Markedly Different Pathogenicity of Four Immunoglobulin G Isotype-Switch Variants of an Antierythrocyte Autoantibody Is Based on Their Capacity to Interact In Vivo with the Low-Affinity Fcγ Receptor III

By Liliane Fossati-Jimack,* Andreea Ioan-Facsinay,‡ Luc Reininger,§ Yves Chicheportiche,* Norihiko Watanabe,‖ Takashi Saito,‖ Frans M. A. Hofhuis,¶ J. Engelbert Gessner,** Carsten Schiller,** Reinhold E. Schmidt,** Tasuku Honjo,‡‡ J. Sjef Verbeek,‡ and Shozo Izui*

*From the *Department of Pathology, University of Geneva, 1211 Geneva 4, Switzerland; the ‡Department of Human and Clinical Genetics, Leiden University Medical Center, 2300 RA Leiden, The Netherlands; the §Institut National de la Santé et de la Recherche Médicale U 399, F-13385 Marseille, France; the ‖Department of Molecular Genetics, Chiba University Graduate School of Medicine, Chiba 260, Japan; the ¶Department of Immunology, University Hospital Utrecht, 3508 GA Utrecht, The Netherlands; the **Department of Clinical Immunology, Hannover Medical School, 30625 Hannover, Germany; and the ‡‡Department of Medical Chemistry, Kyoto University Graduate School of Medicine, Kyoto 606-8501, Japan*

Abstract

Using three different Fcγ receptor (FcγR)-deficient mouse strains, we examined the induction of autoimmune hemolytic anemia by each of the four immunoglobulin (Ig)G isotype-switch variants of a 4C8 IgM antierythrocyte autoantibody and its relation to the contributions of the two FcγR, FcγRI, and FcγRIII, operative in the phagocytosis of opsonized particles. We found that the four IgG isotypes of this antibody displayed striking differences in pathogenicity, which were related to their respective capacity to interact in vivo with the two phagocytic FcγRs, defined as follows: IgG2a > IgG2b > IgG3/IgG1 for FcγRI, and IgG2a > IgG1 > IgG2b > IgG3 for FcγRIII. Accordingly, the IgG2a autoantibody exhibited the highest pathogenicity, ~20–100-fold more potent than its IgG1 and IgG2b variants, respectively, while the IgG3 variant, which displays little interaction with these FcγRs, was not pathogenic at all. An unexpected critical role of the low-affinity FcγRIII was revealed by the use of two different IgG2a anti–red blood cell autoantibodies, which displayed a striking preferential utilization of FcγRIII, compared with the high-affinity FcγRI. This demonstration of the respective roles in vivo of four different IgG isotypes, and of two phagocytic FcγRs, in autoimmune hemolytic anemia highlights the major importance of the regulation of IgG isotype responses in autoantibody-mediated pathology and humoral immunity.

Key words: autoantibody • autoimmune hemolytic anemia • Fc receptor • IgG isotype • knockout mouse

Introduction

NZB mice spontaneously develop an autoimmune hemolytic anemia as a result of production of Coombs' anti-RBC autoantibodies (1). Although the specificity of anti-RBC autoantibodies is of primary importance in the expression of their pathogenic activities in vivo, effector functions associated with the Fc regions of the different Ig isotypes are also likely to play a critical role. Among the various effector functions mediated by the Ig heavy-chain constant regions, it is striking to see that the complement activation plays a minimal, if any, role in the development of anemia induced by anti-RBC antibodies (2, 3). In con-

Address correspondence to Dr. Shozo Izui, Department of Pathology, Centre Médical Universitaire, 1 rue Michel-Servet, Geneva 4, 1211, Switzerland. Phone: 41-22-70-25-741; Fax: 41-22-70-25-746; E-mail: shozo.izui@medecine.unige.ch

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trast, IgG Fc receptor (FcγR)[1]-mediated erythrophagocytosis has been recognized as the major pathogenic mechanism responsible for autoimmune hemolytic anemia in mice (2, 4–7).

Murine phagocytic effector cells express three different classes of FcγR: a high-affinity receptor, FcγRI, and two low-affinity receptors, FcγRII and FcγRIII (for reviews, see references 8–10). FcγRI and FcγRIII are heterooligomeric complexes, in which the respective ligand-binding α chains are associated with the common γ chain (FcRγ). FcRγ is required for their assembly and for the triggering of their various effector functions, including phagocytosis by macrophages, degranulation by mast cells, and antibody-dependent cell-mediated cytotoxicity by NK cells (11). In contrast, FcγRII is a single α chain receptor, with two major isoforms, FcγRIIb1 and FcγRIIb2 (12), both of which apparently lack phagocytosis-inducing capacity (11). The macrophage-specific isoform, FcγRIIb2, is capable of mediating the binding and endocytosis of IgG immune complexes (ICs), thereby facilitating antigen processing and presentation, whereas the b1 isoform, mainly expressed in B lymphocytes, is not efficiently internalized upon binding of IgG ICs, but mediates inhibition of surface IgM–triggered B cell activation after coligation (13–15). The high-affinity receptor, FcγRI, is capable of binding monomeric IgG2a (16–18), and the two low-affinity receptors, FcγRII and FcγRIII, bind polymeric IgG of different IgG isotypes except IgG3 (19). Thus, it has been proposed that IgG2a ICs interact preferentially with the high-affinity FcγRI, IgG1, and IgG2b ICs with the low-affinity FcγR. In addition, a more recent in vitro study has claimed that IgG3 ICs selectively interact with FcγRI (20). By the use of FcγR-deficient mice, it has now been well established that FcγRIII is the sole receptor mediating IgG1-dependent phagocytosis in vivo (6, 7, 21). However, the precise contribution of each of these two FcγR to phagocytize opsonized particles with IgG2a, IgG2b, or IgG3 antibodies remains to be defined.

