

Complement Activation Selectively Potentiates the Pathogenicity of the IgG2b and IgG3 Isotypes of a High Affinity Anti-Erythrocyte Autoantibody

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Abstract

By generating four IgG isotype-switch variants of the high affinity 34–3C anti-erythrocyte autoantibody, and comparing them to the IgG variants of the low affinity 4C8 anti-erythrocyte autoantibody that we have previously studied, we evaluated in this study how high affinity binding to erythrocytes influences the pathogenicity of each IgG isotype in relation to the respective contributions of Fc γ receptor (Fc γ R) and complement. The 34–3C autoantibody opsonizing extensively circulating erythrocytes efficiently activated complement in vivo (IgG2a = IgG2b > IgG3), except for the IgG1 isotype, while the 4C8 IgG autoantibody failed to activate complement. The pathogenicity of the 34–3C autoantibody of IgG2b and IgG3 isotypes was dramatically higher (>200-fold) than that of the corresponding isotypes of the 4C8 antibody. This enhanced activity was highly (IgG2b) or totally (IgG3) dependent on complement. In contrast, erythrocyte-binding affinities only played a minor role in in vivo hemolytic activities of the IgG1 and IgG2a isotypes of 34–3C and 4C8 antibodies, where complement was not or only partially involved, respectively. The remarkably different capacities of four different IgG isotypes of low and high affinity anti-erythrocyte autoantibodies to activate Fc γ R-bearing effector cells and complement in vivo demonstrate the role of autoantibody affinity maturation and of IgG isotype switching in autoantibody-mediated pathology.

Key words: autoimmune hemolytic anemia • complement receptor • Fc receptor • IgG isotype • phagocytosis

Introduction

Autoantibodies are the essential factors for particular clinical manifestations associated with a number of autoimmune diseases. The pathogenic potential of autoantibodies is likely to be determined by the combined action of the self-antigen binding properties (specificity and affinity) of the Fab region and the effector functions associated with the Fc region of the different Ig isotypes. Therefore, it is conceivable that a change of Ig isotype may result in a remarkable change of the autoantibody's pathogenic potential, because Ig class switching can alter Fc-dependent effector functions

and can be accompanied by concomitant changes in autoantibody affinity. To address this question, we have recently analyzed the pathogenic activity of four different IgG switch variants derived from a murine anti-RBC monoclonal autoantibody, 4C8, originally established from autoimmune-prone NZB mice (1, 2). To our surprise, despite its very poor RBC-binding activity, the IgG2a isotype of the low affinity 4C8 mAb was highly pathogenic and capable of inducing anemia at a dose comparable to that of a high affinity 34–3C IgG2a anti-RBC mAb. This high pathogenicity was apparently due to the capacity of the IgG2a isotype to interact very efficiently with the IgG Fc receptor (Fc γ R) involved in erythrophagocytosis, consistent with a poor pathogenicity of the three other IgG iso-

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types (IgG1, IgG2b, and IgG3) of the 4C8 mAb having a limited interaction with Fc γ R. However, it has not been explored whether high affinity anti-RBC autoantibodies of IgG1, IgG2b, and IgG3 isotypes could become more pathogenic as a result of a markedly increased RBC-binding and thereby enhanced interaction with Fc γ R and/or activation of complement.

Among the various effector functions mediated by the Ig heavy-chain constant regions, Fc γ R-mediated erythrophagocytosis has been recognized as the major pathogenic mechanism responsible for autoimmune hemolytic anemia in mice (2–5). Murine phagocytic effector cells express two different classes of phagocytic Fc γ R, high affinity Fc γ RI, and low affinity Fc γ RIII (for a review, see reference 6). These are hetero-oligomeric complexes, in which the respective ligand-binding α -chains are associated with the common γ -chain (FcR γ). FcR γ is required for the assembly of both Fc γ RI and Fc γ RIII and for the triggering of their various effector functions, including phagocytosis (7). Our recent analysis by the use of different strains of Fc γ R-deficient mice revealed that the capacity of each IgG isotype to interact with the low affinity Fc γ RIII is the critical factor determining the pathogenic potency of individual IgG isotypes of the 4C8 anti-RBC mAb (1, 2), as the high affinity Fc γ RI apparently plays a relatively limited role, probably because of the competition by circulating monomeric IgG2a.

