germany; ^{||}Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands; and ^{||}The Rockefeller University, New York, NY 10065

Received for publication April 4, 2008. Accepted for publication July 8, 2008.

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¹ This work was supported by a grant from the Swiss National Foundation for Scientific Research, by Special Coordination Funds for Promoting Science and Technology of the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government, and by a grant from the Roche Research Foundation. F.N. was supported by grants from the German Research Foundation (DFG) and from the Bavarian Genome Research Network (BayGene).

² Address correspondence and reprint requests to Dr. Shozo Izui, Department of Pathology and Immunology, Centre Médicale Universitaire, 1211 Geneva 4, Switzerland. E-mail address: Shozo.Izui@medecine.unige.ch

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mutant lacking these three amino acids. Then, we assessed the abilities of these mutants, in comparison with their wild types (WT),³ to bind different FcγRs, activate complement, and induce anemia. Our results show that IgG effector functions were profoundly affected as a consequence of the three amino acid deletion at positions 233–235, but that the 233–235 deletion alone cannot explain the relative lack of pathogenicity of IgG1.

Materials and Methods

Mice

FcγRIII^{−/−} mice, generated by gene targeting in 129-derived embryonic stem cells (12), were backcrossed for seven generations on a BALB/c background, as described previously (3). BALB/c mice were purchased from The Jackson Laboratory.

Monoclonal Abs

The hybridoma secreting the 34-3C IgG2a anti-RBC monoclonal autoantibody was derived from unmanipulated New Zealand black mice (13), and the generation of IgG1 and IgG2b subclass switch variants was described previously (2). The 34-3C IgG1 insertion mutant carrying three amino acid residues at positions 233–235 of the IgG2b subclass (IgG1[233–235]) and the IgG2b deletion mutant at positions 233–235 (IgG2bΔ233–235) were generated by transfecting a 34-3C H chain-loss cell line, obtained by selective ELISA-guided subcloning of a spontaneous mutant secreting only 34-3C L chains, with VDJ34-3C-Cγ1[233–235] or VDJ34-3C-Cγ2bΔ233–235 mutant plasmid, which was generated by oligonucleotide-directed mutagenesis, according to the method described by Ho et al. (14). Mouse RBC-binding activity of 34-3C mAb was assessed *in vitro* by a flow cytometric analysis using a biotinylated rat anti-mouse κ-chain mAb (H139.52.1.5), followed by PE-conjugated streptavidin, as described previously (15). Hamster IgG 9E9 FcγRIV-blocking mAb was described previously (9). All the transfectoma cells were grown in DMEM supplemented with 1% Ultrosor HY (PALL Life Sciences), and IgG mAb were purified from culture supernatants by protein G column chromatography. The purity of IgG was >95% as documented by SDS/PAGE.

Surface plasmon resonance (SPR) analysis

A Biacore 3000 biosensor system was used to determine the interaction of soluble murine FcγRs (FcγRI, FcγRIIB, FcγRIII, and FcγRIV) with different 34-3C IgG anti-RBC mAb, as described previously (9). In brief, soluble versions of murine FcγRs were injected through flow cells containing immobilized Abs at five different concentrations. Background binding to a reference flow cell containing immobilized BSA was subtracted. Results are expressed as association constant (K_a , M^{-1}). The SD for the different K_a values was below 5%.

Detection of C1q and C3 deposits on RBC *in vivo*

The deposition of C1q and C3 on RBC 24 h after an i.v. injection of 34-3C anti-RBC mAb in BALB/c mice was determined by a flow cytometric assay, using biotinylated goat anti-mouse C1q (16) or goat anti-mouse C3 (Cappel Laboratories), followed by PE-conjugated streptavidin, as previously described (2). The injection of mAb was controlled by assessing the level of Ab opsonization of RBC by using biotinylated rat anti-mouse κ-chain mAb.

Experimental autoimmune hemolytic anemia

Autoimmune hemolytic anemia was induced by a single i.v. injection of purified anti-RBC mAb into 2- to 3-mo-old mice. Blood samples were collected into heparinized microhematocrit tubes every 2 days after the injection, and hematocrit (Ht) values were directly determined after centrifugation. To block FcγRIV, mice were treated with 200 μg of 9E9 anti-FcγRIV mAb 30 min before and 2 days after administration of the 34-3C mAb. As a control, mice were treated with polyclonal hamster IgG (Jackson ImmunoResearch Laboratories). Livers, obtained 8 days after injection of mAb, were processed for histological examination, and the extent of *in vivo* RBC destruction by Kupffer cell-mediated phagocytosis was determined by Perls iron staining.

IgG1	: VP---EVSSVF
IgG1[233–235]	: VPNLEGGPSVF
IgG2b	: APNLEGGPSVF
IgG2bΔ233–235	: AP---GGPSVF
IgG2a	: APNLLGGPSVF
IgG3	: PGNILGGPSVF

FIGURE 1. Amino acid sequences at positions 231–241 in the CH2 domain of four different murine IgG subclasses and two mutants, IgG1[233–235] and IgG2bΔ233–235.