In view of the major role of FcγR-mediated erythrophagocytosis in the pathogenesis of autoimmune hemolytic anemia (4–7), the in vivo pathogenicity of anti-RBC autoantibodies of different IgG isotypes may be critically dependent on the relative affinities of two different phagocytic FcγRs (FcγRI and FcγRIII) to the polymeric form of each IgG isotype. This question cannot be explored through the use of a random panel of monoclonal anti–mouse-RBC autoantibodies differing in Ig isotypes, as they may also differ in antigen-binding specificities and affinities. We have recently prepared an IgG2a class-switch variant from the NZB-derived 4C8 anti-RBC IgM monoclonal autoantibody (2), and found it highly pathogenic as the result of its efficient interaction with phagocytic FcγR (22). Therefore, we have generated three other IgG class-switch variants (IgG1, IgG2b, and IgG3) of this mAb, and compared their pathogenic potency with that of the IgG2a variant in relation to their utilization of the two classes of phagocytic FcγR, as explored by the use of three different strains of FcγR-deficient mice. We observed remarkable differences in the pathogenic potentials of these IgG variant autoantibodies. These differences appear to be determined by the capacity of individual IgG isotypes to interact in vivo with the low-affinity FcγRIII. Thus, the results have defined the respective roles of the two different phagocytic FcγRs in vivo, providing a rational for the IgG isotype–dependent pathogenicity observed in autoimmune hemolytic anemia.

Materials and Methods

Mice. FcγRIII-deficient (FcγRIII$^{-/-}$) mice lacking the α chain of FcγRIII with a mixed genetic background between C57BL/6 and 129 strains, FcRγ-deficient (FcRγ$^{-/-}$) mice (lacking functional expression of both FcγRI and FcγRIII) with a pure C57BL/6 background, and their corresponding wild-type (WT) littermates were developed as described previously (21, 23). FcγRI-deficient (FcγRI$^{-/-}$) mice lacking the α chain of FcγRI were generated in the laboratory of J. S. Verbeek (Leiden University) by homologous recombination, backcrossed for four generations with BALB/c mice, and bred to homozygosity at the *fcgrI* null allele (our unpublished results). BALB/c mice were purchased from Gl. Bomholtgard Ltd.

DNA Constructions. The VDJH4C8-Cγ1, -Cγ2b, and -Cγ3 plasmids containing the complete 4C8 IgG heavy-chain gene of the respective IgG subclass were constructed using the following DNA fragments: the rearranged VDJ region isolated from cDNA encoding the V region of the heavy chain of the 4C8 mAb (24), the promoter region isolated from pSV-Vμ1 (25), the heavy chain enhancer region isolated from pSVE2-neo (26), and the Cγ1, Cγ2b, or Cγ3 region derived from the respective genomic clones, pEVHCγ1 (26), pIgH22 (27), and pJW7 (28).

mAb. The 4C8 IgG1, IgG2b, and IgG3 class-switch variants were obtained by transfecting 4C8 heavy-chain-loss mutant cells by electroporation with the VDJH4C8-Cγ plasmids together with a pSVE2-neo plasmid containing the neomycin-resistant gene, as described for the generation of the 4C8 IgG2a variant (22). Clones secreting ~2–5 μg/ml were selected and used in this study. The 4C8 IgG class-switch variants exhibited a comparable mouse RBC-binding activity in vitro, as assessed by a flow cytometric analysis using a biotinylated rat anti–mouse κ-chain mAb (H139.52.1.5), followed by PE-conjugated streptavidin (22). Notably, the VH4C8 and Vκ4C8 sequences of the 4C8 heavy- and light-chain cDNA derived from a reverse transcriptase PCR amplification of mRNA isolated from the cells secreting 4C8 IgG switch variants were identical to the original published sequence (24). Hybridoma secreting 34-3C IgG2a anti–mouse RBC mAb was derived from nonmanipulated NZB mice (2). S54 IgG1 anti-4C8 idiotypic mAb recognizing the combination of both the heavy and light chains of the 4C8 mAb was obtained as described (29). Other mAbs in use were: IgG2a anti-TNP (Hy1.2), IgG1 anti-SRBC (Sp3HL), IgG2b anti-SRBC (N-S.8.1), rat IgG2b anti-CD4 (GK1.5), and rat IgG2b anti-CD8 (H35-17.2) mAbs. IgG mAbs were purified from culture supernatants by protein A or protein G column chromatography. The purity of IgG was >90% as documented by SDS-PAGE.

[1]*Abbreviations used in this paper:* FcγR, Fc receptor; FcRγ, FcR common γ chain; Ht, hematocrit; IC, immune complex; WT, wild-type.

Reverse Transcriptase PCR and cDNA Sequencing. RNA was prepared from 4C8 IgG transfectomas by RNeasy Mini Kit (Qiagen AG). The first strand of cDNA was synthesized with an oligo(dT) primer and 5 μg of total RNA. For amplification with *Pfu* DNA polymerase (Stratagene Cloning Systems), the following primers were used: 5′-untranslated VH primer (5′-CAGTTCTCTCTACAGTTA-3′), Cγ2b-CH1 primer (5′-GCCAGTGGATAGAC-3′), Cγ1/3-CH1 primer (5′-GGATAGACAGATGG-3′) for the 4C8 heavy-chain, and 5′-untranslated Vκ primer (5′-CAGGGGAAGCAAGATGG-3′) and Cκ primer (5′-TGGATGGTGGGAAGATG-3′) for the 4C8 light chain. The nucleotide sequence corresponding to the V region of the 4C8 heavy or light chain was determined by the dideoxynucleotide chain terminating method (30).

ELISA. Concentrations of mAbs in culture supernatants were quantified by IgG subclass-specific ELISA, as described previously (31). The expression of the 4C8 idiotype in sera was determined by ELISA. In brief, globulins from serum samples were first separated by precipitation in ammonium sulfate at 50% saturation, and the precipitate resuspended in 0.05 M carbonate buffer (pH 9.5) at a dilution of 1:1,000 was used for coating microtiter plates. Then, the assay was developed with alkaline phosphatase–labeled S54 anti-4C8 idiotypic mAb.