In contrast, it has been suggested that complement activation plays a minimal role in the development of anemia induced by anti-RBC antibodies (3, 4, 8). However, the interpretation of these results was still tentative, as murine IgG2a, IgG2b, and IgG3 isotypes (but not IgG1) efficiently activate complement (9, 10). The lack of the involvement of complement in anemia induced by the low affinity 4C8 IgG switch variants could be due to a limited opsonization of RBC in mice injected with this mAb (1, 2). Earlier studies in complement-deficient guinea pigs suggested the synergistic cooperation of Fc γ R and CRs expressed on Kupffer cells in erythrophagocytosis of RBCs sensitized with polyclonal rabbit anti-RBC antibodies (11). Thus, the pathogenicity of high affinity anti-RBC autoantibodies of IgG2b and IgG3 isotypes, which poorly interact with Fc γ R, could be promoted as a result of complement activation, either by acting synergistically with Fc γ R or by triggering CR-dependent erythrophagocytosis.

We have generated IgG class-switch variants (IgG1, IgG2b, and IgG3) of the high affinity 34–3C IgG2a anti-RBC mAb derived from NZB mice (3) in order to better define the role of autoantibody binding affinities in relation to Fc-associated effector functions in the development of autoimmune hemolytic anemia. The level of *in vivo* opsonization of circulating RBCs induced by the high affinity 34–3C mAb was remarkably enhanced, compared with the low affinity 4C8 anti-RBC IgG variants having <1,000 times weaker RBC-binding activity (1). Therefore, by assessing the pathogenic effect of the 34–3C IgG variants in mice deficient in Fc γ R and/or C3, we should be able to study how high affinity binding to RBCs modulates the pathogenic potential of each IgG isotype, and whether the

level of RBC opsonization *in vivo* could influence the extent of complement activation and Fc γ R interaction. We show in this study that the pathogenicity of the high affinity 34–3C anti-RBC mAb is markedly enhanced, compared with that of the low affinity 4C8 mAb, but in an IgG isotype-dependent manner. Remarkable and selective enhancement of the pathogenicity of the IgG2b and IgG3 isotypes (>200-fold increases) was associated with a marked activation of complement, as a result of extensive opsonization of circulating RBCs. In contrast, for the IgG1 and IgG2a isotypes, high affinity binding exhibited only a minimal effect on their hemolytic activities *in vivo*, which were poorly dependent on complement activation.

Materials and Methods

Mice. BALB/c mice were purchased from Gl. Bomholtgard Ltd. FcR γ -deficient (FcR γ ^{-/-}) mice (lacking functional expression of both Fc γ RI and Fc γ RIII) with a pure C57BL/6 (B6) background, and their corresponding wild-type (WT)* littermates were developed as described previously (12). C1q- and C3-deficient mice were backcrossed for 10 and 5 generations with B6 mice, respectively (13, 14). FcR γ and C3 double-deficient mice (FcR γ /C3^{-/-}) were obtained from intercross between FcR γ ^{-/-} and C3^{-/-} B6 mice. Mice deficient in C3 were identified by the absence of serum C3, as determined by ELISA, and the FcR γ ^{-/-} genotype was determined by PCR analysis using a combination of the following sets of primers: WT-specific sense primer (5'-CCAACGCTATGTCCTGATAG-3'), mutant-specific sense primer (5'-TGCTGTCCTGTTTTTGTATGG-3'), and common antisense primer (5'-GCTGCCTTCGGACCTGGAT-3'). μ MT B6 mice deficient in B cells (15) were obtained from B&K Universal.

DNA Constructions. The VD γ H34–3C-C γ 1, -C γ 2b, and -C γ 3 plasmids containing the complete 34–3C IgG heavy-chain gene of the respective IgG subclass were constructed using the following DNA fragments: the rearranged VD γ J (variable-diversity-joining) region isolated from cDNA encoding the variable region of the heavy chain of the 34–3C mAb, the promoter region isolated from pSV-V μ 1 (16), the heavy-chain enhancer region isolated from pSVE2-neo (17) and the C γ 1, C γ 2b, or C γ 3 region derived from the respective genomic clones, pEVHC γ 1 (17), pIgH22 (18), and pJW7 (19).

mAb. The hybridoma secreting the 34–3C IgG2a high affinity anti-RBC monoclonal autoantibody was derived from unmanipulated NZB mice (3). The 34–3C IgG1, IgG2b, and IgG3 class-switch variants were obtained by transfecting 34–3C heavy-chain-loss mutant cells by electroporation with the respective VD γ H34–3C-C γ plasmids together with a pSVE2-neo plasmid containing the neomycin-resistant gene, as described previously (1). IgG mAb were purified from culture supernatants by protein G column chromatography. The purity of IgG was >90% as documented by SDS/PAGE. The 34–3C IgG class-switch variants exhibited comparable mouse RBC-binding activity *in vitro*, as assessed by a flow cytometric analysis (1). The generation of IgG switch variants of the 4C8 low affinity anti-RBC mAb was described previously (1, 2).