Analysis of oligosaccharide structures

Purification of oligosaccharides from 34-3C IgG1 and IgG2b mAb, and from polyclonal human IgG (Sigma-Aldrich), as a control, were performed based on chemoselective glycoblotting technique as described (17, 18). In brief, IgG samples were reductively alkylated under the presence of detergent, then digested successively with trypsin and peptide *N*-glycosidase F (Roche Diagnostics), as previously described (19). The digested sample was mixed with a novel hydrazide-functionalized glycoblotting polymer (18) and, following washing of the unbound substances (e.g., peptides, detergent, enzymes), sialic acids were methyl-esterified to render sialylated oligosaccharides chemically equivalent to neutral oligosaccharides, as described (20). The IgG oligosaccharides were finally recovered as derivatives of αωWR (Nα-((aminoxy)acetyl)tryptophanylarginine methyl ester), an oligosaccharide labeling reagent that allows highly sensitive detection on mass spectrometry (MS) (21). Then, the recovered glycans were subjected to MALDI-TOF MS using an Ultraflex II mass spectrometer (Bruker Daltonik) controlled by the FlexControl 2.0 software package. The used analytical procedure was proven to be reproducible with coefficient of variation less than 15% by analysis of *N*-glycans prepared from normal human serum and from human IgG (18). Estimation of *N*-linked oligosaccharide structures was obtained by input of peak masses into the GlycoMod Tool (<http://au.expasy.org/tools/glycomod/>) or GlycoSuite Tool (<https://glycosuite.proteomesystems.com/glycosuite/glycodb>).

Statistical analysis

Statistical analysis was performed with the Wilcoxon two-sample test. Probability values <5% were considered significant.

Results

Altered bindings of IgG1[233–235] and IgG2bΔ233–235 mutants to FcγRIIB, FcγRIII, and FcγRIV

To investigate the possibility that the presence of the three amino acid deletion at positions 233–235 in the CH2 domain of murine IgG1 is responsible for its weak Fc-associated effector function, we generated an IgG1[233–235] insertion mutant, which carries three amino acid residues present at positions 233–235 of the IgG2b subclass (Fig. 1). Because of the additional differences in the flanking three amino acid residues at positions 236–238 of IgG1 (glutamic acid-valine-serine) from those of the three other IgG subclasses (glycine-glycine-proline), these three amino acid residues were also substituted by those conserved in the other subclasses. In addition, an IgG2bΔ233–235 deletion mutant was also generated. Notably, *in vitro* RBC-binding assays confirmed that these two mutants exhibited mouse RBC-binding activity comparable to WT mAb (data not shown).

By using SPR with soluble forms of FcγRs, we determined the ability of IgG1[233–235] and IgG2bΔ233–235 mutants to bind four different FcγRs (FcγRI, FcγRIIB, FcγRIII, and FcγRIV) in comparison with the respective WT Abs. The IgG1[233–235] mutant displayed ~6-fold higher binding to FcγRIII than WT IgG1 (Table I). In agreement with this finding, the affinity of FcγRIII for the IgG2bΔ233–235 mutant was diminished ~10 times as compared with its WT counterpart. More significantly, FcγRIV bound the IgG1[233–235] mutant with an affinity of 1.0×10^6 (M^{-1}), whereas no measurable affinity for the IgG2bΔ233–235 mutant was detectable, as in the case of WT IgG1 Ab (Table I). Notably, the affinity of

³ Abbreviations used in this paper: WT, wild type; SPR, surface plasmon resonance; Ht, hematocrit; MS, mass spectrometry; MBL, mannose-binding lectin.

Table I. Affinities of FcγRI, FcγRIIB, FcγRIII, and FcγRIV for 34-3C IgG1, IgG2b, and their mutants^a

mAb	FcγRI	FcγRIIB	FcγRIII	FcγRIV
IgG1 WT	NB	1.2 × 10 ⁶	2.1 × 10 ⁵	NB
IgG1[233–235]	NB	1.7 × 10 ⁶	1.3 × 10 ⁶	1.0 × 10 ⁶
IgG2b WT	NB	1.1 × 10 ⁶	6.4 × 10 ⁵	1.6 × 10 ⁷
IgG2bΔ233–235	NB	7.0 × 10 ⁴	6.0 × 10 ⁴	<10 ⁴
IgG2a	3.9 × 10 ⁷	5.6 × 10 ⁵	5.6 × 10 ⁵	1.4 × 10 ⁷

^a Results are expressed as Ka (M^{−1}). NB, No binding.

FcγRIV for the IgG1[233–235] mutant was still more than 10-fold lower than that for IgG2b but comparable to those obtained with the low-affinity FcγRIIB and FcγRIII. In addition, the binding capacity of the IgG2bΔ233–235 mutant to FcγRIIB was also markedly (more than 10-fold) diminished, whereas only a minimal increase in FcγRIIB binding was obtained with the IgG1[233–235] mutant. As expected, none of the WT and mutant mAb of the IgG1 and IgG2b subclasses exhibited any significant binding to FcγRI (Table I).