Experimental Autoimmune Hemolytic Anemia. Autoimmune hemolytic anemia was induced by a single intraperitoneal injection of purified anti-RBC mAb into 2–3-mo-old mice. In some experiments, 10^7 transfectoma or hybridoma cells were injected intraperitoneally into pristane-treated mice. As the transfectoma cells were derived from a fusion of NZB spleen cells with a BALB/c myeloma cell line, expressing the H-2^d haplotype, pristane-treated mice were given a mixture of anti-CD4 (GK1.5) and anti-CD8 (H35-17.2) mAbs (500 μg of each mAb) 1 d previously and 1 d after the transplantation of the transfectoma cells to avoid their rejection, as described previously (32). Blood samples were collected into heparinized microhematocrit tubes, and hematocrits (Ht) were directly determined after centrifugation, as described previously (2).

Histological Studies. Livers were obtained at autopsy, 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.

In Vitro Phagocytosis of IgG-opsonized SRBCs by Macrophages. Peritoneal macrophages were obtained from mice pretreated with thioglycollate, and adhered for 4 h at 37°C on chamber slides (Nunc) at a concentration of 3×10^5 cells per well. Then, aliquots of 200 μl of 5% SRBC opsonized with Sp3HL IgG1 or N-S.8.1 IgG2b anti-SRBC mAb at nonagglutinating titers were added to each well, and the plates were incubated for 60 min at 37°C. Extracellular SRBCs were lysed by hypotonic shock, immediately followed by two washes with PBS. The percentage of positive macrophages (i.e., those containing more than two SRBCs) was determined by light microscopy.

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

Results

Marked Differences in Pathogenic Activities among the 4C8 IgG Isotype-Switch Variants. The role of the IgG heavy-chain C region of anti–mouse RBC autoantibodies on the development of anemia was first analyzed by a single intraperitoneal injection of 1 mg of the four different IgG class-switch variants of the 4C8 mAb into BALB/c mice. The IgG2a variant induced the most severe form of anemia (a decrease in mean Ht values to 21% at day 4), the IgG1 variant a mild anemia (average Ht of 36%), and the IgG2b and IgG3 variants were unable to significantly decrease Ht levels (Fig. 1 A). Quantitative analysis revealed that 50 μg of the 4C8 IgG2a mAb was sufficient to induce anemia (mean Ht values of three mice 4 d after the injection: 36 ± 3%) at a level comparable to that observed with 1 mg of the 4C8 IgG1 isotype. For the 4C8 IgG2b variant, a dose as high as 5 mg caused a significant, though marginal, drop in Ht values (means of four mice: 46 ± 1% at day 0; 41 ± 2% at day 4; $P < 0.01$), whereas up to 5 mg of the 4C8 IgG3 variant had no detectable effects (means of four mice: 47 ± 2% at day 0; 45 ± 2% at day 4), as was the case of mice injected with a control IgG2a anti-TNP mAb (data not shown). Thus, the pathogenic potency of the 4C8 IgG2a isotype was ~20-fold, 100-fold, and still much higher than that of the IgG1, IgG2b, and IgG3 isotypes, respectively.

To compare more precisely the potency of the two less pathogenic isotypes, IgG2b- and IgG3-secreting transfectomas were implanted intraperitoneally into BALB/c mice. The 4C8 IgG2b transfectoma cells provoked severe anemia, with a decrease in mean Ht values to 31 and 18% at day 6 and 8, respectively, whereas Ht values remained within normal limits (>40%) in mice transplanted with 4C8 IgG3 or Hy1.2 IgG2a anti-TNP hybridoma cells (Fig. 1 B). The secretion of excess amounts of the 4C8 IgG3 mAb in vivo was documented by the presence of substantial amounts of free antibodies bearing the 4C8 idiotype (data not shown).

With the three anemia-inducing isotypes, histological examinations showed that the most remarkable pathological change was erythrophagocytosis by hepatic Kupffer cells. The extent of erythrophagocytosis, documented by iron deposits in Kupffer cells, correlated with the level of anemia induced by these three different IgG switch variants (Fig. 2).

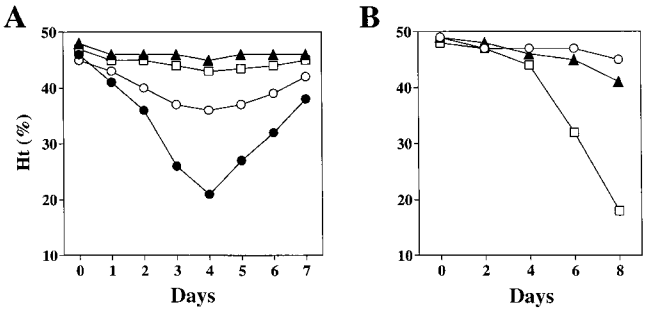

Figure 1. Development of anemia by the 4C8 IgG class-switch variants in BALB/c mice. (A) Mice were injected intraperitoneally with 1 mg of purified 4C8 IgG variants (IgG1, ○; IgG2a, ●; IgG2b, □; IgG3, ▲) on day 0. Results are expressed as mean Ht values of three to five mice. (B) Mice were transplanted intraperitoneally with 10^7 cells secreting 4C8 IgG2b (□), 4C8 IgG3 (▲), or Hy1.2 IgG2a anti-TNP (○) on day 0. Results are expressed as mean Ht values of five to seven mice.