Detection of C3 in Sera and of Opsonized RBCs in the Circulating Blood. The presence or absence of C3 in sera from mice were determined by ELISA. Briefly, IgG goat anti-mouse C3 antibody

*Abbreviations used in this paper: Ht, hematocrit; WT, wild-type.

ies (Capell Laboratories) were used for coating microtiter plates, and incubated overnight at 4°C with serum samples at a dilution of 1/1,000. Then, the assay was developed with alkaline phosphatase-labeled goat anti-mouse C3 conjugates. The presence of opsonized RBCs in mice injected with 34-3C or 4C8 anti-RBC mAb was detected by a similar flow cytometric assay, using biotinylated goat anti-mouse C3 or rat anti-mouse *k* chain mAb (H139.52.1.5), followed by PE-conjugated streptavidin, as described (1). The specificity of the assay for C3 opsonization was confirmed by the absence of staining on circulating RBCs in C3^{-/-} mice.

Experimental Autoimmune Hemolytic Anemia. Autoimmune hemolytic anemia was induced by a single intraperitoneal injection of purified anti-RBC mAb into two to three mo-old mice. Blood samples were collected into heparinized microhematocrit tubes every 2 d after the injection, and hematocrits (Hts) were directly determined after centrifugation, as described previously (2). The injection of mAb was controlled 24 h later by assessing the level of antibody opsonization of circulating RBCs by a flow cytometric analysis using biotinylated rat anti-mouse *k* chain mAb. Livers were obtained 8 d after injection of mAb, processed for histological examination, and stained with hematoxylin and eosin. The extent of in vivo RBC destruction by Kupffer cell-mediated phagocytosis was determined by Perls iron staining.

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

Results

Efficient Activation of Complement by the High Affinity 34-3C IgG Class-switch Variants, but Not by the Low Affinity 4C8 IgG Variants. To assess the ability of individual IgG isotypes of the 34-3C anti-RBC mAb to activate complement in vivo, we analyzed by flow cytometry the extent of C3 deposition on circulating RBCs 24 h after a single intraperitoneal injection of 50 or 200 µg of purified mAb into BALB/c mice. The highest and comparable levels of C3 opsonization were observed in mice injected with the IgG2a and IgG2b isotypes; the IgG3 isotype was less efficient (Fig. 1). As expected, no significant C3 opsonization was observed in mice receiving the IgG1 isotype, which is

known not to activate complement efficiently (9, 10). As it has been claimed that murine IgG3 isotype failed to activate the classical pathway of complement (20), the levels of C3 opsonization were evaluated in C1q-deficient B6 mice. When these mice were injected with 200 µg of the 34-3C IgG3 variant, C3-opsonized RBCs were no longer detectable in the blood (Fig. 1), indicating the activation of the classical complement pathway after the binding of the 34-3C IgG3 mAb to RBCs. In contrast, the injection of the low affinity 4C8 IgG variants of any isotype failed to induce a significant C3 opsonization on circulating RBCs (Fig. 1).

Markedly Enhanced Pathogenicity of IgG2b and IgG3, but not IgG1 and IgG2a Isotypes of the High Affinity 34-3C Anti-RBC mAb, Compared with the Low Affinity 4C8 IgG Variants. The pathogenic activity of individual IgG isotypes of the 34-3C mAb was analyzed by a single intraperitoneal injection of 200 µg of purified mAb into BALB/c mice. The IgG2a and IgG2b isotypes of the 34-3C mAb induced the most severe form of anemia (a decrease in mean Ht values to 10–12% at day 4), the IgG3 variant a mild anemia (average Ht of 30%), and the IgG1 variant was unable to significantly decrease Ht levels (Fig. 2). Notably, the extent of opsonization of circulating RBCs 24 h after mAb injection was comparable in mice treated with different IgG variants, as revealed by rat anti-mouse *k* chain mAb (data not shown).

To compare more quantitatively the pathogenic activity of individual IgG isotypes of the 34-3C mAb, various amounts of mAb were intraperitoneally injected into BALB/c mice and the quantities of mAb required to induce anemia (decreasing Ht values to <40%) were estimated. Mild anemia was induced by the injection of 25 µg of the 34-3C IgG2a and IgG2b mAb, while 100 µg of the IgG3 isotype and 500 µg of the IgG1 isotype were required to provoke a drop of Ht values below 40% (Table I). Thus, the pathogenic potency of the 34-3C IgG2a and IgG2b isotypes was approximately 4- and 20-fold higher than that of the IgG3 and IgG1 isotypes, respectively. Histological examinations showed that the extent of erythrophagocytosis, documented by iron deposits in hepatic