Lack of complement activation by the IgG2bΔ233–235 mutant

To assess the possible implication of the three amino acid deletion at positions 233–235 in the lack of complement activation by the IgG1 subclass, we analyzed by flow cytometry the extent of C1q and C3 deposition on circulating RBC 24 h after a single i.v. injection into BALB/c mice of 200 μg of 34-3C IgG1[233–235] and IgG2bΔ233–235 mutants, in comparison with their respective WT Abs. As expected, no significant deposition of C1q and C3 were detectable on RBC in mice injected with 34-3C WT IgG1 mAb (Fig. 2). However, no appreciable increases in C1q and C3 depo-

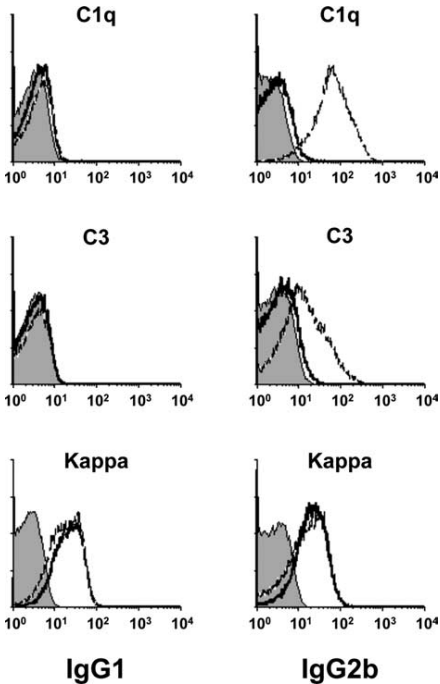


FIGURE 2. Flow cytometric analysis of complement activation in vivo by 34-3C IgG1[233–235], IgG2bΔ233–235, and their respective WT Abs. A total of 24 h after an i.v. injection of 200 μg of 34-3C anti-RBC IgG1 or IgG2b into BALB/c mice, RBC were stained with biotinylated goat anti-mouse C1q, goat anti-C3, or rat anti-mouse κ-chain Abs, followed by PE-conjugated streptavidin. Thick lines: mutant Abs; thin lines: WT Abs. Shaded areas indicate the background staining obtained with untreated BALB/c mice.

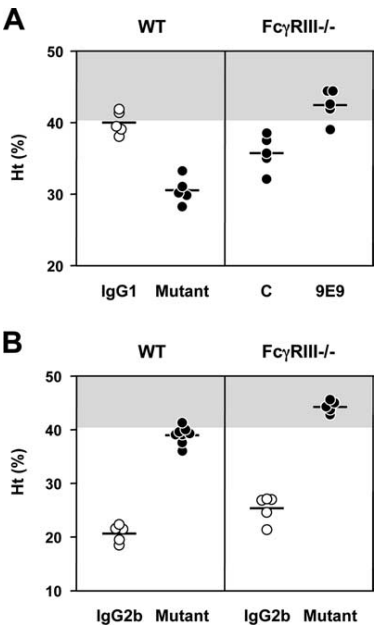


FIGURE 3. Development of anemia in WT and FcγRIII^{−/−} BALB/c mice following the injection of 34-3C IgG1[233–235], IgG2bΔ233–235, and their respective WT Abs. **A**, WT BALB/c mice were i.v. injected with 200 μg of 34-3C IgG1 WT (○) or IgG1[233–235] mutant (●) (left panel), and FcγRIII^{−/−} BALB/c mice treated with 9E9 FcγRIV-blocking mAb or control hamster IgG were injected with 200 μg of 34-3C IgG1[233–235] mutant (●) (right panel). **B**, A total of 200 μg of 34-3C IgG2b WT (○) or IgG2bΔ233–235 mutant (●) were injected i.v. into WT and FcγRIII^{−/−} BALB/c mice. Ht values of individual mice measured 4 days after i.v. injection of 34-3C mAb are shown. Mean Ht values are indicated by horizontal lines. The normal range of Ht values (mean ± 3SD) of 2- to 3-month-old BALB/c mice is represented as shaded area.

sition were observed with the IgG1[233–235] mutant either. Thus, the presence or absence of the three amino acid deletion did not affect the extent of C1q binding and complement activation by the IgG1 subclass. However, this was not the case for the IgG2b subclass. The injection of WT IgG2b mAb induced substantial C1q and C3 deposition, whereas such deposition was hardly detectable in mice receiving the IgG2bΔ233–235 mutant (Fig. 2). This indicated that the presence of the three amino acid deletion at positions 233–235 almost completely abrogated the activation of complement by the IgG2b subclass in vivo.