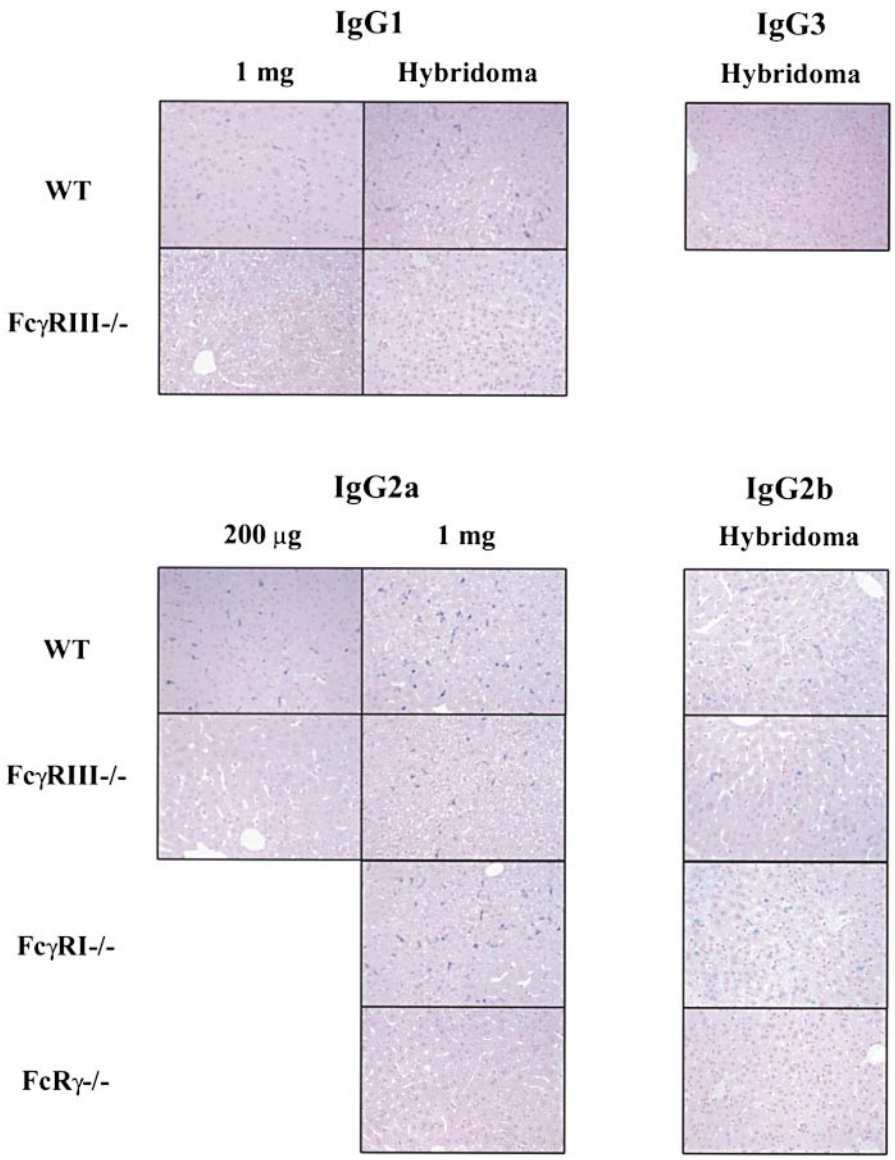

Figure 2. Representative histological appearance of iron deposits in Kupffer cells from FcγR-deficient and WT mice after the injection of the 4C8 IgG class-switch variants. Mice were injected intraperitoneally with either purified antibodies or 10^7 4C8 IgG-secreting cells, and killed at day 8. Extent of in vivo RBC destruction by phagocytosis was revealed by coloration of liver sections with Perls iron staining. Note complete absence of iron deposits in livers from FcγRIII$^{-/-}$ mice injected with the 4C8 IgG1 mAb, from FcRγ$^{-/-}$ mice receiving 4C8 IgG2a or IgG2b variant, and from WT mice transplanted with the 4C8 IgG3 transfectoma (original magnifications: ×200).

It should be mentioned that although Ht values in mice receiving 5 mg 4C8 IgG2b remained within normal limits, significant iron deposits in Kupffer cells were observed; this was markedly augmented in mice implanted with the 4C8 IgG2b transfectoma. In contrast, Kupffer cell–mediated erythrophagocytosis and iron deposits were totally absent in the 4C8 IgG3-injected mice, even following the transplantation of 4C8 IgG3 cells (Fig. 2). Moreover, these mice failed to show a massive accumulation of agglutinated RBCs in spleen and liver, unlike mice injected with the 4C8 IgM mAb (2).

Differential Contributions of FcγRI and FcγRIII to the Development of Anemia in Relation to the 4C8 IgG Isotypes. By using three different mouse strains—strains deficient in FcγRI, FcγRIII, or FcRγ (i.e., lacking functional expression of both FcγRI and FcγRIII)—we next investigated the respective contribution of two different classes of phagocytic FcγR and FcγRIII to the anemia induced by IgG1, IgG2a, and IgG2b isotype variants of the 4C8 mAb.

The development of anemia occurring in WT mice in-

Table I. *Development of Anemia in FcγR-deficient and WT Mice after the Injection of the 4C8 IgG1 and IgG2a Variants*

Isotype	Dose	Mice	Ht*
			%
IgG1	1 mg	WT (4)‡	36.3 ± 2.9
		FcγRIII$^{-/-}$ (5)	46.6 ± 0.9
IgG2a	200 μg	WT (4)	31.0 ± 2.0
		FcγRIII$^{-/-}$ (4)	45.7 ± 2.1
	1 mg	WT (7)	21.5 ± 3.7
		FcγRIII$^{-/-}$ (7)	37.2 ± 2.3
		FcγRI$^{-/-}$ (5)	27.6 ± 3.9
		FcRγ$^{-/-}$ (4)	44.6 ± 1.8

*Ht values (mean ± 1SD) were determined 4 d after the intraperitoneal injection of purified 4C8 IgG variants. Ht values before the injection of anti-RBC mAB in WT and FcγR-deficient mice were in the 44–48% range.
‡Numbers of mice studied are indicated in parentheses.

jected with 1 mg 4C8 IgG1 mAb was completely prevented in FcγRIII$^{-/-}$ mice (Table I), which failed to exhibit erythrophagocytosis, as documented by the lack of iron deposits in their Kupffer cells (Fig. 2). The far more severe erythrophagocytosis observed in WT mice after the transplantation of the 4C8 IgG1 transfectoma was also abolished in FcγRIII$^{-/-}$ mice (Fig. 2), indicating that FcγRIII is the sole receptor involved in the 4C8 IgG1-mediated erythrophagocytosis by Kupffer cells.