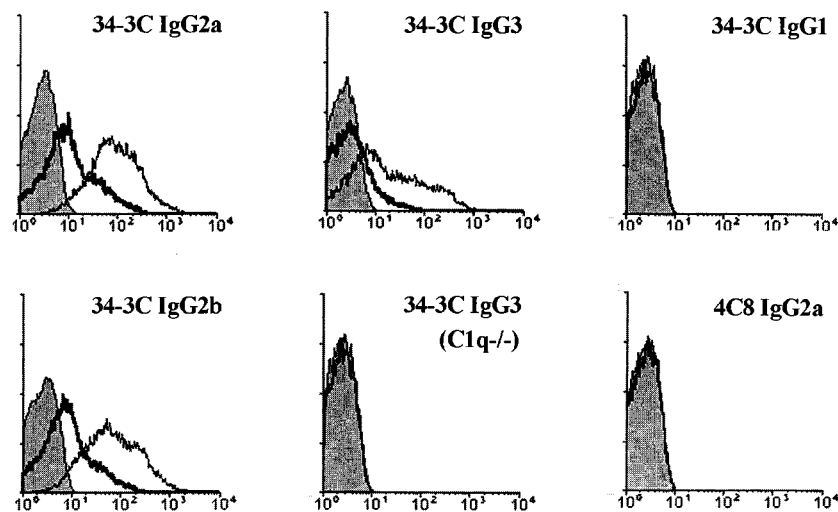


Figure 1. Flow cytometric analysis of complement activation in vivo by the 34-3C and 4C8 IgG class-switch variants. Mouse RBCs were obtained 24 h after an intraperitoneal injection of 50 or 200 µg of 34-3C or 4C8 IgG variants into mice, and then stained with biotinylated goat anti-mouse C3 antibodies, followed by PE-conjugated streptavidin. Results obtained with 50 µg (thick lines) and 200 µg (thin lines) of the 34-3C IgG2a, IgG2b, and IgG3 variants in BALB/c mice, 200 µg of the 34-3C IgG3 variant in C1q^{-/-} mice, and 200 µg of the 34-3C IgG1 or 4C8 IgG2a variant in BALB/c mice are shown. Shaded areas indicate the background staining with PE-conjugated streptavidin.

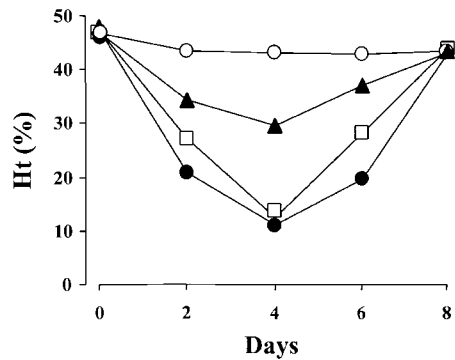


Figure 2. Development of anemia induced by the 34-3C IgG class-switch variants in mice. BALB/c mice were injected intraperitoneally with 200 μ g of purified 34-3C IgG variants (IgG1, ○; IgG2a, ●; IgG2b, □; IgG3, ▲) on day 0. Results are expressed as mean Ht values of five mice.

Kupffer cells, correlated in all these cases with the level of anemia (data not shown).

When the pathogenic activity of the high affinity 34-3C IgG variants was compared with that of the low affinity 4C8 IgG variants (2), the ability to induce mild anemia was dramatically enhanced (200-fold and even more) in the 34-3C IgG2b and IgG3 isotypes (Table I). In contrast, the minimal amount of the IgG1 and IgG2a isotypes required for the induction of mild anemia was almost comparable between the 34-3C and 4C8 mAb. However, for the induction of a severe form of anemia (causing a 50% decrease in Ht values), 10 times lesser amounts of the 34-3C IgG2a mAb (100 μ g) were sufficient, compared with the 4C8 IgG2a mAb (1 mg), indicating that the high affinity 34-3C IgG2a mAb became more pathogenic at higher doses than the low affinity 4C8 IgG2a mAb.

Major Role of Fc γ R and Secondary Role of Complement in the Development of Anemia Induced by 34-3C IgG2a Anti-RBC mAb. As the 34-3C IgG2a mAb efficiently activated complement in vivo, we reevaluated the respective contributions of Fc γ R and complement to the anemia induced by two different doses (50 and 200 μ g) of the 34-3C IgG2a mAb in B6 mice deficient in FcR γ (i.e. lacking functional expression of both phagocytic Fc γ RI and Fc γ RIII) and/or C3. The development of anemia occurring in WT mice injected with 50 μ g of the 34-3C IgG2a mAb was almost completely prevented in FcR γ ^{-/-} mice ($P < 0.01$), while no significant protection was observed in C3^{-/-} mice ($P > 0.05$; Fig. 3 and Table II). Histological analysis still revealed the presence of substantial iron deposits in Kupffer cells in FcR γ ^{-/-} mice receiving the 34-3C IgG2a mAb. However, such deposits were no longer detectable in 34-3C IgG2a mAb-injected FcR γ /C3^{-/-} mice. In contrast, the level of protection from anemia provoked by the injection of 200 μ g mAb was only moderate in FcR γ ^{-/-} mice, while a lower but significant level of protection was observed in C3^{-/-} mice ($P < 0.005$; Fig. 3 and Table II). Notably, the development of anemia was completely prevented in FcR γ /C3^{-/-} mice injected with this high dose of the 34-3C IgG2a mAb ($P < 0.01$). These data indicated