Moderate increase in the pathogenicity of the IgG1[233–235] mutant and marked decrease in the pathogenicity of the IgG2bΔ233–235 mutant

Because of substantial alterations in the Fc-dependent effector functions of 34-3C IgG1[233–235] and IgG2bΔ233–235 mutants as compared with their respective WT Abs, we analyzed their pathogenic activity in BALB/c mice. As shown previously (2), a single injection of 200 μg 34-3C IgG1 WT mAb hardly induced anemia, whereas the IgG1[233–235] mutant provoked mild but significant anemia with maximal drops of Ht values peaking at day 4 (IgG1[233–235]: 30.6 ± 1.8%; IgG1 WT: 40.0 ± 1.6%, *p* < 0.01; Fig. 3A). Since SPR analysis revealed that the IgG1[233–235] mutant newly exhibited substantial binding to FcγRIV (in addition to FcγRIII), we assessed the contribution of FcγRIV to the increased pathogenicity of IgG1[233–235]. FcγRIII^{−/−} mice receiving the IgG1[233–235] mutant were not completely protected and still developed anemia (mean Ht values at day 4: 35.6 ±

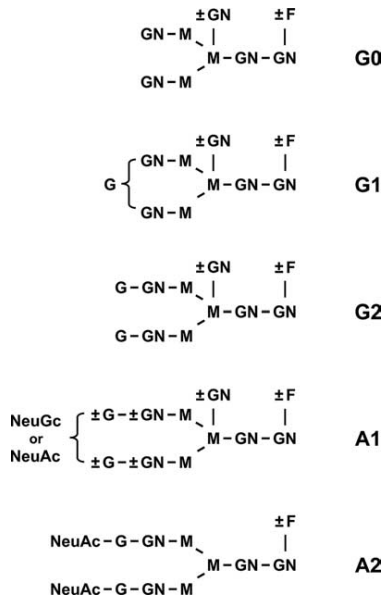


FIGURE 4. Biantennary complex-type oligosaccharide structures released from 34-3C anti-RBC IgG1 and IgG2b Abs. Structures of different sialylated (A1 and A2) and nonsialylated glycoforms (G0, G1, and G2) are summarized. NeuAc: *N*-acetylneuramic acid; NeuGc: *N*-glycolylneuramic acid; G: galactose; GN: *N*-acetylglucosamine; M: mannose; F: fucose.

2.5%, $p < 0.02$; Fig. 3A). However, FcγRIII^{−/−} mice treated with 9E9 FcγRIV-blocking mAb became totally resistant to the pathogenic effect of IgG1[233–235] ($42.5 \pm 2.2\%$, $p < 0.01$; Fig. 3A). Histological analysis confirmed the complete absence of iron deposits by Kupffer cells in 9E9-treated FcγRIII^{−/−} mice, which contrasted with the presence of substantial levels of erythrophagocytosis in control IgG-treated FcγRIII^{−/−} mice (data not shown).

A single injection of 200 μg of 34-3C IgG2b WT mAb induced severe anemia with mean Ht values of $20.7 \pm 1.6\%$ at day 4 in BALB/c mice (Fig. 3B), with three classes of phagocytic receptors (FcγRIII, FcγRIV, and complement receptors) being involved in the development of anemia (3). In contrast, the IgG2bΔ233–235 mutant at a dose of 200 μg barely provoked anemia (mean Ht values at day 4: $39.0 \pm 1.6\%$, $p < 0.002$; Fig. 3B). Notably, FcγRIII^{−/−} mice injected with 34-3C IgG2b WT mAb still developed severe anemia ($25.4 \pm 2.5\%$), whereas the development of anemia by IgG2bΔ233–235 was completely prevented in FcγRIII^{−/−} mice ($44.3 \pm 1.0\%$, $p < 0.002$; Fig. 3B), in which no sign of erythrophagocytosis by Kupffer cells was detectable (data not shown).

Little alterations in levels of sialylation due to the presence of the three amino acid deletion at positions 233–235 in IgG1 and IgG2b subclasses

Asparagine-linked biantennary complex-type oligosaccharide chains attached at position 297 have been shown to be essential for IgG Fc-dependent effector functions (22–24). As described previously (25, 26), most of these oligosaccharide side chains are fucosylated and nonsialylated, ending with either two galactose residues (G2), one galactose and one *N*-acetylglucosamine (G1), or two *N*-acetylglucosamines (i.e., agalactosylated; G0) (Fig. 4). However, a significant, though minor, fraction of G1 and G2 glycoforms bears one or two terminal sialic acids (A1 or A2). In view of the critical role of sialic-acid contents in IgG effector functions (27), we determined whether the modulation of Fc-associated effector functions observed with 34-3C IgG1[233–235] and

Table II. Structural analysis of *N*-linked oligosaccharide chains purified from 34-3C IgG1[233–235], IgG2bΔ233–235, and their respective WT Abs^a