Although FcγRIII$^{-/-}$ mice were also totally resistant to the pathogenic effect of 200 μg 4C8 IgG2a variant, a less severe, but significant anemia with a lower extent of erythrophagocytosis was still induced in these mice by a higher dose (1 mg) of the 4C8 IgG2a ($P < 0.001$) (Table I, and Fig. 2). This indicated that FcγRIII plays a major role in the 4C8 IgG2a-induced anemia, but that FcγRI is also involved in the severe anemia caused by higher amounts of this isotype. This conclusion was confirmed by the use of two other strains of FcγR-deficient mice, in which the level of protection from the pathogenic effect of 1 mg 4C8 IgG2a mAb was found to be more limited in FcγRI-deficient mice than in FcγRIII-deficient mice ($P < 0.005$), but complete in FcRγ$^{-/-}$ mice lacking both receptors (Table I and Fig. 2).

In contrast to the IgG1 and IgG2a variants, the development of a severe anemia provoked by the transplantation of the 4C8 IgG2b transfectoma was almost indistinguishable in kinetics and histological changes among FcγRIII$^{-/-}$, FcγRI$^{-/-}$, and WT mice (Fig. 2 and Fig. 3). However, FcRγ$^{-/-}$ mice deficient in both FcγRI and FcγRIII were resistant to the pathogenic effect of the 4C8 IgG2b, as erythrophagocytosis by Kupffer cells was no longer visible in these mice (Fig. 2 and Fig. 3). Notably, serum levels of antibodies bearing the 4C8 idiotype, measured at killing (6–8 d after the transplantation of the 4C8 IgG2b cells), were comparable between FcγR-deficient mice and their corresponding WT mice (data not shown). It should also be stressed that both FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ mice injected with 5 mg of the 4C8 IgG2b exhibited a modest hepatic erythrophagocytosis at levels comparable to that of WT mice (data not shown). These results indicated that the involvement of both FcγRI and FcγRIII in hemolytic anemia is induced by very high doses of the 4C8 IgG2b variant.

Major Contribution of FcγRIII to the Development of Anemia by 34-3C IgG2a Anti–Mouse RBC mAb. Previous studies have demonstrated the contribution of both FcγRI and FcγRIII to the development of anemia after the injection of a highly pathogenic dose of the 34-3C IgG2a anti–mouse RBC mAb (5, 7). As the present studies revealed a critical role of FcγRIII in the development of anemia induced by a lower dose of the 4C8 IgG2a variant, we reassessed the pathogenic effect of the 34-3C IgG2a mAb in FcγRIII$^{-/-}$ mice compared with WT mice. The development of anemia was partially prevented in FcγRIII$^{-/-}$ mice after the injection of a highly pathogenic dose (200 μg) of the 34-3C mAb, which caused a severe anemia in WT mice ($P < 0.05$). However, it was completely prevented after the injection of a lower dose (50 μg) that still caused anemia in WT mice ($P < 0.005$; Fig. 4). These results confirmed the prominent role of FcγRIII over FcγRI in the IgG2a anti-RBC–induced autoimmune hemolytic anemia.

Contribution of Both FcγRI and FcγRIII to Phagocytosis of IgG2b-opsonized SRBCs by Peritoneal Macrophages In Vitro. An intriguing observation made in this study was the sig-

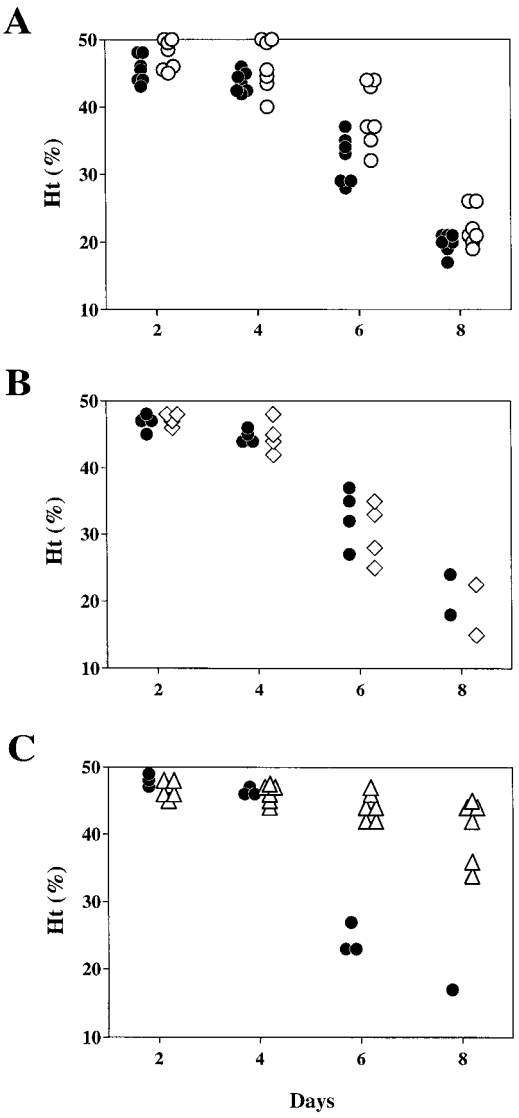

Figure 3. Development of anemia in FcγR-deficient and WT mice after the transplantation of the 4C8 IgG2b transfectoma. 10^7 transfectoma cells were injected into (A) FcγRIII$^{-/-}$ (○) or WT littermates (●); (B) FcγRI$^{-/-}$ (◇) or WT littermates (●); and (C) FcRγ$^{-/-}$ (△) or WT littermates (●) on day 0. Ht values of individual mice measured every 2 d are shown.