Table I. Estimation of Quantities of the High Affinity 34-3C and Low Affinity 4C8 IgG Class-switch Variants Required for Inducing Mild and Severe Anemia

Isotype	34-3C mAb		4C8 mAb	
	Mild anemia ^a	Severe anemia ^b	Mild anemia ^a	Severe anemia ^b
IgG1	500 μ g	ND ^c	1 mg	ND
IgG2a	25 μ g	100 μ g	50 μ g	1 mg
IgG2b	25 μ g	100 μ g	>5 mg	ND
IgG3	100 μ g	ND	Not pathogenic	

^aThe quantity of the 34-3C and 4C8 IgG variants required for inducing mild anemia (decreasing Ht values to <40%) in BALB/c mice.

^bThe quantity of the 34-3C and 4C8 IgG variants required for inducing severe anemia (causing a 50% decrease in Ht values) in BALB/c mice.

^cNot done.

a minor but still significant contribution of CR-mediated erythrophagocytosis to the development of the severe form of anemia induced by a high dose (200 μ g) of the 34-3C IgG2a mAb.

Comparable Contributions of Fc γ R and Complement to the Development of Anemia Induced by 34-3C IgG2b Anti-RBC mAb. We similarly evaluated the respective roles of Fc γ R and complement in the development of anemia induced by the 34-3C IgG2b mAb. This antibody is able to activate complement as strongly as the IgG2a isotype, but interacts less efficiently with Fc γ R (2). The development of anemia occurring in WT B6 mice injected with 50 μ g of the 34-3C IgG2b mAb was completely prevented in FcR γ ^{-/-} B6 mice ($P < 0.005$; Fig. 4 and Table II). Histological analysis confirmed the absence of erythrophagocytosis, as documented by the lack of iron deposits in Kupffer cells. However, unlike after injection of the 34-3C IgG2a mAb, the development of anemia was also markedly prevented in

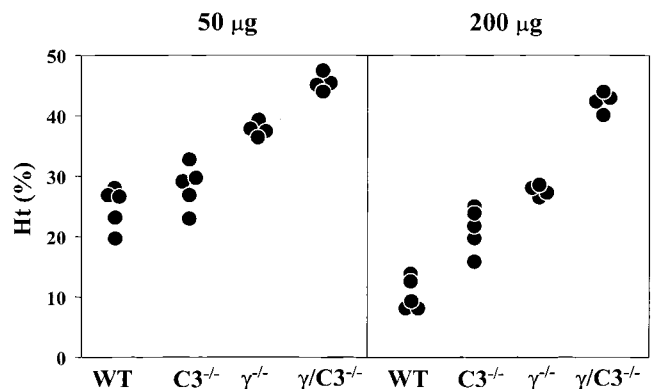


Figure 3. Development of anemia in WT, C3^{-/-}, FcR γ ^{-/-}, or FcR γ /C3^{-/-} mice after the injection of the 34-3C IgG2a mAb. Ht values of individual mice measured 4 d after the intraperitoneal injection of 50 or 200 μ g of the mAb are shown.

Table II. Pathogenic Activities of the 34–3C IgG Class-switch Variants in WT, C3^{-/-}, FcRγ^{-/-}, and FcRγ/C3^{-/-} B6 Mice

Isotype	Dose	Ht (%) ^a				In vivo erythrophagocytosis ^b			
		WT	C3 ^{-/-}	FcRγ ^{-/-}	FcRγ/C3 ^{-/-}	WT	C3 ^{-/-}	FcRγ ^{-/-}	FcRγ/C3 ^{-/-}
IgG2a	50 μg	25 ± 3	28 ± 4	39 ± 1	46 ± 2	++	++	+	-
	200 μg	10 ± 3	21 ± 4	28 ± 1	43 ± 2	+++	+/+++	++	-
IgG2b	50 μg	28 ± 2	38 ± 3	44 ± 1	ND ^c	+/+++	±	-	ND
	200 μg	12 ± 3	36 ± 3	36 ± 1	45 ± 2	+++	+/+++	+/+++	-
IgG1	500 μg	37 ± 1	38 ± 2	46 ± 2	ND	+	+	-	ND
IgG3	200 μg	30 ± 3	40 ± 1	30 ± 3	ND	+	-	+	ND

^aHt values (mean of 4–5 mice ± 1SD) were determined 4 d after the intraperitoneal injection of purified 34–3C IgG variants (data from Figs. 3, 4, and 5). Ht values before the injection of anti-RBC mAb in WT, C3^{-/-}, FcRγ^{-/-}, and FcRγ/C3^{-/-} mice were in a range from 44–48%.