IgG	Sialylated Glycoforms		Nonsialylated Glycoforms		
	A1	A2	G0	G1	G2
IgG1 WT	7.6	0	34.1	38.0	13.0
IgG1[233–235]	3.3	0	27.6	40.7	22.8
IgG2b WT	0.6	0	17.9	48.5	29.0
IgG2bΔ233–235	1.0	0.3	29.5	45.2	15.8

^a Results are expressed as relative abundance of different sialylated (A1 and A2) and nonsialylated (G0, G1, and G2) glycoforms among total oligosaccharides. Some of the carbohydrate moieties were unable to be assigned to the glycoforms defined in Fig. 4. However, these are nonsialylated and represent a very minor fraction: 7.3% for IgG1 WT, 5.6% for IgG1[233–235] mutant, 4.0% for IgG2b WT, and 8.2% for IgG2bΔ233–235 mutant. Notably, this was also the case for polyclonal human IgG (Sigma-Aldrich), in which 4.0% of the glycoforms were unable to be assigned (data not shown).

IgG2bΔ233–235 mutants (i.e., opsonization with complement and erythrophagocytosis by Kupffer cells) could be attributed to a possible change in sialic-acid contents of their carbohydrate side chains.

To this end, the oligosaccharide side chains liberated from 34-3C IgG1 and IgG2b mutants and from their respective WT Abs were subjected to MALDI-TOF MS analysis, and the content of different sialylated (A1 and A2) and nonsialylated (G0, G1 and G2) glycoforms was estimated. As compared with their respective WT counterparts, the contents of sialic acids in IgG1[233–235] and IgG2bΔ233–235 mutants were only slightly reduced and increased, respectively (Table II and Fig. 4), indicating that the extent of sialylation was independent of the presence or absence of the three amino acid deletion at positions 233–235. In contrast, we noted increased contents of the agalactosylated G0 glycoforms, in association with decreases in the G2 glycoforms, in IgG1 WT and IgG2bΔ233–235 mutant, both of which lack the three amino acids at positions 233–235.

Discussion

The present study was designed to define the possible role in down-modulation of IgG Fc-associated effector functions of the three amino acid deletion in the CH2 domain at positions 233–235 uniquely present in murine IgG1 subclass. Comparative analysis of 34-3C anti-RBC IgG2b and its deletion mutant revealed that this deletion resulted in a profound effect on the interaction with FcγRs and on complement activation. Consequently, the pathogenic potential of the IgG2b subclass became as weak as that of the IgG1 subclass. However, the IgG1[233–235] insertion mutant displayed a low-affinity interaction with FcγRIV but no improvement of the binding to C1q. Thus, its pathogenicity was still weaker than that of the IgG2b WT. Our results indicate that in addition to the three amino acid deletion at positions 233–235, the IgG1 subclass lacks additional important amino acid residues implicated in the efficient binding to FcγRIV and C1q.

The three amino acid deletion at positions 233–235 in the 34-3C IgG2b anti-RBC mAb resulted in almost complete loss of its effector functions, as documented by in vitro binding to soluble forms of different FcγRs and by in vivo binding of C1q and subsequent activation of complement. As a consequence, the pathogenic potential of this mutant became as poor as that of the IgG1 subclass, and the induction of anemia by the IgG2bΔ233–235 mutant was mediated only by FcγRIII, but no longer dependent on FcγRIV and complement, as is the case for the IgG1 anti-RBC autoantibody (1, 2, 28). It should be stressed that among different

Fc γ Rs, the binding to Fc γ RIV was the one that was most strongly affected by the introduction of the deletion in the IgG2b subclass. The poor pathogenicity of the IgG2b Δ 233–235 mutant is thus consistent with the finding that Fc γ RIV plays a major role for IgG2b effector functions in vivo in different experimental models of IgG Ab-mediated inflammatory disorders (3, 9, 29). Clearly, the lack of affinity of Fc γ RIV for the IgG1 subclass is primarily explained by the deletion at positions 233–235 in this subclass. However, the binding ability of the IgG1[233–235] mutant to Fc γ RIV was still more than 10 times less than that of the IgG2b subclass. This indicates that the deletion at positions 233–235 does not alone account for the lack of interaction of the IgG1 subclass with Fc γ RIV.

It is somehow unexpected that the 34-3C IgG2b Δ 233–235 mutant failed to activate complement, whereas the IgG1[233–235] mutant was still unable to restore the capacity to efficiently bind C1q and activate complement. This strongly suggests that in addition to the deletion at positions 233–235, the IgG1 subclass lacks an additional amino acid motif critically involved in the activation of complement. Previous mutagenesis analysis of an IgG2b Ab has identified that glutamic acid at position 318 and two lysine residues at positions 320 and 322 are essential in the binding to C1q and subsequent complement activation (30). Indeed, IgG2a, IgG2b, and IgG3 subclasses bearing this C1q-binding motif efficiently activate complement, whereas a lysine at position 322 is replaced by an arginine in the IgG1 subclass. However, the analysis of an IgG2b point mutant showed that both arginine and lysine at position 322 confer equally well the ability of C1q binding and complement activation (30). This argues against the possibility that the lysine-to-arginine substitution in the IgG1 subclass affects the efficient interaction with C1q. In this regard, it is worth mentioning that residues at positions 318, 320, and 322 are conserved in all four human IgG subclasses, independently of their capacity to activate complement (31). Mutagenesis analysis revealed that a serine at position 331 in human IgG4, instead of a proline in the three other subclasses, critically determined the inability of IgG4 to bind C1q and activate complement. However, this is clearly not the case in mouse IgG1, since all four mouse IgG subclasses carry a proline at position 331. All these data suggest that the mouse IgG1 subclass lacks an as yet unknown sequence motif critically involved in the binding to C1q.