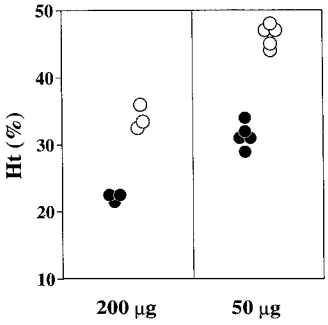

Figure 4. Development of anemia in FcγRIII$^{-/-}$ and WT mice after the injection of the 34-3C IgG2a mAb. 200 or 50 μg of the mAb was injected intraperitoneally into FcγRIII$^{-/-}$ (○) or WT littermates (●) on day 0. Ht values of individual mice measured 4 d after the mAb injection are shown. Note the complete prevention of anemia in FcγRIII$^{-/-}$ mice injected with 50 μg of the 34-3C mAb.

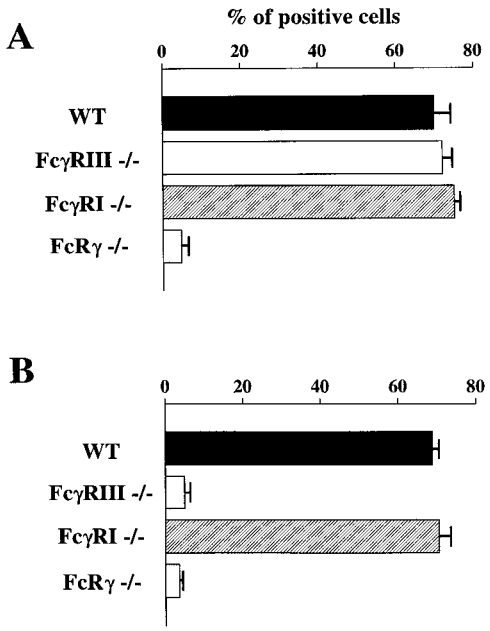

Figure 5. In vitro phagocytosis of IgG2b- and IgG1-opsonized SRBCs by macrophages from FcγR-deficient and WT mice. Adherent thioglycollate-elicited peritoneal macrophages from WT, FcγRIII$^{-/-}$, FcγRI$^{-/-}$, and FcRγ$^{-/-}$ mice were incubated with SRBCs opsonized with N-S.8.1 IgG2b (A) or Sp3HL IgG1 (B) anti-SRBC mAb. Phagocytosis was determined after lysing extracellular SRBCs by a hypotonic shock. Results are expressed as the percentage (means ± SEM of triplicate cultures) of positive macrophages that had ingested more than two SRBCs.

nificant contribution of FcγRI to the development of IgG2b-induced erythrophagocytosis, as it has been thought that murine IgG2b ICs are unable to interact with the high-affinity FcγRI (33). As no other IgG2b anti–mouse RBC mAb capable of inducing erythrophagocytosis in vivo was available, we analyzed the in vitro phagocytosis of SRBCs opsonized with murine IgG2b anti-SRBC mAb by thioglycollate-elicited peritoneal macrophages derived from mice deficient in FcγRI and/or FcγRIII. Phagocytosis of IgG2b-opsonized SRBCs by macrophages lacking either FcγRI or FcγRIII was not affected at all, whereas FcRγ-deficient macrophages failed to display significant phagocytosis of these opsonized SRBCs (Fig. 5 A). Macrophages from FcγRI$^{-/-}$ mice exhibited phagocytosis of IgG1-opsonized SRBCs as strong as those from WT mice, whereas this phagocytic activity was hardly detectable by macrophages lacking FcγRIII (Fig. 5 B).

Discussion

We have generated four IgG class-switch variants bearing identical VH and Vκ regions, those of a pathogenic 4C8 IgM anti–mouse RBC autoantibody derived from lupus-prone NZB mice, and determined their potency in the induction of autoimmune hemolytic anemia in relation to the respective contributions of two different classes of phagocytic FcγR, namely, the high-affinity FcγRI and the low-affinity FcγRIII. The IgG2a isotype interacting most efficiently with both FcγRI and FcγRIII exhibited the highest hemolytic activity, followed by the IgG1 isotype having a substantial affinity to FcγRIII, and then by the IgG2b isotype capable of interacting only weakly with both receptors. The IgG3 isotype lacking significant binding to these FcγRs displayed no pathogenicity at all. Furthermore, our results have demonstrated preferential utilization of FcγRIII by the IgG2a isotype in vivo, despite their low-affinity interaction compared with FcγRI, revealing the major role of the low-affinity FcγRIII in autoimmune hemolytic anemia.