^bThe extent of erythrophagocytosis by Kupffer cells in the liver is arbitrarily graded, in a blinded fashion, on the basis of the intensity of Perls iron staining on liver sections.

^cNot done.

C3^{-/-} mice ($P < 0.005$), which exhibited only a limited extent of erythrophagocytosis. At a highly pathogenic dose (200 μg) of the 34–3C IgG2b mAb, the development of anemia was strongly, though not completely, inhibited in both FcRγ^{-/-} and C3^{-/-} mice ($P < 0.005$; Fig. 4 and Table II). As in the case of the IgG2a isotype, FcRγ/C3^{-/-} mice were totally resistant to the pathogenic effect of 200 μg of the 34–3C IgG2b mAb ($P < 0.01$), and did not show any significant iron deposits in Kupffer cells.

Differential Role of FcγR and Complement in the Development of Anemia Induced by 34–3C IgG1 and IgG3 Anti-RBC mAb. Using high affinity 105–2H IgG1 and low affinity 4C8 IgG1 anti-RBC mAb derived from NZB mice, it has been well established that FcγRIII is the sole FcγR mediating IgG1-dependent phagocytosis in vivo (2, 5, 21). In agreement with these prior observations, FcRγ^{-/-} mice were completely resistant to the pathogenic effect of the 34–3C IgG1 variant ($P < 0.01$), and failed to show any sign of erythrophagocytosis (Fig. 5 and Table II). Notably,

the development of anemia was not prevented in C3^{-/-} mice after the injection of the 34–3C IgG1 isotype.

Unlike the IgG1, IgG2a, and IgG2b isotypes of the 34–3C mAb, FcRγ-deficient mice were not resistant at all to the pathogenic effect of 200 μg of the 34–3C IgG3 variant (Fig. 5 and Table II). In contrast, the development of anemia was completely prevented in C3^{-/-} mice ($P < 0.01$), in which erythrophagocytosis by Kupffer cells was no longer detectable.

A recent in vitro study has reported that polymeric IgG3 is capable of interacting with FcγRI (22). Thus, an only limited usage of FcγRI in vivo for phagocytosis of 34–3C IgG3-opsonized RBCs could be due to competition with excess amounts of circulating monomeric IgG2a having a high affinity interaction with FcγRI (23). If this were the case, one could expect an enhancement of the pathogenic effect of the 34–3C IgG3 mAb in Ig-deficient μMT B6 mice because of the absence of competition with IgG2a. However, after injection of 50 μg of the 34–3C IgG3

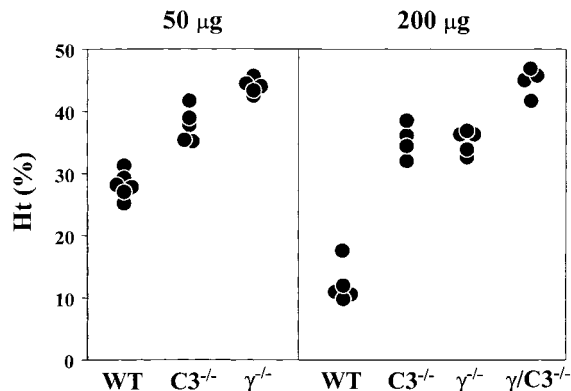


Figure 4. Development of anemia in WT, C3^{-/-}, FcRγ^{-/-}, or FcRγ/C3^{-/-} mice after the injection of the 34–3C IgG2b variant. Ht values of individual mice measured 4 d after the intraperitoneal injection of 50 or 200 μg of the mAb are shown.

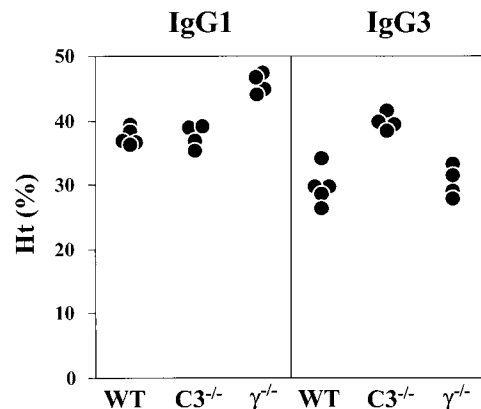


Figure 5. Development of anemia in WT, C3^{-/-}, or FcRγ^{-/-} mice after the injection of 34–3C IgG1 or IgG3 variant. Ht values of individual mice measured 4 d after the intraperitoneal injection of 500 μg of 34–3C IgG1 or 200 μg of 34–3C IgG3 mAb are shown.

mAb, μ MT mice failed to show significant drops in Ht values and increased sign of erythrophagocytosis, compared with WT mice (mean Ht values of four mice 4 d after the injection: μ MT mice, $46.9 \pm 1.3\%$; WT mice, $45.5 \pm 0.5\%$).