It is worth noting that the affinity of the inhibitory Fc γ RIIB for the IgG1 subclass was little affected by the presence of the three amino acid deletion, whereas the binding of the IgG2b subclass to Fc γ RIIB was markedly (more than 10-fold) reduced by the presence of this deletion. This suggests that the IgG1 subclass carries a unique sequence, which promotes the interaction with Fc γ RIIB, thus additionally contributing to the poor effector function of this subclass. As expected, no effect on the binding to Fc γ RI by both IgG1 and IgG2b mutants was observed, since the motif responsible for the high-affinity interaction with Fc γ RI is LLGGP at positions 234–238 of the IgG2a subclass (3, 11). Indeed, the replacement of a leucine by a glutamic acid at position 235 (i.e., the sequence present in the IgG2b subclass) resulted in the loss of high-affinity interaction of IgG2a with Fc γ RI (3).

The remarkable effects of the deletion at positions 233–235 on IgG effector functions could be attributed to a possible modification in the structure of oligosaccharide side chains attached to the CH2 domain of IgG. The introduction of mutations in the CH2 domain led to marked changes in the levels of galactosylation and sialylation, when expressed in Chinese hamster ovary cells, and consequently modulated IgG effector functions (32). Indeed, it has recently been shown that nonsialylated IgG can more efficiently interact with activating Fc γ Rs (27). However, only a minimal in-

crease in the content of sialic acids of the IgG2b Δ 233–235 mutant ruled out the implication of sialylated glycoforms in its markedly reduced effector functions. Furthermore, it has been reported that galactose-less IgG can more efficiently activate complement through the lectin pathway in vitro, in which mannose-binding lectin (MBL) can interact with *N*-acetylglucosamines exposed as a result of absence of galactosylation (G0 in Fig. 4) (33). However, we observed an increased content of galactose-less glycoforms in both IgG1 and IgG2b Abs with the three amino acid deletion, which failed to activate complement. Thus, our present results argue against the implication of MBL in the activation of complement by galactose-less IgG in vivo. This is in line with recent results obtained in MBL-deficient mice, which demonstrated a dominant role of activating Fc γ Rs in mediating the pathogenicity of galactose-less IgG Abs (34). Collectively, our results rather suggest that the deletion at positions 233–235 could lead to a conformational change in the Fc region, thereby down-modulating IgG effector functions.

In conclusion, our present study revealed that the initiation of Fc γ R- and complement-mediated effector functions of IgG2b was profoundly affected by the three amino acid deletion at positions 233–235. In this regard, it should be mentioned that introduction of mutations at positions 234 and 245 of human IgG1 also led to the abrogation of binding to human Fc γ RI and Fc γ RIIa (35–37), highlighting the importance of these three amino acid residues in human IgG effector functions as well. Notably, the three amino acid deletion at positions 233–235 is not alone responsible for the lack of efficient interaction of the IgG1 subclass with Fc γ RIV and C1q, which results in poor effector functions. In addition, this subclass apparently carries a unique sequence motif to interact more efficiently with the inhibitory Fc γ RIIB. Since IgG bind to both activating Fc γ Rs and the inhibitory Fc γ RIIB, competitive engagement of these two types of Fc γ Rs is critical for the effector functions of individual IgG subclasses in vivo (4, 38). In view of a growing interest in therapeutic applications of mAb, identifying the precise amino acid residues implicated in the binding to C1q as well as activating and inhibitory Fc γ Rs would provide useful guiding principles for the engineering of mAb for in vivo applications.

Acknowledgments

We thank Dr. T. Moll for critical reading of the manuscript, and G. Celetta, G. Brighouse, G. Sealy, and T. Le Minh for technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