Differential Contributions of FcγRI and FcγRIII to IgG Isotype-dependent Anti-RBC Pathogenicity. It was striking to observe how different the utilization by individual IgG isotypes of the two different types of FcγR involved in erythrophagocytosis in vivo is, as reflected in the pathogenesis of autoimmune hemolytic anemia (Table II). The complete absence of erythrophagocytosis by the 4C8 IgG1 variant in FcγRIII$^{-/-}$ mice confirmed the critical role of FcγRIII in the IgG1-mediated erythrophagocytosis, as shown recently by using another 105-2H IgG1 anti–mouse RBC monoclonal autoantibody (6, 7). Significantly, our study disclosed that the contribution of FcγRIII to the development of IgG2a-induced autoimmune hemolytic anemia is more prominent than that of FcγRI. This was somehow unexpected, as the high-affinity binding of FcγRI to the IgG2a isotype has been well established (16–18, 33). However, it should be stressed that FcγRI contributes to IgG2a-dependent erythrophagocytosis, but only when higher doses of IgG2a anti–mouse RBC mAb were injected. A limited utilization of FcγRI for phagocytosis of IgG2a-opsonized RBCs may be due to the competition by excess amounts of unbound circulating monomeric IgG2a having a high-affinity interaction with FcγRI. In this regard, it may be worth noting that a significant anemia was still observed in FcγRIII$^{-/-}$ mice injected with 200 μg of the 34-3C IgG2a, but not with the same amount of the 4C8 IgG2a. Thus, it appears that the contribution of the high-affinity FcγRI to pathogenicity is more influenced by the antigen-binding properties of the IgG2a antibodies. In agreement with this conclusion, we have recently shown that in vivo bindings to circulating RBCs were much stronger with the 34-3C mAb than with the 4C8 IgG2a, reflecting marked differences in RBC-binding affinities of these two antibodies (22). Thus, it is likely that higher densities of the 34-3C IgG2a bound on RBCs may efficiently compete with circulating monomeric IgG2a for FcγRI binding on phagocytes. An alternative, or additional possibility is that the IgG2a anti-RBC mAb at higher doses could mediate erythrophagocytosis by their direct binding to FcγRI, followed by subsequent interaction of cell-bound antibodies with circulating RBCs. Owing to its higher RBC-binding capacity, the FcγRI-bound 34-3C mAb on the surface of phagocytes may be much more efficient to capture circulating RBCs, causing erythrophagocytosis, compared with the low-affinity 4C8 IgG2a.

An additional and unexpected observation was that of a significant role of FcγRI in the pathogenesis of 4C8

Table II. *Pathogenetic Activities of the 4C8 IgG Class-Switch Variants, Respective Contributions of FcγRI and FcγRIII to the Development of Anemia Induced by the 4C8 IgG Variants, and Their Relative Affinities to ICs of the Four IgG Isotypes*

Isotype	Pathogenicity*	Contribution of FcγR to 4C8 IgG-induced anemia	Affinity of	
			FcγRI‡	FcγRIII‡
IgG1	++ (1 mg)	FcγRIII	−	++
IgG2a	+++ (50 μg)	FcγRIII > FcγRI	++	+++
IgG2b	+ (>5 mg)	FcγRI/FcγRIII	+	+
IgG3	−	None	−	−

*Minimum amounts of mAb required for inducing anemia (decreasing Ht values <40%) are indicated in parentheses.
‡The relative affinity of FcγRI and FcγRIII to ICs of the four different IgG isotypes is arbitrarily graded on the base of in vivo evidence of erythrophagocytosis by Kupffer cells.

IgG2b-induced autoimmune hemolytic anemia. Indeed, it has long been believed that the IgG2b isotype is unable to interact with the high-affinity FcγRI (33). However, it should be emphasized that the latter conclusion was based on the results obtained with COS cells expressing FcγRI α chains in absence of the FcR γ chain (33, 34). The Fc receptors expressed on those transfected cells exhibit binding to monomeric IgG2a and IgG2a-coated RBCs. However, these receptors do not appear to behave as do heterooligomeric complexes of the native receptor normally borne on phagocytic effector cells, as FcγRI is functionally absent in FcRγ-deficient peritoneal macrophages and Kupffer cells (5, 11). It has also been noted that the affinity of the FcγRI expressed on the transfected COS cells is two- to fivefold lower than that of the native receptor, but is restored at normal levels through their association with FcRγ (18, 35, 36). This could account for the lack of detectable binding of IgG2b-opsonized RBCs to COS cells transfected with murine FcγRIα cDNA (33), because of a weak affinity of FcγRI to IgG2b ICs, as discussed below. On the other hand, our demonstration that 4C8 IgG2b-dependent erythrophagocytosis by Kupffer cells was little affected by the absence of FcγRIII is in good agreement with in vitro studies using peritoneal macrophages isolated from mice deficient in FcγRIII (6, 21). However, it should be emphasized that Kupffer cell–mediated erythrophagocytosis in WT mice injected with 5 mg of purified antibodies or transplanted with the 4C8 IgG2b-secreting cells was not affected in FcγRI$^{-/-}$ mice, but completely protected in FcRγ$^{-/-}$ mice lacking both FcγRI and FcγRIII. The presence of very high concentrations of the 4C8 IgG2b mAb in these experimental conditions may promote the utilization of FcγRI, as discussed above for the IgG2a isotype. Consequently, FcγRI and FcγRIII are able to efficiently compensate each other to mediate the phagocytosis of IgG2b-opsonized RBCs in vivo. As we could not assess the respective role of both receptors at lower concentrations of the IgG2b isotype, in contrast with the situation with the 4C8 IgG2a isotype, our present conclusion, that the comparable contribution of FcγRI and FcγRIII to the IgG2b-induced anemia, is still tentative (Table II). Only experiments with a highly pathogenic IgG2b anti-RBC mAb would provide definitive conclusions on this issue.

The complete dependency of IgG-mediated erythrophagocytosis on FcγRI and/or FcγRIII confirms a lack of phagocytosis-inducing capacity by FcγRII, though capable of mediating endocytosis of polymeric IgG (13, 14). On the other hand, it has been demonstrated that FcγRII-deficient mice exhibited higher humoral, anaphylactic, and inflammatory immune responses that underline the importance of FcγRII for the negative regulation of B cell receptor–, FcεRI-, and FcγRIII-dependent effector functions in vivo (15, 37, 38). However, we have recently shown that FcγRII is unable to downregulate FcγRIII-mediated phagocytosis of RBCs opsonized with 105-2H IgG1 anti-RBC mAb in vivo (39). Although the possible negative effect of FcγRII on FcγRI-dependent erythrophagocytosis cannot be excluded, our preliminary analysis with the 34-3C IgG2a mAb in FcγRII-deficient mice argues against such a possibility.