Discussion

We have generated three IgG class-switch variants bearing identical VH and V k regions of a high affinity 34–3C IgG2a anti-RBC autoantibody derived from lupus-prone NZB mice, and determined how a high binding affinity to circulating RBCs influenced the pathogenicity of individual IgG isotypes in relation to the respective contributions of Fc γ R and complement. Strikingly, the pattern of the IgG isotype-dependent pathogenicity of the 34–3C mAb (IgG2a = IgG2b > IgG3 > IgG1) was totally different from that of the low affinity 4C8 mAb (IgG2a > IgG1 > IgG2b > IgG3) (2). The combined analysis in mice deficient in FcR γ or C3 revealed that this difference was primarily due to a marked enhancement of the pathogenic effect of the IgG2b and IgG3 isotypes (>200-fold increases) as a result of complement activation, which not only triggered CR-dependent erythrophagocytosis but also promoted Fc γ R-mediated erythrophagocytosis through synergistic cooperation with CR. In contrast, a high affinity binding to RBCs barely augmented in vivo hemolytic activities of the IgG1 and IgG2a isotypes, in which complement played no or only a minimal role. Our data thus define the role of autoantibody affinity maturation and IgG isotype switching in relation to the activation of Fc γ R and complement in vivo in the pathogenesis of autoimmune hemolytic anemia.

The analysis of IgG switch variants of the high affinity 34–3C mAb has clarified the issue concerning the role of complement in the development of autoimmune hemolytic anemia (Table III). As already described previously (2, 4, 5), Fc γ R-mediated erythrophagocytosis was the major mechanism for the induction of anemia induced by the IgG2a isotype, which most efficiently interacts with both classes of phagocytic Fc γ R, Fc γ RI, and Fc γ RIII (2). C3 deficiency only minimally affected the development of mild anemia at a low dose (50 μ g) of this isotype, despite its efficient activation of complement. This indicates that CR-mediated erythrophagocytosis apparently requires an extensive opsonization of C3 fragments on RBCs. On the contrary, a limited opsonization with IgG2a antibodies is sufficient to trigger Fc γ R-dependent erythrophagocytosis, as observed in mice injected with the low affinity 4C8 IgG2a mAb, which very poorly opsonizes circulating RBCs (1). Notably, CR-dependent erythrophagocytosis significantly contributed to the development of the severe form of anemia induced by a high dose (200 μ g) of the 34–3C IgG2a mAb. Thus, differential contribution of complement to the development of anemia induced by low and high doses of the high affinity 34–3C IgG2a mAb would explain why the 34–3C mAb became more pathogenic only at higher doses, but not at lower doses, compared with the low affinity 4C8

Table III. Pathogenic Activities of the 34–3C IgG Class-switch Variants and Respective Contributions of Fc γ R and Complement to the 34–3C IgG-induced Anemia

Isotype	Pathogenicity	Effector functions
IgG2a	+++	Fc γ R > complement
IgG2b	+++	Fc γ R and complement
IgG3	++	Complement
IgG1	+	Fc γ R

mAb, which poorly activated complement in vivo. The latter observation also indicates that relatively high density of IgG is required for efficient binding and activation of C1. Thus, complement activation by the classical pathway could be markedly influenced by antibody affinity, in addition to the density and distribution of epitope, which is responsible for creating an appropriate angle of the Fab arms of IgG required for C1 activation (24, 25).

In contrast to the IgG2a isotype, both complement and Fc γ R contributed almost equally well to the development of anemia induced by the IgG2b isotype (Table III). However, the role of complement is apparently different between anemia induced at low and high doses of this isotype. The development of mild anemia provoked by a low dose (50 μ g) was markedly prevented in C3^{-/-} mice, but completely prevented in Fc γ R-deficient mice. This last observation suggests that Fc γ R is the sole receptor involved in erythrophagocytosis, but that, because of a very weak affinity of Fc γ R to the IgG2b isotype (2), moderately opsonized RBCs require an additional involvement of complement to trigger optimally Fc γ R-dependent phagocytosis. This interpretation is consistent with previous reports suggesting a synergistic cooperation of CR and Fc γ R for phagocytosis in vivo and in vitro (11, 26, 27). However, this was no longer the case for severe anemia after injection of a high dose (200 μ g) of the IgG2b isotype, as anemia developed in mice deficient in either Fc γ R or C3, but not in those deficient in both Fc γ R and C3. It is possible that the extensive opsonization resulting from the injection of this high dose could not only overcome the low avidity interaction between Fc γ R and IgG2b isotype to provoke Fc γ R-mediated phagocytosis, independently of complement activation, but also trigger CR-mediated erythrophagocytosis. Our data thus indicate a critical role for the autoantibody affinity in the pathogenic activity of the IgG2b isotype by promoting IgG Fc-associated effector functions.