1. Fossati-Jimack, L., A. Ioan-Facsinay, L. Reininger, Y. Chicheportiche, N. Watanabe, T. Saito, F. M. Hofhuis, J. E. Gessner, C. Schiller, R. E. Schmidt, et al. 2000. Markedly different pathogenicity of four IgG isotype-switch variants of an anti-erythrocyte autoantibody is based on their respective capacity to interact in vivo with the low-affinity Fc γ RIII. *J. Exp. Med.* 191: 1293–1302.
2. Azeredo da Silveira, S., S. Kikuchi, L. Fossati-Jimack, T. Moll, T. Saito, J. S. Verbeek, M. Botto, M. J. Walport, M. Carroll, and S. Izui. 2002. Complement activation selectively potentiates the pathogenicity of the IgG2b and IgG3 isotypes of a high affinity anti-erythrocyte autoantibody. *J. Exp. Med.* 195: 665–672.
3. Baudino, L., F. Nimmerjahn, S. Azeredo da Silveira, E. Martinez-Soria, T. Saito, M. Carroll, J. V. Ravetch, J. S. Verbeek, and S. Izui. 2008. Differential contribution of three activating IgG Fc receptors (Fc γ RI, Fc γ RIII, and Fc γ RIV) to IgG2a- and IgG2b-induced autoimmune hemolytic anemia in mice. *J. Immunol.* 180: 1948–1953.
4. Nimmerjahn, F., and J. V. Ravetch. 2006. Fc γ receptors: old friends and new family members. *Immunity* 24: 19–28.
5. Takai, T., M. Li, D. Sylvestre, R. Clynes, and J. V. Ravetch. 1994. Fc γ chain deletion results in pleiotropic effector cell defects. *Cell* 76: 519–529.
6. Takai, T., M. Ono, M. Hikida, H. Ohmori, and J. V. Ravetch. 1996. Augmented humoral and anaphylactic responses in Fc γ RII-deficient mice. *Nature* 379: 346–349.