Relative Affinities of FcγRI and FcγRIII to the Polymeric Forms of the Four Different Murine IgG Isotypes. Our results help clarify the issue regarding the relative affinity of each of these two FcγRs to the different IgG isotypes in vivo, and hence, the respective roles of the four different IgG isotypes in the pathogenesis of autoimmune hemolytic anemia. With respect to the affinity of FcγRI to polymeric form of murine IgG isotypes, our study reveals that FcγRI has a significant affinity to IgG2b ICs, as discussed above. However, its affinity to IgG2b ICs is much lower than that to IgG2a ICs, as FcγRI-mediated erythrophagocytosis was observed in FcγRIII$^{-/-}$ mice with 1 and 5 mg of the 4C8 IgG2a and IgG2b variants, respectively. Recently, using bone marrow–derived macrophages from FcγRI-deficient mice, FcγRI has been reported to be the sole receptor for the IgG3 isotype (20). However, we did not observe any significant erythrophagocytosis by Kupffer cells in WT mice, even after the implantation of 4C8 IgG3 cells. In agreement with this observation, a recent study has reported that an IgG3 mAb against cryptococcal capsular

polysaccharide failed to provoke phagocytosis through FcγRI and FcγRIII in vitro and in vivo (40). Together with the complete protection from the pathogenic effect of the 4C8 IgG1 variant in FcγRIII$^{-/-}$ mice, we propose that the relative in vivo binding activity of FcγRI to antigen–antibody complexes of different murine IgG isotypes is in the order of IgG2a > IgG2b > IgG3/IgG1 (Table II).

Based on in vitro studies using macrophages or transfected cell lines (17, 19), it has been proposed that FcγRIII has a comparable affinity to IgG1, IgG2b, and IgG2a, but little affinity to IgG3. Although we confirmed that FcγRIII is capable of mediating phagocytosis of RBCs opsonized with IgG1, IgG2b, and IgG2a, but not with IgG3, our analysis has clearly demonstrated marked differences in the relative affinity of FcγRIII to these three IgG isotypes; highest for IgG2a, intermediate for IgG1, and lowest for IgG2b (Table II). This conclusion is based on the finding that FcγRIII-dependent erythrophagocytosis was inducible at a dose of 50 μg, 1 mg, and 5 mg of the 4C8 IgG2a, IgG1, and IgG2b isotypes, respectively. However, it should be mentioned that IgG2b ICs are potent to induce passive cutaneous anaphylaxis upon triggering FcγRIII expressed on mast cells (6). An efficient activation of mast cells by IgG2b ICs may be related to the fact that unlike macrophages, mast cells express a unique form of FcγRIII associated with the FcR β chain, which functions as an amplifier of FcγRIII responses by enhancing FcRγ-mediated signaling (41).

Does Complement Play Any Role in Autoimmune Hemolytic Anemia? The present results are consistent with the previous conclusion that complement-mediated hemolysis and complement receptor–dependent erythrophagocytosis may play but a minor, if any, role in this model of autoimmune hemolytic anemia. The development of 34-3C IgG2a-induced anemia was indeed not affected at all in C5-deficient DBA/2 mice or in C3-depleted mice by the treatment with cobra venom factor (2, 7). This is further supported by the recent demonstration that mice genetically deficient in C3 were not protected from anemia caused by polyclonal rabbit IgG anti–mouse RBC antibodies, whereas loss of both FcγRI and FcγRIII prevented the anemia (3). However, these results cannot completely exclude the possible role of C4 in autoimmune hemolytic anemia, as the C4b fragment is recognized by the complement receptor type I (CR1), which stimulates phagocytosis (for a review, see reference 42). In addition, in vivo clearance experiments of RBCs sensitized with polyclonal rabbit IgG anti-RBC antibodies in C4-deficient guinea pigs have shown that erythrophagocytosis can be mediated by the synergistic cooperation of FcγR and complement receptors expressed on Kupffer cells (43). Such a mechanism could be operative under certain conditions, depending on the extent of opsonization and the IgG isotypes of anti-RBC autoantibodies. If so, the differential ability of individual IgG isotypes to activate the complement pathway may additionally contribute to the remarkable differences in the pathogenicity observed in this report. Clearly, more detailed analysis on C3- and C4-deficient mice in relation to the IgG isotypes of anti-RBC autoantibodies, their RBC-binding affinities, and the extent of RBC opsonization could help to define a role for complement in the development of autoimmune hemolytic anemia.

Concluding Remarks. The use of the four different anti-RBC IgG switch variants bearing identical VH and Vκ regions has provided a unique opportunity to define the respective roles of two different phagocytic FcγRs in IgG isotype–dependent effector functions, and hence, the pathogenic potency of individual murine IgG isotypes. Strikingly, the capacity of each IgG isotype to interact with the low-affinity FcγRIII is the critical factor determining the pathogenic potency of individual IgG isotypes, as the high-affinity FcγRI apparently plays a relatively limited role, probably because of the competition by circulating monomeric IgG2a. In addition, our results should provide useful guiding principles for the engineering of mAbs for in vivo applications.

The demonstration of the highest pathogenic potency of the IgG2a isotype highlights the importance of the regulation of IgG isotype responses in both autoantibody-mediated pathology and IC-mediated inflammatory disorders. A recent study has shown that FcγR-mediated inflammatory responses play an important role in the pathogenesis of lupus-like glomerulonephritis (44), supporting the possibility of a higher nephritogenic potential for autoantibodies of the IgG2a isotype. Although anti-RBC autoantibodies of the IgG3 isotype are poorly pathogenic, nephritogenic activities of IgG3 autoantibodies have also been well established, on the basis of a cryoglobulin activity uniquely associated with the IgG3 heavy-chain C region (45–48). These findings are consistent with the observation that the progression of murine lupus-like autoimmune syndrome is correlated with the relative dominance of Th1 autoimmune responses promoting the production of IgG2a and IgG3 autoantibodies (49–53). Clearly, further studies on the pathogenic role of autoantibodies according to their Ig isotypes and in relation with the Th subset responses would help establish new strategies for the development of therapeutic approaches in autoantibody-mediated autoimmune diseases.

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