As expected from our previous studies (2, 5), the 34–3C IgG1 isotype triggered erythrophagocytosis only by activating Fc γ R, but not complement (Table III). It is somehow surprising to see that the pathogenic potential of the high affinity 34–3C IgG1 mAb was poor and almost comparable to that of the low affinity 4C8 IgG1 variant. This is likely to result from the lack of complement activation, which is, in contrast, partly responsible for the markedly enhanced

pathogenicity of the IgG2b isotype of the high affinity 34–3C mAb, as discussed above. Another important difference is that the IgG1 isotype interacts only with Fc γ RIII (2, 5, 21), while the IgG2b isotype is apparently able to interact not only with Fc γ RIII but also with another phagocytic receptor, Fc γ RI (2). In this respect, we recently observed that the development of anemia triggered by the 34–3C IgG2b variant was significantly inhibited in Fc γ RI-deficient mice as well as in Fc γ RIII-deficient mice (unpublished data). Accordingly, RBCs highly opsonized with 34–3C IgG2b mAb could interact with three different receptors, Fc γ RI, Fc γ RIII, and CR, thereby potentiating erythrophagocytosis through synergistic cooperation among these three receptors.

In marked contrast to the three other IgG isotypes, the development of anemia provoked by the high affinity 34–3C IgG3 variant was totally dependent on complement activation, and this isotype apparently fails to interact with phagocytic Fc γ R (Table III). There has been a controversy about the presence of any receptor for murine IgG3 isotype. Diamond and Yelton have proposed the presence of an IgG3-specific phagocytic Fc γ R expressed on macrophages (28). This has also been supported by a recent study, demonstrating the possible involvement of nonconventional Fc γ R in the phagocytosis of IgG3-opsonized cryptococci by peritoneal macrophages *in vitro* (29). In contrast, using bone marrow-derived macrophages from Fc γ RI-deficient mice, Fc γ RI has been claimed to be the sole receptor for the IgG3 isotype (22). The present results showing that Kupffer cell-mediated phagocytosis of RBCs highly opsonized with the 34–3C IgG3 mAb is not inhibited in FcR γ -deficient mice, but completely abrogated in C3-deficient mice strongly argue not only against any significant affinity of conventional Fc γ R to murine IgG3 isotype *in vivo*, but also against the presence of a novel IgG3-specific Fc γ R mediating phagocytosis. The absence of interaction of 34–3C IgG3-opsonized RBCs with Fc γ RI *in vivo* could be explained by competition with circulating monomeric IgG2a having a high affinity interaction with Fc γ RI (23). However, the lack of enhanced erythrophagocytosis by Kupffer cells in Ig-deficient μ MT mice after injection of the 34–3C IgG3 mAb argues against this hypothesis.

The use of four different anti-RBC IgG switch variants of the high affinity 34–3C and the low affinity 4C8 mAb in this and previous studies (2) has provided a unique opportunity to define the pathogenic potency of individual murine IgG isotypes in relation to autoantibody affinity and to IgG Fc-dependent effector functions in the development of autoimmune hemolytic anemia. Taken together, our studies have demonstrated the importance of the autoantigen-binding affinity for the pathogenicity of certain IgG isotypes (IgG2b and IgG3) by promoting the activation of complement and/or Fc γ R-bearing effector cells. Studies with an IgG2a anti-platelet monoclonal autoantibody have shown a minimal role of complement in immune elimination of platelets (4, 8, 30); however, more extensive analysis with other IgG isotypes of anti-platelet autoantibodies is awaited to elucidate the potential role of complement

in immune thrombocytopenic purpura. Furthermore, it should also be stressed that, in addition to the role of Fc γ R in activation of inflammatory effector cells (12, 31, 32), complement is apparently required for the full-blown expression of immune complex-mediated inflammatory reactions in several experimental models including Arthus reaction, immune complex alveolitis, and anti-glomerular basement membrane nephritis (33–37). Thus, complement is actively implicated in autoantibody- and immune complex-triggered inflammatory cascades, in addition to its essential role in innate immune responses (14, 38, 39). Given the role of Fc γ R and complement in phagocytosis, cellular cytotoxicity, and inflammatory reactions, further analysis of autoantibody-triggered cellular and tissue injuries in relation to the respective roles of Fc γ R and complement would help establish new strategies for the development of therapeutic approaches in autoantibody- and immune complex-mediated inflammatory disorders.

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