7. Sears, D. W., N. Osman, B. Tate, I. F. C. McKenzie, and P. M. Hogarth. 1990. Molecular cloning and expression of the mouse high affinity Fc receptor for IgG. *J. Immunol.* 144: 371–378.
8. Weinshank, R. L., A. D. Luster, and J. V. Ravetch. 1988. Function and regulation of a murine macrophage-specific IgG Fc receptor, FcγR-α. *J. Exp. Med.* 167: 1909–1925.
9. Nimmerjahn, F., P. Bruhns, K. Horiuchi, and J. V. Ravetch. 2005. FcγRIV: a novel FcR with distinct IgG subclass specificity. *Immunity* 23: 41–51.
10. Klaus, G. G., M. B. Pepys, K. Kitajima, and B. A. Askonas. 1979. Activation of mouse complement by different classes of mouse antibody. *Immunology* 38: 687–695.
11. Duncan, A. R., J. M. Woof, L. J. Partridge, D. R. Burton, and G. Winter. 1988. Localization of the binding site for the human high-affinity Fc receptor on IgG. *Nature* 332: 563–564.
12. Hazenbos, W. L., J. E. Gessner, F. M. Hofhuis, H. Kuipers, D. Meyer, I. A. F. M. Heijnen, R. E. Schmidt, M. Sandor, P. J. Capel, M. Daëron, et al. 1996. Impaired IgG-dependent anaphylaxis and Arthus reaction in FcγRIII (CD16) deficient mice. *Immunity* 5: 181–188.
13. Shibata, T., T. Berney, L. Reininger, Y. Chicheportiche, S. Ozaki, T. Shirai, and S. Izui. 1990. Monoclonal anti-erythrocyte autoantibodies derived from NZB mice cause autoimmune hemolytic anemia by two distinct pathogenic mechanisms. *Int. Immunol.* 2: 1133–1141.
14. Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77: 51–59.
15. Fossati-Jimack, L., L. Reininger, Y. Chicheportiche, R. Clynes, J. V. Ravetch, T. Honjo, and S. Izui. 1999. High pathogenic potential of low-affinity autoantibodies in experimental autoimmune hemolytic anemia. *J. Exp. Med.* 190: 1689–1696.
16. Petry, F., M. Botto, R. Holtappels, M. J. Walport, and M. Loos. 2001. Reconstitution of the complement function in C1q-deficient (C1qa^{-/-}) mice with wild-type bone marrow cells. *J. Immunol.* 167: 4033–4037.
17. Nishimura, S.-I., K. Niikura, M. Kuroguchi, T. Matsushita, M. Fumoto, H. Hinou, R. Kamitani, H. Nakagawa, K. Deguchi, N. Miura, et al. 2004. High-throughput protein glycomics: combined use of chemoselective glycoblotting and MALDI-TOF/TOF mass spectrometry. *Angew. Chem. Int. Ed. Engl.* 44: 91–96.
18. Furukawa, J. I., Y. Shinohara, H. Kuramoto, Y. Miura, H. Shimaoka, M. Kuroguchi, M. Nakano, and S.-I. Nishimura. 2008. Comprehensive approach to structural and functional glycomics based on chemoselective glycoblotting and sequential tag conversion. *Anal. Chem.* 80: 1094–1101.
19. Kita, Y., Y. Miura, J. Furukawa, M. Nakano, Y. Shinohara, M. Ohno, A. Takimoto, and S.-I. Nishimura. 2007. Quantitative glycomics of human whole serum glycoproteins based on the standardized protocol for liberating N-glycans. *Mol. Cell. Proteomics* 6: 1437–1445.
20. Miura, Y., Y. Shinohara, J. Furukawa, N. Nagahori, and S. Nishimura. 2007. Rapid and simple solid-phase esterification of sialic acid residues for quantitative glycomics by mass spectrometry. *Chemistry* 13: 4797–4804.
21. Uematsu, R., J. Furukawa, H. Nakagawa, Y. Shinohara, K. Deguchi, K. Monde, and S.-I. Nishimura. 2005. High throughput quantitative glycomics and glycoform-focused proteomics of murine dermis and epidermis. *Mol. Cell. Proteomics* 4: 1977–1989.
22. Nose, M., and H. Wigzell. 1983. Biological significance of carbohydrate chains on monoclonal antibodies. *Proc. Natl. Acad. Sci. USA* 80: 6632–6636.
23. Wright, A., and S. L. Morrison. 1994. Effect of altered CH-associated carbohydrate structure on the functional properties and in vivo fate of chimeric mouse-human immunoglobulin G1. *J. Exp. Med.* 180: 1087–1096.
24. Nandakumar, K. S., M. Collin, A. Olsen, F. Nimmerjahn, A. M. Blom, J. V. Ravetch, and R. Holmdahl. 2007. Endoglycosidase treatment abrogates IgG arthritogenicity: importance of IgG glycosylation in arthritis. *Eur. J. Immunol.* 37: 2973–2982.
25. Parekh, R. B., R. A. Dwek, B. J. Sutton, D. L. Fernandes, A. Leung, D. Stanworth, T. W. Rademacher, T. Mizuochi, T. Taniguchi, K. Matsuta, et al. 1985. Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature* 316: 452–457.
26. Mizuochi, T., J. Hamako, M. Nose, and K. Titani. 1990. Structural changes in the oligosaccharide chains of IgG in autoimmune MRL/Mp-lpr/lpr mice. *J. Immunol.* 145: 1794–1798.
27. Kaneko, Y., F. Nimmerjahn, and J. V. Ravetch. 2006. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* 313: 670–673.
28. Meyer, D., C. Schiller, J. Westermann, S. Izui, W. L. W. Hazenbos, J. S. Verbeek, R. E. Schmidt, and J. E. Gessner. 1998. FcγRIII (CD16)-deficient mice show IgG isotope-dependent protection to experimental autoimmune hemolytic anemia. *Blood* 92: 3997–4002.
29. Kaneko, Y., F. Nimmerjahn, M. P. Madaio, and J. V. Ravetch. 2006. Pathology and protection in nephrotoxic nephritis is determined by selective engagement of specific Fc receptors. *J. Exp. Med.* 203: 789–797.
30. Duncan, A. R., and G. Winter. 1988. The binding site for C1q on IgG. *Nature* 332: 738–740.
31. Tao, M. H., R. I. Smith, and S. L. Morrison. 1993. Structural features of human immunoglobulin G that determine isotype-specific differences in complement activation. *J. Exp. Med.* 178: 661–667.
32. Lund, J., N. Takahashi, J. D. Pound, M. Goodall, and R. Jefferis. 1996. Multiple interactions of IgG with its core oligosaccharide can modulate recognition by complement and human Fcγ receptor I and influence the synthesis of its oligosaccharide chains. *J. Immunol.* 157: 4963–4969.
33. Malhotra, R., M. R. Wormald, P. M. Rudd, P. B. Fischer, R. A. Dwek, and R. B. Sim. 1995. Glycosylation changes of IgG associated with rheumatoid arthritis can activate complement via the mannose-binding protein. *Nat. Med.* 1: 237–243.
34. Nimmerjahn, F., R. M. Anthony, and J. V. Ravetch. 2007. Agalactosylated IgG antibodies depend on cellular Fc receptors for in vivo activity. *Proc. Natl. Acad. Sci. USA* 104: 8433–8437.
35. Chappel, M. S., D. E. Isenman, M. Everett, Y. Y. Xu, K. J. Dorning, and M. H. Klein. 1991. Identification of the Fcγ receptor class I binding site in human IgG through the use of recombinant IgG1/IgG2 hybrid and point-mutated antibodies. *Proc. Natl. Acad. Sci. USA* 88: 9036–9040.
36. Canfield, S. M., and S. L. Morrison. 1991. The binding affinity of human IgG for its high affinity Fc receptor is determined by multiple amino acids in the CH2 domain and is modulated by the hinge region. *J. Exp. Med.* 173: 1483–1491.
37. Wines, B. D., M. S. Powell, P. W. Parren, N. Barnes, and P. M. Hogarth. 2000. The IgG Fc contains distinct Fc receptor (FcR) binding sites: the leukocyte receptors FcγRI and FcγRIIa bind to a region in the Fc distinct from that recognized by neonatal FcR and protein A. *J. Immunol.* 164: 5313–5318.
38. Nimmerjahn, F., and J. V. Ravetch. 2005. Divergent immunoglobulin-G subclass activity through selective Fc receptor binding. *Science* 310: 1510–1512.